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Caffeine Induces Ca^{2+} Release by Reducing The Threshold for Luminal Ca^{2+} Activation of the Ryanodine Receptor

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Abstract

Caffeine has long been used as a pharmacological probe for studying ryanodine receptor (RyR)-mediated Ca^{2+} release and cardiac arrhythmias. However, the precise mechanism by which caffeine activates RyRs is elusive. Here we investigated the effects of caffeine on spontaneous Ca^{2+} release and on the response of single cardiac RyR (RyR2) channels to luminal or cytosolic Ca^{2+}. We found that HEK293 cells expressing RyR2 displayed partial or "quantal" Ca^{2+} release in response to repetitive additions of submaximal concentrations of caffeine. This quantal Ca^{2+} release was abolished by ryanodine. Monitoring of endoplasmic reticulum luminal Ca^{2+} revealed that caffeine reduced the luminal Ca^{2+} threshold at which spontaneous Ca^{2+} release occurs. Interestingly, spontaneous Ca^{2+} release in the form of Ca^{2+} oscillations persisted in the presence of 10 mM caffeine, and was diminished by ryanodine, demonstrating that unlike ryanodine, caffeine, even at high concentrations, does not hold the channel open. At the single channel level, caffeine markedly reduced the threshold for luminal Ca^{2+} activation, but had little effect on the threshold for cytosolic Ca^{2+} activation, indicating that the major action of caffeine is to reduce the luminal, but not the cytosolic, Ca^{2+} activation threshold. Furthermore, as with caffeine, the clinically relevant, pro-arrhythmic methylxanthines aminophylline and theophylline potentiated luminal Ca^{2+} activation of RyR2, and increased the propensity for spontaneous Ca^{2+} release, mimicking the effects of diseased-linked RyR2 mutations. Collectively, our results demonstrate that caffeine triggers Ca^{2+} release by reducing the threshold for luminal Ca^{2+} activation of RyR2, and suggest that disease-linked RyR2 mutations and RyR2-interacting pro-arrhythmic agents may share the same arrhythmogenic mechanism.

Keywords

Ryanodine Receptor; Spontaneous Ca^{2+} release; Quantal Ca^{2+} release; Cardiac arrhythmias; Methylxanthines

INTRODUCTION

A number of naturally-occurring mutations in the cardiac Ca^{2+} release channel/ryanodine receptor (RyR2) have been linked to at least two forms of cardiac arrhythmias: catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular displaysia type 2 (ARVD2), but their causal mechanisms have not been completely

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defined [1]. We have recently shown that disease-causing RyR2 mutations enhance the sensitivity of the channel to activation by luminal Ca^{2+} and reduce the threshold for spontaneous Ca^{2+} release, also known as store-overload-induced Ca^{2+} release (SOICR)[2,3]. It is well known that spontaneous Ca^{2+} release can alter membrane potential by generating delayed afterdepolarizations (DADs), which in turn can lead to triggered arrhythmias [4]. Alternatively, it has also been proposed that disease-linked RyR2 mutations alter protein-protein or inter-domain interactions that are important for stabilizing the closed state of the channel, thus rendering the channel hyperactive and leaky [5,6].

In addition to RyR2 mutations, RyR2-interacting drugs, including caffeine and other methylxanthines (aminophylline and theophylline), have been shown to promote catecholamine-induced arrhythmias, but by itself caffeine is not arrhythmogenic and does not have a sustained impact on stimulated Ca^{2+} release [7–14]. This pro-arrhythmic characteristic of caffeine resembles that of the RyR2 CPVT mutations, which predispose patients to exercise or stress-induced cardiac arrhythmias, but cause no obvious structural or functional cardiac defects at rest [1]. These observations suggest that caffeine and CPVT mutations may affect the RyR2 channel in a similar manner. Consistent with this hypothesis, caffeine has been shown to reduce the threshold for spontaneous Ca^{2+} release [2,7,15]. However, exactly how caffeine reduces the threshold for spontaneous Ca^{2+} release is not well understood.

Caffeine has commonly been used as an RyR agonist for inducing Ca^{2+} release from intracellular Ca^{2+} stores [16–20]. A unique feature of caffeine-induced Ca^{2+} release from RyR-gated Ca^{2+} stores is its lack of desensitization. Multiple additions of caffeine at submaximal concentrations can each induce a partial and transient Ca^{2+} release from intracellular Ca^{2+} stores in cells expressing RyRs or from sarcoplasmic reticulum membrane vesicles [17–20], a phenomenon known as “quantal” Ca^{2+} release. The partial or quantal Ca^{2+} release induced by incremental concentrations of caffeine was thought to result from the sequential activation of different populations of RyRs expressed in the same cell with different sensitivities to caffeine in an all-or-none fashion [19,20]. However, it has also been shown that the ability of caffeine to induce Ca^{2+} release is dependent on the store Ca^{2+} content [17,21,22]. When the store Ca^{2+} level is below a threshold level, caffeine is no longer able to induce Ca^{2+} release despite its continued presence. Hence, the partial or quantal Ca^{2+} release is believed to result from store-dependent negative feedback regulation of caffeine activation of the channel [17,22]. Similar to the phenomenon of quantal Ca^{2+} release, caffeine at low concentrations has also been shown to only transiently potentiate stimulated Ca^{2+} release in cardiac cells [15]. This transient effect of caffeine is believed to be due to the auto-regulation of SR Ca^{2+} release by the SR Ca^{2+} content [23]. Furthermore, Ca^{2+} release studies using SR membrane vesicles have also shown that a certain level of store Ca^{2+} content must be present before caffeine-induced Ca^{2+} release can occur [24–26]. Together, these observations clearly indicate that SR luminal Ca^{2+} plays an important role in the action of caffeine, but the molecular basis of this luminal Ca^{2+} dependence is unclear.

Caffeine is commonly thought to sensitize the RyR2 channel to activation by cytosolic Ca^{2+}, leading to an increase in the open probability (Po) of the channel [27,28]. An enhanced Po of RyR2 would result in a decreased SR Ca^{2+} content, which would, in turn, reduce the Po of RyR2. As a result of this counteractive reduction in luminal Ca^{2+}, caffeine, despite its continued presence, only causes a transient effect on SR Ca^{2+} release [15]. However, recent studies revealed that single RyR2 channels are rather insensitive to caffeine in the absence of luminal Ca^{2+} [29]. Alternatively, since RyR2 is also regulated by luminal Ca^{2+} [30–32], caffeine may alter the response of the channel to luminal Ca^{2+}. Based on our observation that CPVT RyR2 mutations reduce the threshold for spontaneous Ca^{2+} release by increasing the sensitivity of the channel to luminal Ca^{2+} activation, we reasoned that caffeine, which also reduces the threshold for spontaneous Ca^{2+} release, might sensitize the channel to luminal Ca^{2+} activation.

To test this hypothesis, in the present study we investigated the impact of caffeine on the sensitivity of single RyR2 channels to activation by cytosolic or luminal Ca^{2+}. We found that caffeine preferentially potentiated the luminal Ca^{2+} activation of RyR2 at low cytosolic Ca^{2+} concentrations. Similar effects were also observed with two other methylxanthines, aminophylline and theophylline. These observations suggest that the pro-arrhythmic action of clinically relevant methylxanthines likely results from their luminal Ca^{2+} activating properties.

EXPERIMENTAL PROCEEDURES

Materials

Soybean phosphatidylcholine, heart phosphatidylethanolamine, and brain phosphatidylserine were obtained from Avanti Polar Lipids, Inc (Alabaster, AL). [^{3}H]ryanodine was from PerkinElmer Life Sciences. CHAPS and other reagents were purchased from Sigma.

Ca^{2+} release measurements—The free cytosolic Ca^{2+} concentration in transfected HEK293 cells was measured using the fluorescent Ca^{2+} indicator dye fluo-3-AMas described previously [33].

Generation of stable, inducible HEK293 cells—HEK293 cells expressing RyR2 (wt) were generated and characterized previously [2].

Single cell Ca^{2+} imaging (luminal Ca^{2+})—Luminal Ca^{2+} transients in HEK293cells expressing RyR2 were measured using single-cell Ca^{2+} imaging and the Ca^{2+} sensitive FRET-based cameleon protein D1ER [34]. Stable, inducible HEK293 cells expressing RyR2 were used, but were additionally transfected, using the Ca^{2+} phosphate precipitation method, with D1ER cDNA 24 hr before RyR2 expression was induced. The cells were continuously perfused with Krebs-Ringer- Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6 mM glucose, 1.2 mM MgCl2, 25 mM Hepes, pH 7.4) containing 2 mM $CaCl_2$, various concentrations of caffeine (0, 0.3, 1, 10 mM) and in the presence or absence of 20 μM ryanodine at room temperature. Images were captured with Compix Inc. Simple PCI 6 software at 470 nm and 535 nm emission, with excitation at 430 nm, every 2 s using an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20x/0.75 objective. The amount of FRET was determined from the ratio of the emissions at 535 and 470 nm.

Single channel recordings—Single-channel analyses were carried out as described previously [3]. Briefly, RyR2 proteins were partially purified from cell lysate by sucrose density gradient centrifugation. Heart phosphatidylethanolamine and brain phosphatidylserine (Avanti Polar Lipid), dissolved in chloroform, were combined in a 1:1 ratio (w/w), dried under nitrogen gas and suspended in 30 μl of n-decane at a concentration of 12 mg lipid/ml. Bilayers were formed across a 250-μm hole in a Delrin partition separating two chambers. The *trans* chamber (800 μl) was connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments Inc.). The *cis* chamber (1.2 ml) was held at virtual ground. A symmetrical solution containing 250 mM KCl and 25 mM Hepes (pH 7.4), was used for all recordings, unless indicated otherwise. A 4 μl aliquot (~1 μg of protein) of the sucrose density gradient-purified RyR2 protein was added to the *cis* chamber. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca^{2+}. Only those single channels that are EGTA sensitive and display a stable open probability were used for analyses. The chamber to which the addition of EGTA inhibited the activity of the incorporated channel was presumed to correspond to the cytosolic side of the channel. The direction of single channel currents was always measured from the luminal to the cytosolic side of the channel, unless mentioned otherwise. Recordings were filtered at 2,500 Hz. Free Ca^{2+} concentrations were calculated using the computer

program of Fabiato and Fabiato [35]. Data analyses were carried out using the pClamp 8.1 software (Axon Instruments Inc.).

Single cell Ca^{2+} imaging of HEK293 cells—Intracellular Ca^{2+} transients in stable, inducible HEK293 cells expressing RyR2 were measured using single-cell Ca^{2+} imaging and the fluorescent Ca^{2+} indicator dye fura-2 acetoxymethyl ester (fura-2 AM) as described previously [2]. Cells grown on glass coverslips for 24 hr after induction by 1 μg/ml tetracycline were loaded with 5 μM fura-2 AM in Krebs- Ringer- Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 6 mM glucose, 1.2 mM, $MgCl_2$, 25 mM Hepes, pH 7.4) plus 0.02% pluronic F-127 (Molecular Probes) and 0.1 mg/ml BSA for 20 min at room temperature. The coverslips were then mounted in a perfusion chamber (Warner Instruments, Hamden, CT) on an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20x/0.75 objective. The cells were continuously perfused with KRH buffer containing 0, 0.1, 0.2, 0.3, 0.5, 1 mM $CaCl_2$ and 0.3 mM caffeine, aminophylline or theophylline at room temperature. 10 mM caffeine was applied at the end of each experiment. Time-lapse images (0.25 frames s^{-1}) were captured and analyzed with the Compix Inc. Simple PCI 6 software. Fluorescent intensities were measured from regions of interest centered on individual cells. Only those cells that responded to caffeine were used in analysis (60–80%).

Isolation of adult rat ventricular myocytes—All studies with rats were approved by the Animal Care Committee of the University of Calgary and complied with *the Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996). Single rat ventricular myocytes were isolated as described previously [36]. Isolated cells were stored at room temperature in a solution containing 20 mM taurine, 5 mg/ml albumin, and 0.5 mM $CaCl_2$, until used for single cell Ca^{2+} imaging studies.

Single cell Ca^{2+} imaging of rat ventricular myocytes—Freshly isolated rat ventricular myocytes were placed on glass coverslips coated with 0.02% (w/v) gelatin and 10 μg/ml fibronectin, and loaded with 5 μM fluo-4-AM Ca^{2+} (Molecular Probes) plus 0.02% pluronic F-127 in Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 6 mM glucose, 1.2 mM $MgCl_2$ and 25 mM Hepes, pH 7.4) (without KH_2PO_4) in the presence of 1.0 mM Ca^{2+} for 20 min at room temperature. The coverslips were mounted in a perfusion chamber on an inverted microscope (Nikon TE2000-S) equipped with an S-Fluor 20x/0.75 objective. The [Ca^{2+}] was then stepped to 5 mM for 5 min before further increasing it to 10 mM. The cells were then continuously perfused with KRH buffer containing 10 mM $CaCl_2$ at room temperature in the absence and presence of 0.5 mM caffeine, aminophylline, or theophylline. Time-lapse images were captured every ~1.3 sec, during the excitation periods, and analyzed using Compix Inc. Simple PCI 6 software.

[^{3}H]Ryanodine binding—Equilibrium [^{3}H]ryanodine (NEN Life Science) binding to cell lysate was performed as described previously [33]. Briefly, a binding mixture (300 μl) containing 30 μl of cell lysate (3–5 mg/ml), 25 mM Tris/50 mM Hepes (pH 7.4), 5 nM [^{3}H] ryanodine, a protease inhibitor mix, and various concentrations of $CaCl_2$, KCl and 2.5 mM caffeine, aminophylline or theophylline as indicated, was incubated at 37°C for 2.5–3.5 hr. The binding mixture was diluted with 5 ml of ice-cold washing buffer containing 25 mM Tris (pH 8.0), and 250 mM KCl, and immediately filtered through Whatman GF/B filters presoaked with 1% polyethylenimine. The filters were washed four times with 5 ml of ice-cold washing buffer and the radioactivity associated with the filters was determined by liquid scintillation counting. Nonspecific binding was determined by measuring [^{3}H]ryanodine binding in the presence of 50 μM unlabeled ryanodine. All binding assays were done in duplicate. Data shown are mean ± SEM for *n* experiments. Statistical significance was evaluated using the unpaired Student's *t* test. A P value of 0.05 is considered to be statistically significant.

RESULTS

Caffeine induces "quantal" Ca^{2+} release in HEK293 cells expressing RyR2

Partial or quantal Ca^{2+} release in response to incremental concentrations of caffeine has been observed with native cells expressing RyRs and SR vesicles isolated from skeletal muscle [17–20]. To determine whether partial or quantal Ca^{2+} release occurs in a heterologous expression system, we assessed the response of HEK293 cells transfected with mouse RyR2 cDNA to multiple additions of caffeine. As shown in Fig. 1A, the addition of 0.2 mM caffeine induced a transient Ca^{2+} release in HEK293 cells expressing RyR2. In the continued presence of caffeine, a second addition of 0.2 mM caffeine was able to trigger another transient Ca^{2+} release in these cells. This partial Ca^{2+} release was clearly observed even after the 7^{th} consecutive addition of 0.2 mM caffeine, although the amplitude of each Ca^{2+} release was progressively reduced. Hence, partial or quantal Ca^{2+} release also occurs in a heterologous expression system, suggesting that the quantal nature of caffeine-induced Ca^{2+} release reflects an intrinsic property of its activation of RyR2.

The partial Ca^{2+} release induced by a submaximal concentration of caffeine (Fig. 1A) could be due to the opening of a subpopulation of the RyR2 channels. To test this hypothesis, we pretreated HEK293 cells expressing RyR2 with 100μM ryanodine before multiple additions of caffeine. Since ryanodine only binds to the open channel and the binding of ryanodine converts the channel to a fully open state [37,38], the ryanodine-modified channel is no longer sensitive to caffeine. If the first addition of 0.2 mM caffeine only activates a subpopulation of RyR2, one would expect that the ryanodine pretreatment could only modify that subpopulation of RyR2 that was opened by the first addition of caffeine, and that HEK293 cells pretreated with ryanodine would still respond to multiple additions of caffeine, as each addition of caffeine would activate a new subpopulation of channels. In contrast to this prediction, we found that cells pretreated with ryanodine only responded to the first addition of caffeine.

As shown in Fig. 1B, in the absence of caffeine, the addition of ryanodine caused a slow release of Ca^{2+}. This is likely due to the binding of ryanodine to a small population of RyR2 channels that are open under these conditions and consequently an increase in Po of these channels. This slow release of Ca^{2+} would be equilibrated at some point with Ca^{2+} uptake into the endoplasmic reticulum (ER) or Ca^{2+} extrusion into the extracellular space, leading to a steady-state cytosolic Ca^{2+} level corresponding to the plateau in the fluorescent signal. The subsequent addition of 0.2 mM caffeine activated the remaining ryanodine-unmodified RyR2 channels and caused a large Ca^{2+} release. The caffeine-activated channels would then be modified by ryanodine into a fully activated state, leading to a depletion of intracellular Ca^{2+} store. The released Ca^{2+} would be extruded into the extracellular space, resulting in a transient Ca^{2+} release. Importantly, unlike those seen in Fig. 1A, 6 subsequent additions of caffeine yielded little or no Ca^{2+} release. The overall decline of fluorescent signals throughout the recordings is due to quenching of the fluo-3 fluorescent dye by caffeine. This caffeine-dependent quenching can clearly be seen in Fig. 1B, where every addition of caffeine caused an immediate drop in the fluorescent signal, while the fluorescent signals between two additions of caffeine are relatively constant. Furthermore, due to the difference in the release kinetics, the amplitudes of ryanodine-induced Ca^{2+} release and caffeine-induced Ca^{2+} release under these different conditions may not be directly comparable. The difference in the decay kinetics of the caffeine-induced Ca^{2+} transients in the presence and absence of ryanodine is likely the result of the modification of the RyR2 channel by ryanodine. These observations suggest that the first addition of 0.2 mM caffeine was able to open nearly all the RyR2 channels, which were subsequently converted by ryanodine into a fully open state and thus became unresponsive to further additions of caffeine. Hence, the partial or quantal Ca^{2+} release in HEK293 cells transfected with a single class of RyR2 cDNA is unlikely to be due to the existence of different populations of RyR2 with various caffeine sensitivities.

Caffeine reduces the threshold at which spontaneous Ca^{2+}-release occurs

It has been shown that caffeine-induced Ca^{2+} release is dependent on the ER/SR luminal Ca^{2+} concentration [17,21,22]. To further investigate the luminal Ca^{2+} dependence of caffeine activation, we directly monitored the ER luminal Ca^{2+} dynamics in HEK293 cells expressing RyR2 before and after the additions of various concentrations of caffeine using a luminal Ca^{2+} indicator protein (D1ER). HEK293 cells expressing RyR2 were transfected with D1ER, a soluble fluorescence resonance energy transfer (FRET) based Ca^{2+} indicator protein that is expressed within the lumen of the ER due to a KDEL retention motif [34]. As seen in Fig. 2A, in the absence of caffeine and the presence of 2 mM external Ca^{2+}, HEK293 cells expressing RyR2 displayed spontaneous Ca^{2+} release, which is reflected by the transient downward deflections in the FRET signal, similar to results reported previously [39]. It is worth noting that spontaneous Ca^{2+} release occurs when the ER Ca^{2+} reaches a certain level (represented by a dash-line at 0 mM caffeine). We referred to this luminal Ca^{2+} level as the luminal Ca^{2+} threshold at which spontaneous Ca^{2+} release occurs. In the presence of 0.3 mM caffeine, the luminal Ca^{2+} level (represented by a dash-line at 0.3 mM caffeine) at which spontaneous Ca^{2+} release occurred was reduced to 86.3 ± 0.9% (n = 34, $P < 0.001$) of that in the absence of caffeine (Fig. 2B). Similarly, after perfusing the cells with 1 mM caffeine, the luminal Ca^{2+} threshold (represented by a dash-line at 1mM caffeine) at which spontaneous Ca^{2+} release occurred was further reduced to 61.1 ± 1.5% (n = 34, $P < 0.001$) of that in the absence of caffeine (Fig. 2B). Interestingly, even in the presence of 10 mM caffeine, spontaneous Ca^{2+} release in the form Ca^{2+} oscillations still persisted despite a markedly reduced luminal Ca^{2+} threshold (21.4 ± 1.1%, n = 34, $P < 0.001$) (Fig. 2B). This observation indicates that the RyR2 channel is activated only when the luminal Ca^{2+} reaches a threshold level, even in the presence of 10 mM caffeine. On the other hand, the addition of 20μM ryanodine abolished Ca^{2+} oscillations. Ryanodine is known to dramatically sensitize the channel to cytosolic Ca^{2+} activation and convert the channel to a persistent activated state [37,38]. As a result, the ER Ca^{2+} store would be completely depleted in the presence of 10 mM caffeine and 20 μM ryanodine. Taken together, these observations demonstrate that unlike ryanodine, caffeine, even at high concentrations, does not always open the RyR2 channel, and that the action of caffeine is to reduce the luminal Ca^{2+} threshold at which spontaneous Ca^{2+} release occurs. The fact that the amplitude of spontaneous Ca^{2+} oscillations is also reduced as the caffeine concentration is increased further supports this view. This is because a reduced threshold at which spontaneous Ca^{2+} release occurs will reduce the maximal SR Ca^{2+} loading as Ca^{2+} will be released from the SR when it reaches the threshold level. A reduced level of SR Ca^{2+} loading will, in turn, decrease the amount of Ca^{2+} release and thus the amplitude of Ca^{2+} oscillations.

Caffeine preferentially sensitizes the luminal Ca^{2+} activation of RyR2 at low cytosolic Ca^{2+} concentrations

To understand how caffeine reduces the threshold for spontaneous Ca^{2+} release, we assessed the impact of caffeine on single RyR2 channels. As shown in Fig. 3A, a single RyR2 channel exhibited little activity at low luminal (45 nM) and cytosolic (45 nM) Ca^{2+} concentrations. Increasing the luminal Ca^{2+} to 300 μM slightly activated the channel (Fig. 3B). A subsequent addition of 2 mM caffeine to the cytosolic side of the channel markedly increased the channel activity (Fig. 3C). The average Po after the addition of caffeine was 0.094 ± 0.025 in the presence of 300 μM luminal Ca^{2+}, which was significantly greater than that before the addition of caffeine (0.006 ± 0.001) (n = 4) ($P < 0.02$). Importantly, this caffeine activation was dependent on luminal Ca^{2+}. Reducing the luminal Ca^{2+} from 300 μM to ~45 nM decreased the activity of the channel to the basal level with Po of 0.002 ± 0.001 (n = 4) ($P < 0.05$) (Fig. 3D). These data indicate that caffeine preferentially potentiates the luminal Ca^{2+} response of RyR2 at low cytosolic Ca^{2+} levels.

To further characterize the luminal and cytosolic Ca^{2+} dependence of caffeine activation, we determined the effect of caffeine on the sensitivity of single RyR2 channels to activation by luminal or cytosolic Ca^{2+}. As shown in Fig. 3E, in the absence of caffeine, single RyR2 channels were activated by luminal Ca^{2+} with a threshold of ~3 mM (n =8), similar to that shown previously [40]. In the presence of 2 mM caffeine, single RyR2 channels were much more sensitive to activation by luminal Ca^{2+}. Caffeine markedly reduced the threshold for luminal Ca^{2+} activation to ~0.1 mM (n =5) (Fig. 3E). On the other hand, caffeine (2 mM) only slightly reduced the EC_{50} for activation of the RyR2 channel by cytosolic Ca^{2+} from 0.31 μM (n=5) to 0.17 μM (n = 7) (Fig. 3F). It should be noted that caffeine has little effect on the threshold for activation by cytosolic Ca^{2+} (~100 nM in the presence and absence of caffeine) (Fig. 3F). These data suggest that at low cytosolic and high luminal Ca^{2+} concentrations, a condition resembling that seen in resting cells, caffeine preferentially sensitizes the RyR2 channel to activation by luminal Ca^{2+}.

Other methylxanthines also potentiate the response of RyR2 to luminal Ca^{2+}

Caffeine is a member of the methylxanthine family of compounds. To determine whether other methylxanthines also preferentially sensitize the luminal Ca^{2+} activation of RyR2, we assessed the effect of the clinically relevant methylxanthines aminophylline and theophylline on the luminal Ca^{2+} response of single RyR2 channels. As shown in Fig. 4A, aminophylline (2 mM) markedly activated the RyR2 channel in the presence of 45 nM cytosolic Ca^{2+} and 300 μM luminal Ca^{2+}. The average Po was significantly increased after the addition of aminophylline, increasing from 0.015 ± 0.008 to 0.102 ± 0.026 (n = 6, $P < 0.02$). As seen with caffeine, this aminophylline-induced enhancement of channel activity depends on luminal Ca^{2+}. Reducing the luminal Ca^{2+} concentration to 45 nM abolished the effect of aminophylline by decreasing the Po to 0.002 ± 0.0003 (n = 6, $P < 0.02$) (Fig. 4Ad). Fig. 4B shows the impact of theophylline. Again and as with caffeine and aminophylline, theophylline activated single RyR2 channels in a luminal Ca^{2+} dependent manner. The average Po was significantly augmented by theophylline from 0.009 ± 0.003 to 0.241 ± 0.03 (n = 5, $P < 0.01$), and was reduced to the basal level (Po = 0.005 ± 0.003, n = 5, $P < 0.01$) when luminal Ca^{2+} was removed. Therefore, like caffeine, aminophylline and theophylline also preferentially sensitize the RyR2 channel to luminal Ca^{2+} activation at low cytosolic Ca^{2+} concentrations.

Methylxanthines increase the propensity for spontaneous Ca^{2+} release in HEK293 cells expressing RyR2

Considering their enhancement of luminal Ca^{2+} activation, which is closely linked to spontaneous Ca^{2+} release, it follows that methylxanthines should likewise increase spontaneous Ca^{2+} release. To test this possibility, we determined the impact of caffeine, aminophylline, and theophylline on spontaneous Ca^{2+} release in HEK293 cells expressing RyR2. Spontaneous Ca^{2+} release was induced in these cells by elevating the external Ca^{2+} concentrations in the absence or presence of caffeine (0.3 mM), aminophylline (0.3 mM), or theophylline (0.3 mM), and was monitored using single cell Ca^{2+} imaging and the fluorescent Ca^{2+} indicator, fura-2 AM. Analyzing the fraction of cells that displayed spontaneous Ca^{2+} release in the form of Ca^{2+} oscillations at each external Ca^{2+} concentration showed that all three methylxanthines increased the propensity for spontaneous Ca^{2+} release (Fig. 5A). For instance, the fraction of oscillating cells was 49.1 ± 4.4% (mean ± SEM) in the presence of 0.3 mM caffeine, 44.6 ± 4.6% in 0.3 mM aminophylline, or 49.3 ± 2.6% in 0.3 mM theophylline, significantly higher than that in the absence of methylxanthines (control, 14.9 ± 5.6%) ($P < 0.003$). The frequency of spontaneous Ca^{2+} oscillations was increased to 143.9 ± 4.1% by caffeine ($P < 0.001$), 145.1 ± 4.4% by aminophylline ($P < 0.005$), and 166.7 ± 10.2% by theophylline ($P < 0.001$). The store level was reduced to 74.7 ± 2.0% by caffeine ($P < 0.001$), 78.0 ± 7.0% by aminophylline ($P < 0.05$), and 69.9 ± 3.0% by theophylline ($P < 0.001$) (Fig. 5B). The number of HEK293 cells used for analyses was 377 for the control, 507 for caffeine,

417 for aminophylline, and 370 for theophylline. These results are consistent with the notion that methylxanthines reduce the threshold for spontaneous Ca^{2+} release.

Methylxanthines enhance the propensity for spontaneous Ca^{2+} release in isolated rat cardiac myocytes

Methylxanthines have been shown to promote cardiac arrhythmias under some conditions [7–14]. It is possible that their pro-arrhythmic nature is related to their enhancing effect on spontaneous Ca^{2+} release. To test this possibility, we determined whether methylxanthines are able to enhance spontaneous Ca^{2+} release in cardiac myocytes. As with HEK293 cells, spontaneous Ca^{2+} release was induced in rat cardiac cells by increasing the external Ca^{2+} concentration and was monitored using single cell Ca^{2+} imaging and the Ca^{2+} indicator, fluo-4-AM. As shown in Fig. 6, spontaneous Ca^{2+} release in the form of Ca^{2+} waves was observed in cardiac cells in the presence of 10 mM external Ca^{2+}. The addition of caffeine (0.5 mM) (Fig. 6A), aminophylline (0.5 mM) (Fig. 6B), or theophylline (0.5 mM) (Fig. 6C) increased the frequency and decreased the amplitude of spontaneous Ca^{2+} waves. The average frequency was 126.9 ± 2.6% of control ($P < 0.0001$) for caffeine, 146.1 ± 15.5% ($P < 0.01$) for aminophylline, and 108.2 ± 6.4 ($P < 0.01$) for theophylline. The average amplitude was 59.7 ± 6.0% of control ($P < 0.0001$) for caffeine, 69.8 ± 6.1% ($P < 0.0001$) for aminophylline, and 66.9 ± 5.3% ($P < 0.001$) for theophylline. The number of cardiac cells used for analyses was 9 for caffeine, 10 for aminophylline, and 16 for theophylline. These data indicate that, as with HEK293 cells, methylxanthines increase the frequency and decrease the amplitude of Ca^{2+} waves, which is consistent with the hypothesis that methylxanthines reduce the threshold for spontaneous Ca^{2+} release in cardiomyocytes.

Methylxanthines increase the basal activity of RyR2, resembling the effect of disease-linked RyR2 mutations

We have shown that disease-linked RyR2 mutations enhance the sensitivity of the channel to luminal Ca^{2+} activation and reduce the threshold for spontaneous Ca^{2+} release. These are the same properties shared by methylxanthines. We have also shown that a number of RyR2 mutations linked to cardiac arrhythmias display an increased activity at high KCl concentrations in the near absence of Ca^{2+} [2,3]. This basal activity likely reflects the stability of the closed state of the channel. To determine whether methylxanthines have any effects on the basal activity of the channel, we performed [^{3}H]ryanodine binding assays. Fig. 7 shows that all three methylxanthines significantly increase the basal activity of the channel. For instance, the basal level of [^{3}H]ryanodine binding at 800 mM KCl was significantly increased from 7.9 ± 1.0 % (control) to 20.2 ± 1.5 % ($n = 3$, $P < 0.05$) by caffeine (2.5 mM), 22.5 ± 0.9 % ($n = 3$, $p < 0.001$) by aminophylline (2.5 mM), or 23.9 ± 0.4 % ($n = 3$, $P < 0.001$) by theophylline (2.5 mM). These data suggest that methylxanthines destabilize the closed state of the channel in a manner similar to those RyR2 mutations known to cause cardiac arrhythmias.

DISCUSSION

Caffeine has widely been used as a probe to study the mechanism of RyR2-associated catecholamine-induced cardiac arrhythmias, but the molecular basis of caffeine activation of RyR2 is unclear. Based on our recent finding that disease-linked RyR2 mutations enhance the luminal Ca^{2+} activation of RyR2 and reduce the threshold for spontaneous Ca^{2+} release or store-overload-induced Ca^{2+} release (SOICR) [2,3], we propose that caffeine promotes catecholamine-induced arrhythmias by sensitizing the RyR2 channel to activation by luminal Ca^{2+}. In support of this hypothesis, we have found that caffeine reduces the threshold for luminal, but not cytosolic, Ca^{2+} activation and the threshold for spontaneous Ca^{2+} release. In addition, we have found that as with caffeine, two other methylxanthine compounds, aminophylline and theophylline, also potentiate the channel to luminal Ca^{2+} activation and

increase the propensity for spontaneous Ca^{2+} release. Our results suggest that altered luminal Ca^{2+} activation of RyR2 underlies a common arrhythmogenic mechanism of inherited and drug-induced arrhythmias associated with RyR2.

How does caffeine trigger Ca^{2+} release: cytosolic Ca^{2+} activation vs luminal Ca^{2+} activation?

Although caffeine has been widely used as an agonist of RyRs to induce Ca^{2+} release from intracellular stores in various muscle and non-muscle cells, it is not clear how caffeine triggers the opening of RyRs and consequently Ca^{2+} release. It is commonly believed that caffeine triggers Ca^{2+} release by sensitizing the channel to cytosolic Ca^{2+} activation [27,28]. In other words, the RyR channels are activated by the resting cytosolic Ca^{2+} upon the addition of caffeine. However, if the activation of RyRs by caffeine were mediated by the resting cytosolic Ca^{2+}, one would expect that in a steady state the activation of RyRs would be maintained in the continuing presence of caffeine, as the resting cytosolic Ca^{2+} after the addition of caffeine would be similar to or greater than that before caffeine stimulation. Such a sustained activation of RyRs by cytosolic Ca^{2+} in the presence of caffeine would lead to Ca^{2+} release in an all-or-none fashion and deplete the intracellular Ca^{2+} stores. In contrast to this prediction, caffeine at submaximal concentrations is able to repetitively trigger partial Ca^{2+} release in a number of cell types, a phenomenon known as “quantal” Ca^{2+} release [16–20]. Consistent with these observations, we found that HEK293 cells expressing recombinant RyR2 also displayed partial or quantal Ca^{2+} release in response to repetitive additions of caffeine (0.2 mM) (Fig. 1A). On the other hand, when these RyR2-expressing HEK293 cells were pretreated with ryanodine, which is known to drastically (>1,000 fold) sensitize the RyR2 channel to activation by cytosolic Ca^{2+} [37], they only responded to the first addition of caffeine, but not to subsequent additions, in an all-or-none manner (Fig. 1B). It is difficult to reconcile these observations with the idea that caffeine-induced Ca^{2+} release is mediated via the activation of the RyR channel by cytosolic Ca^{2+}.

However, the RyR channel can also be activated by luminal Ca^{2+}. So if, alternatively, caffeine induces intracellular Ca^{2+} release by sensitizing the channel to activation by luminal Ca^{2+}, this apparent paradox would be resolved. In this scheme, upon the addition of caffeine the RyR channel is opened by the store luminal Ca^{2+}. Therefore, one would expect that caffeine-induced Ca^{2+} release would be partial and dependent on luminal Ca^{2+}. This is because a certain concentration of caffeine will sensitize the channel to activation by a certain level of luminal Ca^{2+}. As a result of Ca^{2+} release, the store luminal Ca^{2+} level will decrease. The activation of the channel by luminal Ca^{2+} and thus Ca^{2+} release would cease when the luminal Ca^{2+} concentration falls below a threshold level. However, upon increasing the caffeine concentration by a subsequent addition of caffeine, the channel will be further sensitized and again activated by the luminal Ca^{2+} until the luminal Ca^{2+} level decreases to a new steady state. Indeed, it has been shown that caffeine decreased the ER luminal Ca^{2+} level in a concentration dependent manner. The steady state luminal Ca^{2+} level was progressively decreased with increased concentrations of caffeine [17]. Interestingly, caffeine failed to trigger Ca^{2+} release if the luminal Ca^{2+} concentration was lower than the steady state level corresponding to that concentration of caffeine [17]. Similarly, caffeine was found to be unable to trigger Ca^{2+} release from SR membrane vesicles that were loaded with Ca^{2+} below a threshold level [24–26]. The failure of caffeine to trigger Ca^{2+} release in the presence of a normal resting cytosolic Ca^{2+}, but a reduced luminal Ca^{2+} level, further indicates that luminal Ca^{2+}, but not cytosolic Ca^{2+}, is the major mediator of caffeine induced-Ca^{2+} release.

We have previously shown that when the luminal Ca^{2+} level reaches a threshold level, spontaneous Ca^{2+} release occurs in HEK293 cells expressing RyR2 [2,3,39]. In the present study, we investigated the impact of caffeine on the threshold for spontaneous Ca^{2+} release. We found that in the presence of 0.3 mM caffeine, spontaneous Ca^{2+} release occurred at a

lower luminal Ca^{2+} level compared to that in the absence of caffeine (Fig. 2). The threshold for spontaneous Ca^{2+} release was further reduced after the addition of 1 mM caffeine. Interestingly, despite the markedly reduced threshold and amplitude, spontaneous Ca^{2+} release in the form of Ca^{2+} oscillations persisted even in the presence of 10 mM caffeine, and was only abolished by the addition of ryanodine. These observations indicate that, unlike ryanodine, caffeine, even at high concentrations, does not cause a sustained activation of the RyR2 channel. Caffeine only activates the channel when the luminal Ca^{2+} reaches a certain threshold. Hence, the continued presence of caffeine is not always associated with an increased Po of RyR2. Collectively, the action of caffeine is to reduce the threshold for luminal Ca^{2+} activation of RyR2, but not necessarily to increase the Po of RyR2.

How does caffeine reduce the threshold for spontaneous Ca^{2+} release?

Early studies on the effect of caffeine on the cytosolic Ca^{2+} dependent activation of single RyR2 channels were performed in planar lipid bilayers using Ca^{2+} as the charge carrier. These studies demonstrated that caffeine markedly enhanced the Po of single RyR2 channels in the presence of submicromolar concentrations of cytosolic Ca^{2+} and millimolar concentrations of luminal Ca^{2+} [27,28]. Since both cytosolic and luminal Ca^{2+} were present, it is not clear whether the activation of RyR2 by caffeine under these conditions resulted from the sensitization of the channel to cytosolic Ca^{2+} or luminal Ca^{2+} or both. To distinguish these possibilities, we determined the impact of caffeine on the cytosolic Ca^{2+} dependence of activation in the near absence of luminal Ca^{2+} or on the luminal Ca^{2+} dependence of activation in the near absence of cytosolic Ca^{2+}. We found that at low concentrations of cytosolic Ca^{2+}, caffeine markedly reduced the threshold for luminal Ca^{2+} activation, whereas, at low concentrations of luminal Ca^{2+}, caffeine had little effect on the threshold for cytosolic Ca^{2+} activation (Fig. 3). These data indicate that at submicromolar levels of cytosolic Ca^{2+} and millimolar levels of luminal Ca^{2+}, caffeine preferentially sensitizes the channel to luminal Ca^{2+} activation. Consistent with this view, it has recently been shown that in the presence of 100 nM cytosolic Ca^{2+} caffeine readily activated single RyR2 channels using Ca^{2+} as the charge carrier, but had little effect on single RyR2 channel when Ba^{2+} was used as the charge carrier [29]. These observations demonstrate that the activation of single RyR2 channels by caffeine at submicromolar levels of cytosolic Ca^{2+} is dependent on the presence of luminal Ca^{2+}. Taken together, these single channel studies indicate that caffeine reduces the threshold for spontaneous Ca^{2+} release by decreasing the threshold for luminal Ca^{2+} activation of the RyR2 channel.

Caffeine mimics the actions of disease-linked RyR2 mutations

We have recently demonstrated that augmented luminal, but not cytosolic, Ca^{2+} activation of RyR2 is a common feature of a number of disease-linked RyR2 mutations [2,3,39]. Our observation that caffeine reduces the threshold for luminal, but not cytosolic, Ca^{2+} activation of single RyR2 channels indicates that caffeine mimics the effect of disease-linked RyR2 mutations. Indeed, as with disease-linked RyR2 mutations, caffeine at low concentrations reduces the threshold for spontaneous Ca^{2+} release, but has no sustained effect on Ca^{2+}-induced Ca^{2+} release (CICR) [2,7,15,23]. Patients with CPVT RyR2 mutations show no structural or functional cardiac abnormalities at rest, but are predisposed to catecholamine-induced cardiac arrhythmias [1]. Similarly, caffeine alone does not induce spontaneous Ca^{2+} release, DADs, or cardiac arrhythmia, but promotes catecholamine-induced triggered activities [7–10]. Furthermore, as with disease-linked RyR2 mutations, caffeine increases the basal level of [^{3}H]ryanodine binding (Fig. 7). Hence, caffeine and disease-linked RyR2 mutations alter the properties of the channel in the same manner.

Other methylxanthine compounds aminophylline and theophylline have been used clinically for the treatment of pulmonary diseases. However, their use has recently been limited due

largely to their pro-arrhythmic properties [13,14,41,42]. We have found that, like caffeine, both aminophylline and theophylline preferentially potentiate luminal Ca^{2+} activation of RyR2, reduce the threshold for spontaneous Ca^{2+} release, and increase the basal activity of RyR2 (Figs. 4–7). These effects likely underlie the arrhythmogenic mechanism of these methylxanthines.

Luminal Ca^{2+} activation of RyR, a common target for regulation

It has been proposed that under normal SR Ca^{2+} loading the sensitivity of RyR2 to cytosolic Ca^{2+} activation is extremely low at resting cytosolic Ca^{2+} [43]. However, during SR Ca^{2+} overload, RyR2 becomes much more sensitive to activation [44]. This observation suggests that RyR2 is readily regulated by luminal Ca^{2+}. We have recently shown that the activation of the channel by luminal Ca^{2+} is distinct from its activation by cytosolic Ca^{2+} [45]. An increased body of evidence indicates that luminal Ca^{2+} activation of RyR2 is an important target for regulation by endogenous and exogenous effectors [2,45–47]. In addition to methylxanthines, a number of drugs, such as sulmazole, thymol, doxorubicin, ethanol, and shingosyl phosphorylcholine have been found to induce partial or quantal Ca^{2+} release from RyR-gated intracellular Ca^{2+} stores [18]. It is possible that these drugs also induce quantal Ca^{2+} release by sensitizing the channel to activation by luminal Ca^{2+}. Hence, modulating the sensitivity of the channel to luminal Ca^{2+} activation may be a common mechanism of regulation of RyRs.

Mechanisms underlying spontaneous Ca^{2+} release

The phenomenon of spontaneous SR Ca^{2+} release in cardiac cells has been known for decades. However, the exact mechanism underlying this process has not been completely defined. In early studies using skinned cardiac cells, Fabiato demonstrated that there are two kinds of Ca^{2+}-induced release of Ca^{2+} from the SR [48,49]. One is termed Ca^{2+}-induced Ca^{2+} release (CICR), which has a time and Ca^{2+} dependence of activation and inactivation by cytosolic Ca^{2+}. The other is known as spontaneous SR Ca^{2+} release, which has no time dependence of activation and is not inactivated by high concentrations of cytosolic Ca^{2+}, but requires SR Ca^{2+} overload. A key feature of the activation of SR Ca^{2+} release by cytosolic Ca^{2+} or CICR is its dependence on the rate of increase in the cytosolic Ca^{2+} concentration. A high rate of increase in the cytosolic Ca^{2+} concentration triggers CICR, whereas a low rate of increase in the cytosolic Ca^{2+} concentration inhibits CICR and causes SR Ca^{2+} accumulation. Importantly, when the SR Ca^{2+} content has accumulated to a critical level, spontaneous SR Ca^{2+} release occurs [48,49].

Consistent with Fabiato's early observations in skinned cardiac cells, Eisner and his colleagues have shown in intact cardiac myocytes that elevated external Ca^{2+} concentrations cause a slight increase in the cytosolic Ca^{2+} level and lead to SR Ca^{2+} accumulation [50]. Similarly, they found that when the SR Ca^{2+} content reaches a threshold level, spontaneous SR Ca^{2+} release occurs in the absence of membrane depolarization. Spontaneous SR Ca^{2+} release was not observed when the SR Ca^{2+} content was below this threshold level. After spontaneous SR Ca^{2+} release occurred, further elevation of external Ca^{2+} concentration increased the frequency of spontaneous Ca^{2+} release, but had little effect on its amplitude. In other words, the spontaneous SR Ca^{2+} release induced by elevated external Ca^{2+} concentrations occurs only when the SR Ca^{2+} content reaches a threshold level. Although the cytosolic Ca^{2+} concentration also increases slightly during external Ca^{2+} elevation, the rate of increase in the cytosolic Ca^{2+} concentration may be too slow to trigger CICR. Hence, spontaneous SR Ca^{2+} release induced by elevated external Ca^{2+} concentrations is likely the result of SR Ca^{2+} overload, rather than the consequence of cytosolic Ca^{2+} activation or CICR.

We have previously demonstrated that elevating the external Ca^{2+} concentration also increases the store Ca^{2+} content in HEK293 cells expressing RyR2 [2,3], similar to those observed in

cardiac cells. More importantly, and as with cardiac cells, when the store Ca^{2+} reaches a threshold level, spontaneous Ca^{2+} oscillations occur in these RyR2-expressing HEK293 cells, but not in RyR2-non-expressing cells. Recently, using an ER Ca^{2+} sensor, D1ER, we were able to directly show that spontaneous Ca^{2+} release or Ca^{2+} oscillations occur in HEK293 cells when the ER Ca^{2+} reaches a threshold level [39]. Therefore, as with cardiac cells, the spontaneous Ca^{2+} release observed in HEK293 cells is likely the result of store Ca^{2+} overload.

What then is the role of cytosolic Ca^{2+} activation or CICR in spontaneous Ca^{2+} release induced by elevated external Ca^{2+}? We have recently demonstrated that a disease-associated RyR2 mutation, A4860G, abolishes the luminal Ca^{2+} activation of RyR2, but has little effect on the sensitivity of the channel to activation by cytosolic Ca^{2+} [45]. Importantly, this A4860G mutation also abolishes spontaneous Ca^{2+} oscillations in HEK293 cells, despite its normal sensitivity to cytosolic Ca^{2+} activation. These observations indicate that spontaneous Ca^{2+} release is closely linked to the luminal, but not the cytosolic, Ca^{2+} activation of the RyR2 channel, which is consistent with the fact that spontaneous Ca^{2+} release occurs only when the SR Ca^{2+} content reaches a threshold level. Based on these recent observations and those of previous studies, it is likely that spontaneous Ca^{2+} release is initiated by the luminal Ca^{2+} activation of the RyR2 channel. However, since CICR is known to be involved in the propagation of Ca^{2+} waves, spontaneous SR Ca^{2+} release in the form of propagating Ca^{2+} waves is likely the combined product of spontaneous Ca^{2+} release and CICR.

Molecular basis of luminal Ca^{2+} regulation of RyR2

It has been proposed that luminal Ca^{2+} activates RyRs by passing through the open channel and acting on the cytosolic Ca^{2+} activation site (a “feed-through” hypothesis) [31,51]. However, Gyorke *et al*. and Ching *et al*. found that RyR2 could still be activated by luminal Ca^{2+} in the absence of luminal-to-cytosolic Ca^{2+} flux [52,53]. Furthermore, the application of trypsin to the luminal side of the RyR2 channel diminishes luminal Ca^{2+} activation, but not Ca^{2+} fluxes, arguing against the “feed-through” mechanism and suggesting the existence of a luminal Ca^{2+} activation site distinct from the cytosolic Ca^{2+} activation site [53]. Recently, a third model incorporating both the feed-through and true luminal Ca^{2+} activation mechanisms, called the luminal-triggered Ca^{2+} feed-through mechanism, has been proposed. In this model, luminal-to-cytosolic Ca^{2+} flux is required for a full activation of the channel by luminal Ca^{2+} [54]. However, we have recently shown that elevating the luminal Ca^{2+} concentration to 50 mM did not activate the A4860G mutant channel, despite the presence of luminal-to-cytosolic Ca^{2+} flux and the normal activation of the channel by cytosolic Ca^{2+}. These observations indicate that luminal-to-cytosolic Ca^{2+} flux does not activate the RyR2 channel, and suggest that the activation of RyR2 by luminal Ca^{2+} is mediated by a luminal Ca^{2+} sensor.

The identity of this putative luminal Ca^{2+} sensor is also controversial. It has been proposed that calsequestrin, a low affinity, high capacity SR Ca^{2+} binding protein, acts as a luminal Ca^{2+} sensor and is responsible for the activation of RyR2 by luminal Ca^{2+} [55]. According to this theory, the complex of calsequestrin, triadin and junctin confers the sensitivity of RyR2 to luminal Ca^{2+}. At low concentrations of SR luminal Ca^{2+}, calsequestrin binds to the triadin/junctin/RyR2 complex in a Ca^{2+}-sensitive manner and suppresses the stimulatory effect of triadin/junctin on the RyR2 channel. At high SR luminal Ca^{2+} concentrations, calsequestrin dissociates from triadin/junctin/RyR2, so that triadin/junctin activates RyR2 in the absence of calsequestrin [55]. Hence, calsequestrin, by virtue of its Ca^{2+} dependent association with and dissociation from the triadin/junctin/RyR2 complex, servers as a luminal Ca^{2+} sensor for the luminal Ca^{2+} regulation of RyR2.

Recently, Qin et al. have proposed that RyR2 is regulated by luminal Ca^{2+} through a calsequestrin-independent and a calsequestrin-dependent mechanism [56]. Different from the mechanism proposed by Gyorke et al., the calsequestrin-dependent mechanism proposed by

Qin et al. does not involve the association or dissociation of calsequestrin. Instead, the Ca^{2+}-sensitivity of the interaction between a calsequestrin monomer and the triadin/junctin/RyR2 complex is the key in conferring the responsiveness of RyR2 to luminal Ca^{2+} activation. The removal of calsequestrin from the triadin/junctin/RyR2 complex completely abolishes the luminal Ca^{2+} response of RyR2, but does not lead to the activation of RyR2 by luminal Ca^{2+}, as would be expected based on the mechanism proposed by Gyorke et al. [55]. Thus, exactly how calsequestrin is involved in the regulation of RyR2 by luminal Ca^{2+} is unclear.

The view that calsequestrin serves as the luminal Ca^{2+} sensor for RyR2 is also apparently inconsistent with the observation that purified native RyRs remain sensitive to luminal Ca^{2+} activation [31,40,45,57]. Moreover, recent studies have shown that SR Ca^{2+} release in cardiac myoctyes isolated from calsequestrin knock-out mice remains steeply nonlinear with increasing SR Ca^{2+} content, indicating that the RyR2 channel can sense luminal Ca^{2+} in the absence of calsequestrin [58]. Another important observation is that calsequestrin knockout cardiac myocytes display largely unaltered SR Ca^{2+} release and SR Ca^{2+} content under basal conditions, suggesting that calsequestrin does not play an essential role in modulating the gating of RyR2 and SR Ca^{2+} leak at rest or at low SR Ca^{2+} concentrations [58]. These observations have led to the conclusion that calsequestrin, although it may modulate SR Ca^{2+} release, is not required for luminal Ca^{2+} sensing [58]. Consistent with this finding, we found that recombinant RyR2 expressed in HEK293 cells, which lack calsequestrin, is activated by luminal Ca^{2+} [40,45]. The reasons for these apparently controversial findings regarding the role of calsequestrin in the luminal Ca^{2+} regulation of RyR2 from different groups are not clear and further studies are needed.

Summary

In summary, our data demonstrate that caffeine triggers Ca^{2+} release by reducing the threshold for luminal, but not cytosolic, Ca^{2+} activation of the RyR2 channel. Unlike ryanodine, which induces a full activation of the channel, caffeine, even at high concentrations, does not always hold the channel in the open state. Rather, the action of caffeine is to reduce the luminal Ca^{2+} threshold at which spontaneous Ca^{2+} release occurs. As with caffeine, the clinically relevant methylxanthines aminophylline and theophylline preferentially potentiate luminal Ca^{2+} activation, reduce the threshold for spontaneous Ca^{2+} release, and increase the basal channel activity, mimicking the actions of disease-linked RyR2 mutations.

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ABREVIATIONS USED

RyR2
: Cardiac Ryanodine Receptor

SOICR
: Store Overload Induced Ca^{2+} Release

SR
: Sarcoplasmic Reticulum

DAD
: Delayed Afterdeplolarization

CPVT

Catecholaminergic Polymorphic Ventricular Tachycardia

ARVD2

Arrhythmogenic Right Ventricular Cardiomyopathy type 2

FRET

Fluorescence resonance energy transfer

HEK293

Human Embryonic Kidney Cells

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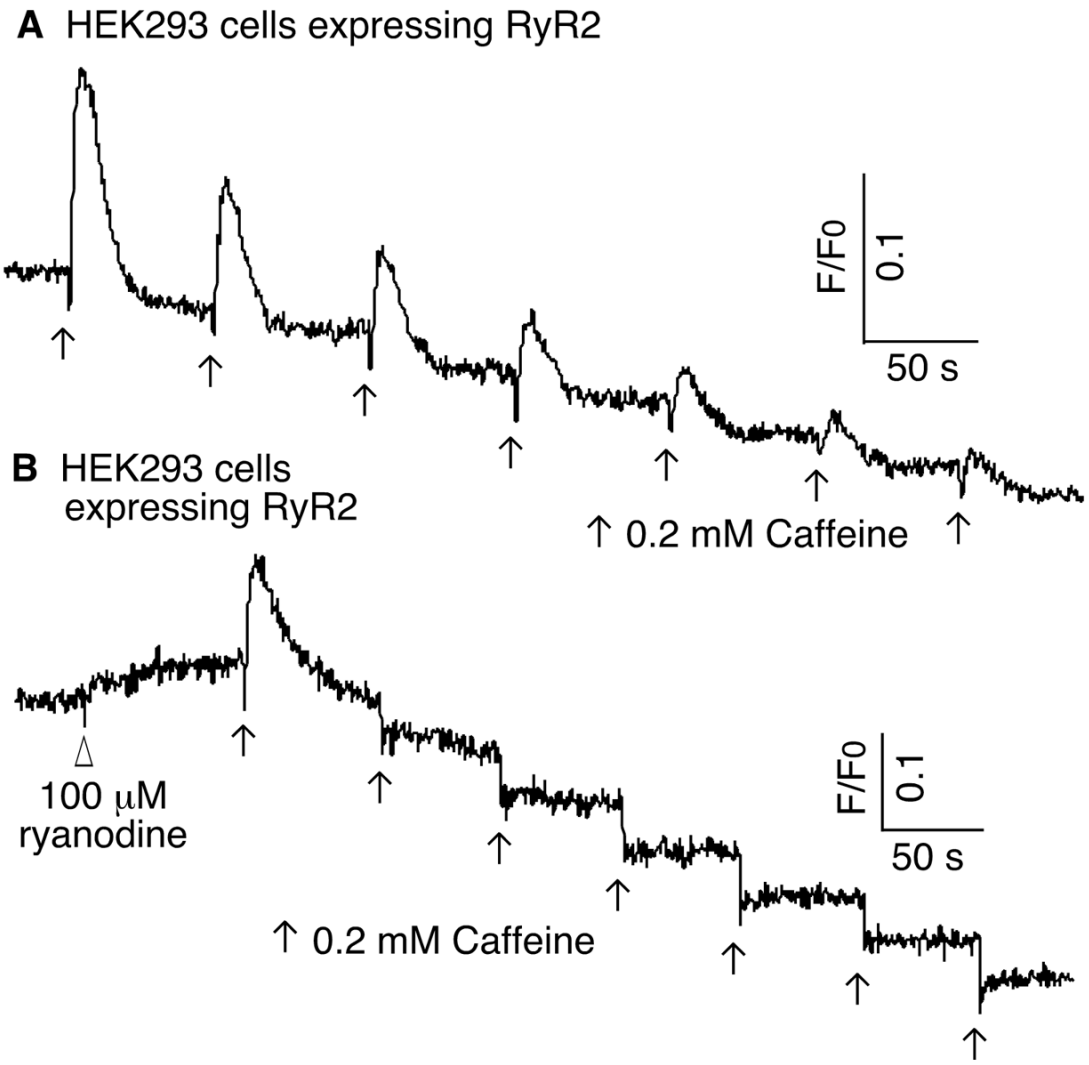

Fig. 1. Caffeine-induced "quantal" Ca^{2+} release in HEK293 cells expressing RyR2

HEK293 cells expressing RyR2 were loaded with 5 μM fluo-3 AM. (*A*) The fluorescent intensity of the cells was monitored continuously before and after repeated additions of 0.2 mM caffeine. (*B*) The fluorescent signals from the fluo-3-loaded cells were measured before and after the addition of ryanodine (100 μM) followed by the repeated additions of caffeine (0.2 mM). Traces shown are from representative experiments that have been repeated three times with similar results.

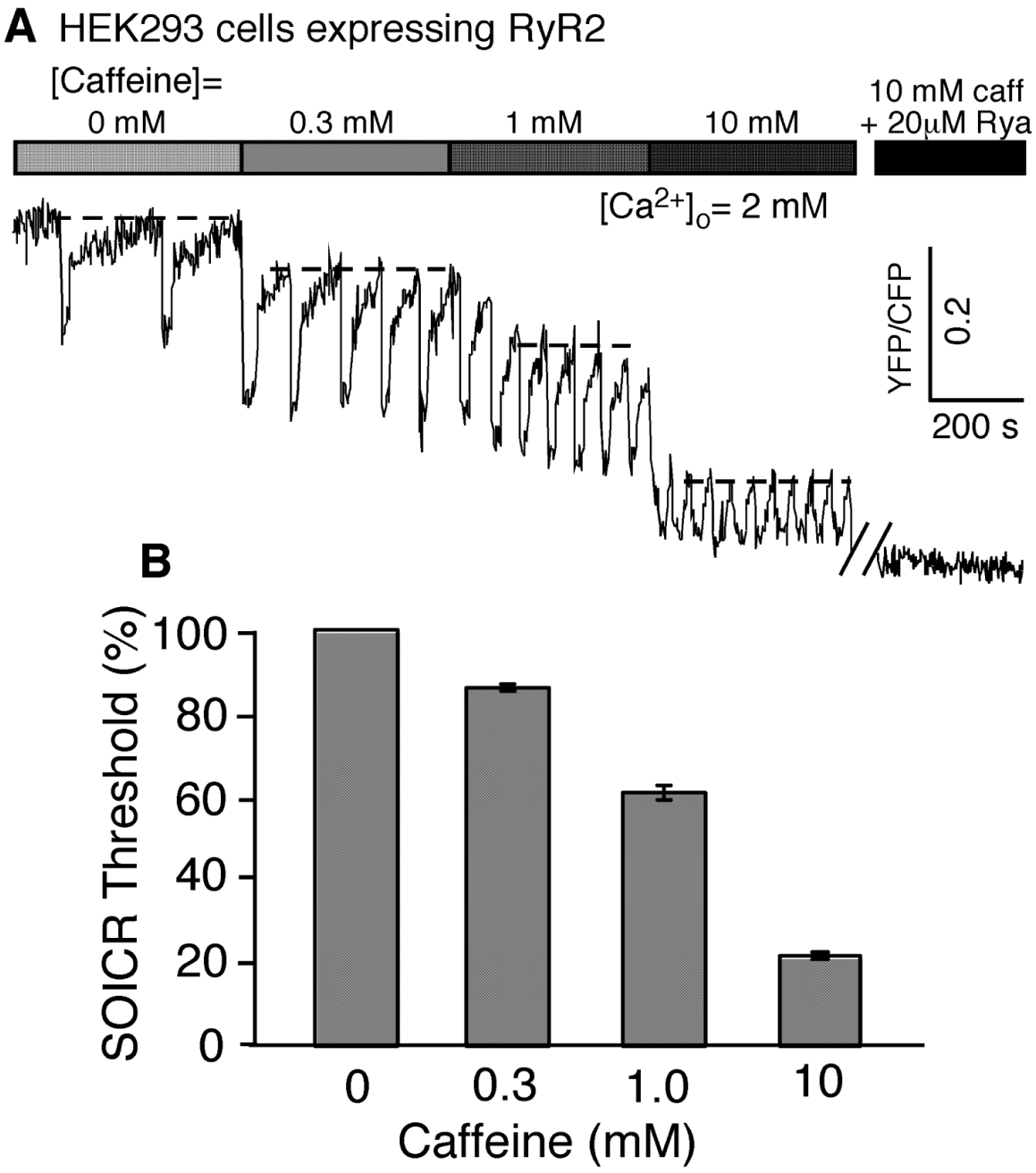

Fig. 2. Caffeine reduces the luminal Ca^{2+} threshold level at which spontaneous Ca^{2+} release occurs

HEK293 cells expressing RyR2 were grown on glass coverslips. Cells were transfected with D1ER cDNA 48 hr before imaging and RyR2 expression was induced 24 hr before imaging. The cells were perfused with KRH buffer containing 2 mM Ca^{2+} and 0, 0.3, 1 or 10 mM caffeine with or without 20μM ryanodine. (A) A representative trace captured using single cell imaging. The dash-lines illustrate the relative luminal Ca^{2+} threshold for spontaneous Ca^{2+} release at each concentration of caffeine. (B) The relative luminal Ca^{2+} threshold for spontaneous Ca^{2+} release at various caffeine concentrations. The threshold for spontaneous Ca^{2+} release is expressed as a percentage of the threshold in the absence of caffeine. Data represents the mean ± SEM of 34 cells from 3 separate experiments.

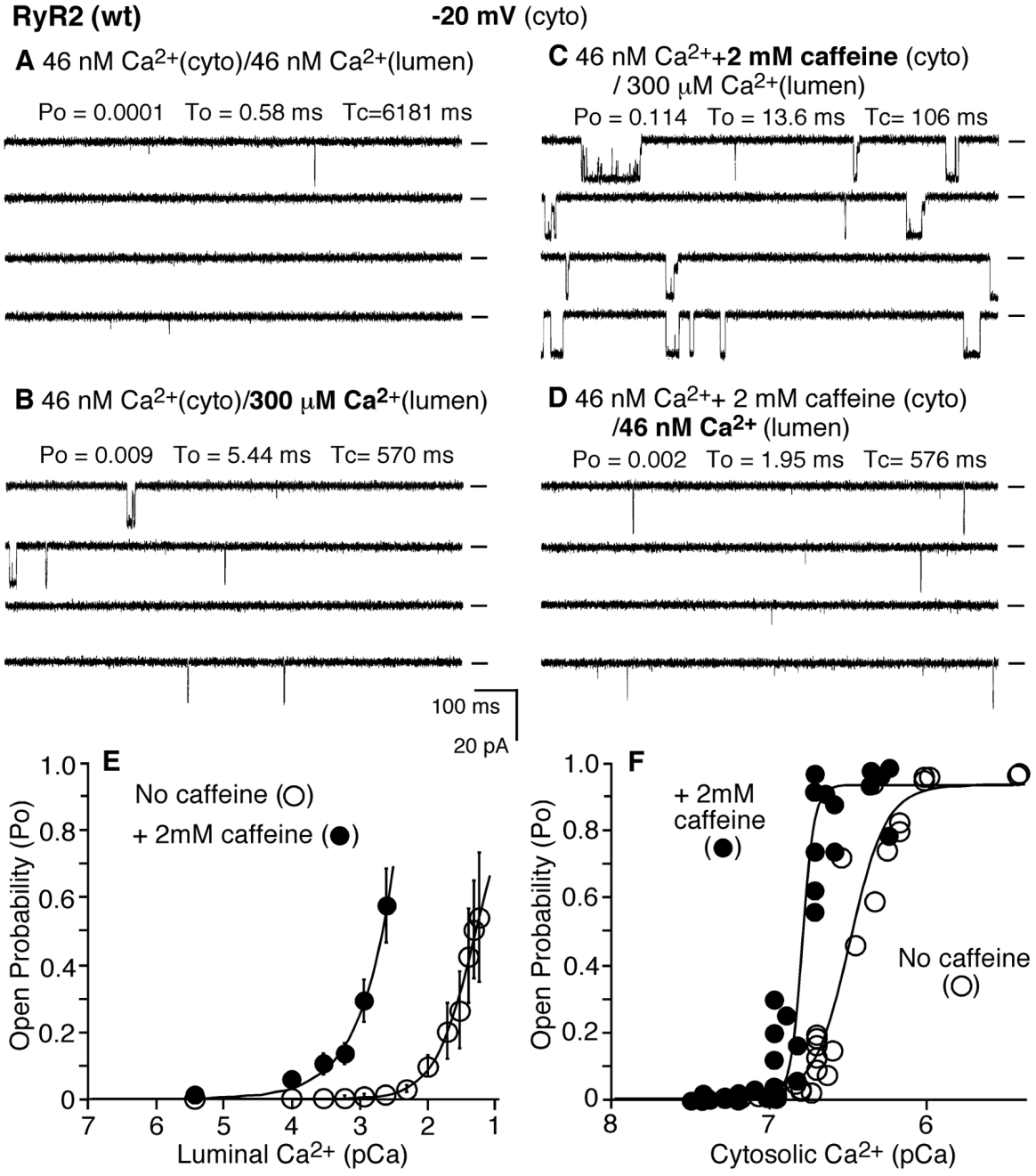

Fig. 3. Caffeine enhances the response of single RyR2 channels to luminal Ca^{2+} activation

Single-channel activities of RyR2 were recorded in a symmetrical recording solution containing 250 mM KCl and 25mM HEPES (pH 7.4) at a holding potential of −20 mV. EGTA was added to either the *cis* or *trans* chamber to determine the orientation of the incorporated channel. The side of the channel to which an addition of EGTA inhibited the activity of the incorporated channel presumably corresponds to the cytosolic face. The Ca^{2+} concentration on both the cytosolic and luminal sides of the incorporated channel was first adjusted to ~46 nM (*A*). The channel was activated by 300 μM luminal Ca^{2+} (*B*). Caffeine was then added to the cytosolic side of the channel in the presence of 300 μM luminal Ca^{2+} (*C*), followed by a decrease of luminal Ca^{2+} to ~46 nM (*D*). Openings are downward. Open probability (*Po*),

arithmetic mean open time (*To*), and arithmetic mean closed time (*Tc*) are indicated at the top of each panel. A short line to the right of each current trace indicates the baseline. A continuous recording is shown. The average recording time for each condition shown in panels A–D from 4 channels is 103 s. The relationship between Po and luminal Ca^{2+} concentration is shown in E, and the relationship between Po and cytosolic Ca^{2+} concentration is shown in F. Data points shown in E are means ± SEM from 5 RyR2 channels in the presence of 2 mM caffeine (solid circles) and 8 RyR2 channels in the absence of caffeine (open circles), and those shown in F are individual measurements obtained from 7 RyR2 channels in the presence of 2 mM caffeine (solid circles) and 5 RyR2 channels in the absence of caffeine. The average recording time is 107 s for E and 93 s for F.

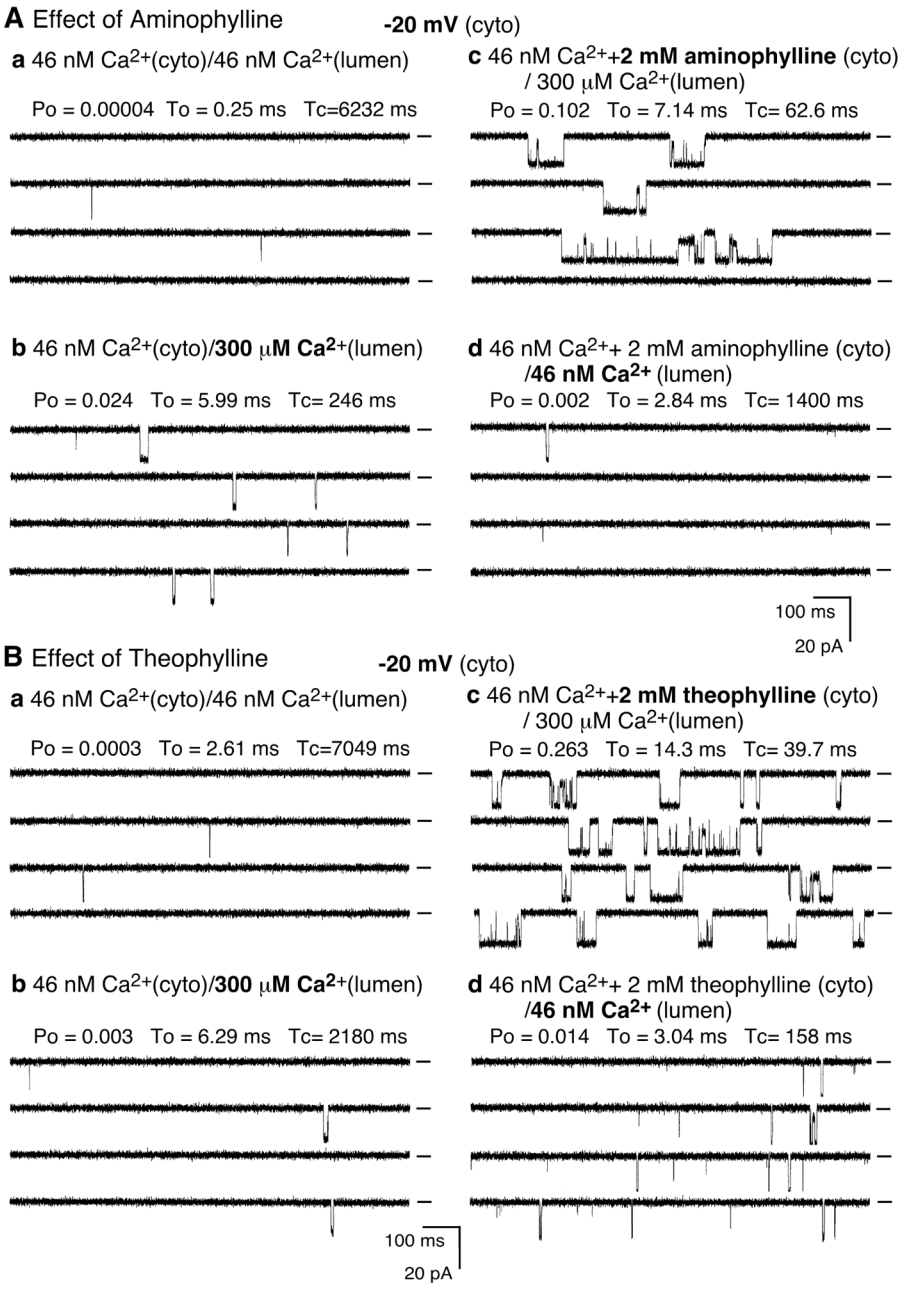

Fig. 4. Aminophylline and theophylline enhance the luminal Ca^{2+} activation of RyR2

The effects of aminophylline (*A*) and theophylline (*B*) on single-channel activities of RyR2 were recorded in a symmetrical recording solution as described in Fig. 3. The Ca^{2+} concentration on both the cytosolic and luminal sides of the incorporated channel was first adjusted to ~46 nM (*Aa*, *Ba*). The channel was activated by 300 μM luminal Ca^{2+} (*Ab*, *Bb*). Aminophylline (*Ac*) or theophylline (*Bc*) was then added to the cytosolic side the channel in the presence of 300 μM luminal Ca^{2+} followed by a decrease of luminal Ca^{2+} to ~46 nM (*Ad*, *Bd*). A similar luminal Ca^{2+} dependence of activation by aminophylline or theophylline was observed in 5–6 RyR2 channels. A continuous recording is shown for each condition. The average recording time is 134 s for panel A and 96 s for panel B.

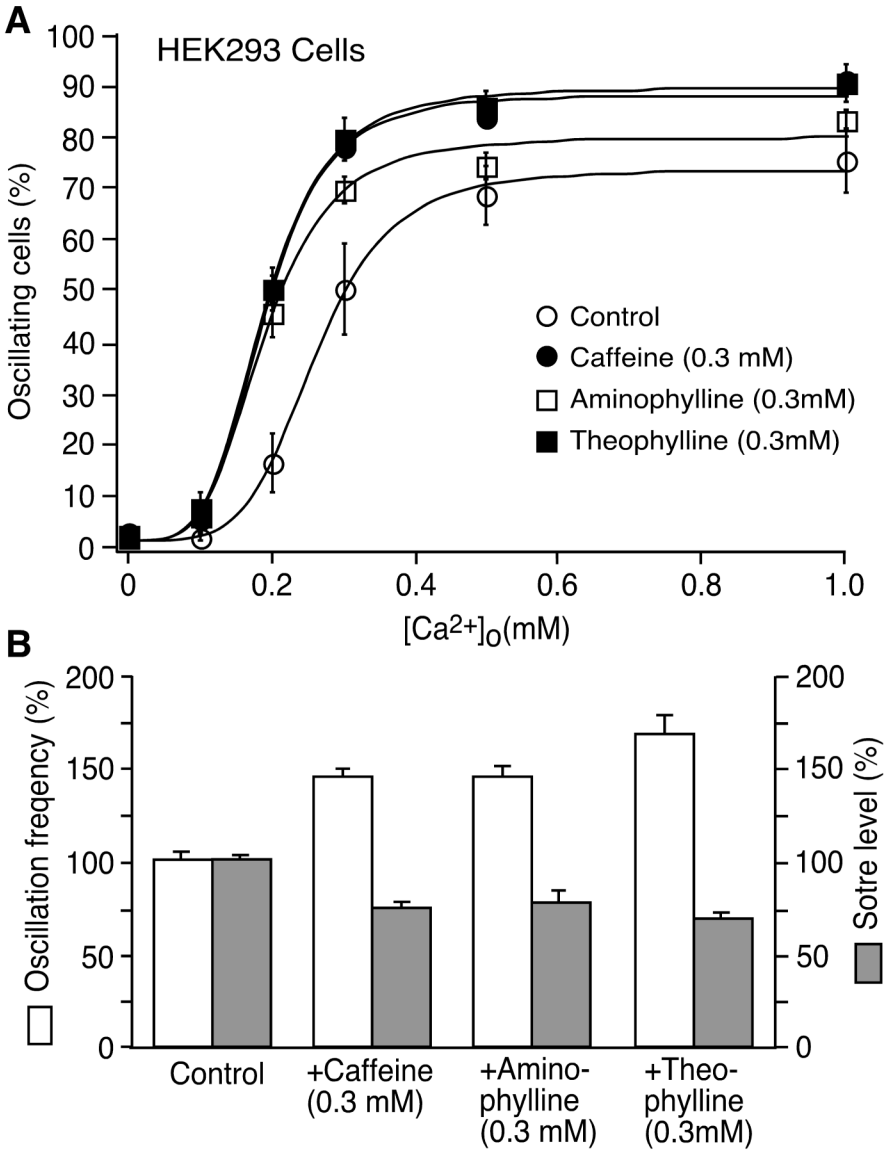

Fig. 5. Methylxanthines increase the propensity for spontaneous Ca^{2+} release in HEK293 cells

(*A*) The fraction (%, mean ± SEM) of RyR2-expressing cells that display Ca^{2+} oscillations at various $[Ca^{2+}]_o$ is shown in the absence of methylxanthines (control, open circles) and in the presence of 0.3 mM caffeine (filled circles), aminophylline (open squares) or theophylline (filled squares). The total numbers of cells analyzed for Ca^{2+} oscillations were 377 for the control, 507 for caffeine, 417 for aminophylline, and 370 for theophylline from 4–6 separate experiments. (*B*) The frequency (white) of spontaneous Ca^{2+} oscillations and the store Ca^{2+} level (grey) (%, mean ± SEM) in HEK293 cells in the absence of methylxanthines (control) or presence of caffeine, aminophylline or theophylline are shown. Both the frequency of Ca^{2+} oscillations and the store Ca^{2+} content were determined at 1 mM $[Ca^{2+}]_o$. The store Ca^{2+} levels were estimated by measuring the peak of Ca^{2+} release induced by 10 mM caffeine. The average frequency and store level observed in the presence of methylxanthines were normalized to the control values (100%).

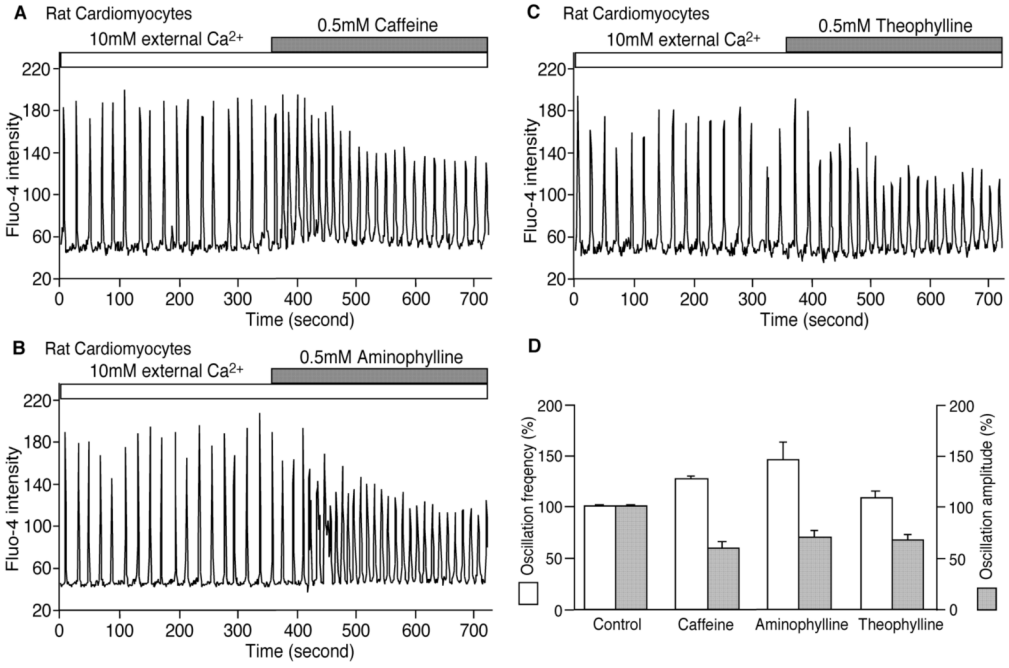

Fig. 6. Methylxanthines enhance spontaneous Ca^{2+} release in rat cardiomyocytes

Rat cardiac myocytes attached to gelatin-fibronectin pre-treated glass coverslips were loaded with 5 μM fluo-4 AM. The cells were then continuously perfused with 10 mM $[Ca^{2+}]_o$ plus 0.5 mM caffeine (*A*), aminophylline (*B*) or theophylline (*C*). Fluo-4 fluorescent intensities of representative myocytes are shown. (*D*) The frequency (white) and amplitude (grey) of spontaneous Ca^{2+} waves (%, mean ± SEM) in rat cardiac myocytes in the absence of methylxanthines (control) or presence of caffeine, aminophylline or theophylline are shown. The frequency and amplitude of spontaneous Ca^{2+} release observed for the control was normalized to 100%. The total numbers of cardiac myocytes analyzed for spontaneous Ca^{2+} release were 9 for caffeine, 10 for aminophylline and 16 for theophylline from 3 separate experiments.

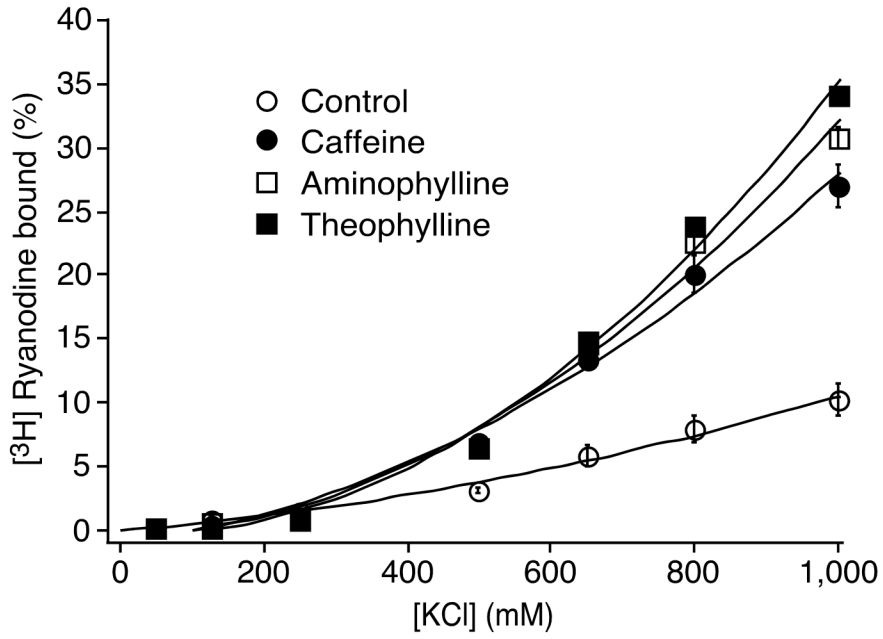

Fig. 7. Effects of methylxanthines on the basal activity of [^{3}H]ryanodine binding

[^{3}H]ryanodine binding to cell lysate prepared from HEK293 cells expressing RyR2 was carried out at ~3 nM Ca^{2+} (pCa 8.49), various concentrations of KCl (50–1000 mM), 2.5 mM caffeine (filled circles), aminophylline (open squares) or theophylline (filled squares), and 5 nM [^{3}H] ryanodine. The channel activity in the absence of methylxanthines (control) is also shown (open circles). The amount of [^{3}H]ryanodine binding at various Ca^{2+} concentrations was normalized to the maximal binding at 1000 mM KCl and pCa 4. Data points shown are mean ± SEM from 3 separate experiments.