Caffeine-activated large-conductance plasma membrane cation channels in cardiac myocytes: characteristics and significance

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Submitted 9 January 2007; accepted in final form 1 May 2007

Zhang Y-a, Tuft RA, Lifshitz LM, Fogarty KE, Singer JJ, Zou H. Caffeine-activated large-conductance plasma membrane cation channels in cardiac myocytes: characteristics and significance. Am J Physiol Heart Circ Physiol 293: H2448–H2461, 2007. First published May 4, 2007; doi:10.1152/ajpheart.00032.2007.—Caffeine-activated, large-conductance, nonselective cation channels (LCCs) have been found in the plasma membrane of isolated cardiac myocytes in several species. However, little is known about the effects of opening these channels. To examine such effects and to further understand the caffeine-activation mechanism, we carried out studies using whole-cell patch-clamp techniques with freshly isolated cardiac myocytes from rats and mice. Unlike previous studies, thapsigargin was used so that both the effect of opening LCCs and the action of caffeine were independent of Ca2+ release from intracellular stores. These Ca2+-permeable LCCs were found in a majority of the cells from atria and ventricles, with a conductance of ~370 pS in rat atria. Caffeine and all its direct metabolic products (theophylline, theobromine, and paraxanthine) activated the channel, while isocaffeine did not. Although they share some similarities with ryanodine receptors (RyRs), the openings of which give rise to Ca2+ sparks), LCCs also showed some different characteristics. With simultaneous Ca2+ imaging and current recording, the localized fluorescence increase due to Ca2+ entry through a single opening of an LCC (SCCaFT) was detected. When membrane potential, instead of current, was recorded, SCCaFT-like fluorescence transients (indicating single LCC openings) were found to accompany membrane depolarizations. To our knowledge, this is the first report directly linking membrane potential changes to a single opening of an ion channel. Moreover, these events in cardiac cells suggest a possible additional mechanism by which caffeine and theophylline contribute to the generation of cardiac arrhythmias. For example, caffeine can cause Ca2+ influx through the plasma membrane. Experiments where cytosolic Ca2+ changes were monitored provided evidence for the existence of such a pathway for leech P neurons (43), cultured DDT1MF-2 smooth muscle cells (45), jejunal smooth muscle cells (37), and aortic endothelial cells (9). Furthermore, recordings of a caffeine-activated current passing through the plasma membrane have been reported (e.g., see Refs. 16, 26, and 33).

In cardiac myocytes isolated from different animals, there have been several reports of caffeine-activated, large-conductance plasma membrane channels (LCCs). Because of their large conductance and long open times with few channels simultaneously open, the unitary currents have been recorded using whole-cell patch-clamp techniques. The first set of reports was obtained from experiments with isolated cardiac myocytes [some from short-term cultures (33, 39) and some from freshly isolated cells (38)] from guinea pig atria and ventricles. These authors concluded that the LCCs were activated by Ca2+ release from stores, either spontaneously or induced by caffeine (33, 39) or halothane (38). Therefore, the effect of caffeine appeared to be indirect, and the authors suggested that it was the increase in intracellular Ca2+ produced by caffeine, not caffeine itself, that activated the channels. From current-voltage relationships of the unitary currents under various ionic conditions, these channels were shown to have a unitary conductance of ~300 pS and appeared to be nonselective among monovalent cations. These authors did not test for divergent cation permeability. Although the question was raised as to whether they might be gap junction hemichannels (39), there was no solid evidence provided for this possibility.

A second set of studies was carried out on freshly isolated rabbit (22) and rat (46) ventricular cells. LCCs from these preparations have a unitary conductance of ~300 pS or greater and were also nonselective for monovalent cations. In addition, these authors showed that the LCCs were permeable to Ca2+ and Ba2+.

Kondo et al. (22) showed that, in rabbit ventricular cells, raising the internal Ca2+ levels could increase the response of the LCCs to caffeine. However, because they were not able to demonstrate that raising internal Ca2+ levels alone could activate the channel, they concluded that the effect of caffeine was directly on the channel. Based on this conclusion, on the results showing that in the presence of ryanodine the channel would go into a long-lasting substate, on the observation that application of extracellular ruthenium red could decrease the unitary current, and on other characteristics of the channel, they concluded the channel was a sarcolemmal RyR.

On the other hand, Volk et al. (46) showed that the LCC in rat ventricular cells, which was activated by caffeine, also

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appeared to be activated by increases in intracellular Ca$^{2+}$. Therefore, they concluded that increases in internal Ca$^{2+}$ could modulate the direct effect of caffeine and directly activate the channel. Because they could not achieve the effects with ruthenium red and ryanodine that were described by Kondo et al. (22), they concluded that the LCC in rat ventricular cells was not a plasma membrane RyR. Instead, based on the finding from RT-PCR that polycystin channels might exist in cardiac cells and on the effects of different cation channel inhibitors (Gd$^{3+}$, La$^{3+}$, and amiloride), they suggested that the LCC in their preparation was “polycystin-2-like.”

All these cardiac LCCs share some common features other than their large conductance: they appear to be nonselective cation channels, they can be activated (directly or indirectly) by caffeine, they are sensitive to cytosolic Ca$^{2+}$ increases, they can have long openings, and they appear to have a low density in the cell plasma membrane or a low open probability based on whole-cell current recordings. The differences among the LCCs cited above might be due to variations in cell isolation procedures or in experimental protocols. They may also reflect true differences among tissue or animal types.

To better understand these channels and examine their involvement in cell function, we carried out a set of experiments on LCCs using atrial and ventricular myocytes isolated from mice and rats. We have focused our studies on the channel in rat atrial cells. Unlike the previous work by others, we carried out many of our experiments in the presence of thapsigargin to block the confounding effect of Ca$^{2+}$ release from internal stores on channel activation. This experimental condition also allowed us to examine the LCC current in isolation from the Na$^{+}$/Ca$^{2+}$ exchange transient inward current caused by caffeine-induced store release of Ca$^{2+}$ (7, 8, 44). Also, we simultaneously used Ca$^{2+}$ imaging and patch-clamp techniques to follow the Ca$^{2+}$ that passed through the channel to obtain a better idea of channel density and the effect of channel openings on changes in intracellular Ca$^{2+}$ and membrane potential.

Based on our results from rat atrial cells, although the LCCs have many properties associated with RyRs, there are also some differences. We found that these LCCs are activated by xanthine analogs other than caffeine and that this activation appears to be a direct effect that does not require an increase in cytosolic Ca$^{2+}$. With simultaneous Ca$^{2+}$ imaging and patch-clamp current recording, we were able to detect the localized fluorescence transients due to Ca$^{2+}$ influx through single openings of these channels [single-channel Ca$^{2+}$ fluorescence transients (SCCaFTs)]. Using SCCaFTs, we further demonstrated that even a single opening of an LCC can significantly depolarize the cell membrane and, sometimes, induce an action potential. Therefore, if caffeine or theophylline opens these channels in vivo, such alterations in membrane potential may contribute to the arrhythmogenic effects of these methylxanthines.

**MATERIALS AND METHODS**

**Single-Cardiac Myocyte Preparation**

Cardiac myocytes were enzymatically dispersed from the heart of either rat or mouse and used on the same day. The methods for excision of the heart for isolation of cardiac myocytes were approved by the Institutional Animal Care and Usage Committee. For most of these studies, we employed a new method for isolating cardiac myocytes (details of the isolation method for this article are available online at the American Journal of Physiology-Heart and Circulatory Physiology website). The procedure for isolating rat atrial cells (from which we obtained most of our data) is briefly described below. Some of the rat ventricular cells were isolated using a conventional Langendorff procedure described by Woodiwiss et al. (48). All experiments were carried out at room temperature.

After isolation of the heart with descending aorta from the animal, the aorta was cannulated with an infusion set connected to a peristaltic pump. Dithiothreitol-activated papain solution (20 ml) consisting of 10–15 U/ml papain in BDM-HEPES solution supplemented with 50 μM MgSO$_4$ and 50 μM EDTA was perfused through the heart at a rate of 1 ml/min. [BDM-HEPES solution contained (in mM) 120 NaCl, 5.4 KCl, 1.2 NaH$_2$PO$_4$, 0.1 MgSO$_4$, 15 NaHCO$_3$, 5 butanediol monoxime (BDM), 10 HEPES (sodium salt), 5 taurine, and 5 glucose. The pH was adjusted to 7.4 with HCl at room temperature.] After perfusion, left and right atria were removed from the heart, cut into pieces, and put into a petri dish containing 5 ml of the perfusion solution for 30 min. The tissue was further cut into smaller (~1 x 2 mm) pieces, washed twice with 5 ml of BDM-HEPES-Mg solution (BDM-HEPES solution supplemented with 1 mM MgSO$_4$), and transferred to 5 ml of BDM-HEPES-Mg solution for 10 min. All the above-described steps were carried out at room temperature. The BDM-HEPES-Mg solution was replaced with 5 ml of digestion solution [BDM-HEPES-Mg solution supplemented with 478 U/ml collagenase (type II, Worthington), 2 mg/ml BSA (Sigma), and 50 U/ml DNase I (Sigma)], and the tissue pieces were incubated for 30 min at 32°C, with gentle agitation every 10 min. (This was the only step carried out at an elevated temperature.) The tissue pieces were then transferred to 3 ml of glutamate-K solution [in mM: 120 glutamic acid, 0.3 KH$_2$PO$_4$, 1 EGTA, 5 MgSO$_4$, 20 taurine, 10 HEPES (potassium salt), 10 ATP, and 10 glucose. The pH adjusted to 7.4 with KOH at room temperature]. The cells were dispersed with trituration using a fire-polished Pasteur pipette (with a ~2-mm-diameter opening). The dispersed cell suspension was transferred to another tube, and another 3 ml of glutamate-K solution were added to the tube containing the tissue. The trituration step was repeated three times, and the pooled cell suspension (10–15 ml total) was centrifuged at 500 rpm for 10 min. The supernatant was carefully removed, and the cells were resuspended into 10 ml of glutamate-K solution supplemented with 3 U/ml papain and 50 U/ml DNase I for 30 min at room temperature. Cells were centrifuged again at 500 rpm for 10 min. The cell pellet was washed with glutamate-K solution, centrifuged, and resuspended in glutamate-K solution. The cells could be stored at room temperature for use within the next 4–6 h.

For experiments with the cells in a solution containing physiological concentrations of Ca$^{2+}$, after the final centrifugation step above, the cells were washed with 10–15 ml of BDM-HEPES-Mg solution, centrifuged, and resuspended in 5 ml of BDM-HEPES-Mg solution. The Ca$^{2+}$ concentration was adjusted stepwise to 0.05, 0.1, 0.2, 0.3, 0.5, 0.7, and 1.0 mM by addition of aliquots of 100 mM CaCl$_2$ solution at 7-min intervals. Cells were then placed on ice for use within the next 4–6 h.

**Patch-Clamp Recordings and Data Processing**

The currents passing through caffeine-activated channels were recorded with an Axopatch-1D amplifier (Axon Instruments) using the whole-cell configuration of the patch clamp. Currents were low-pass filtered at 500 Hz and sampled at 2 kHz. When recording membrane potential, the whole-cell current was usually clamped to zero; i.e., we usually did not apply a steady-state holding current. For most of the cells from which membrane potential was recorded, whole-cell current was also recorded to verify the presence of LCCs. For clarity of presentation, sometimes digital filtering or baseline correction was applied to the current trace (e.g., Figs. 7 and 9D).
The standard bath solution contained (in mM) 130 NaCl, 5.4 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES. Thapsigargin (1 μM) was also included, and pH was adjusted to 7.4 using NaOH. The standard pipette solution contained (in mM) 137 KCl, 3 MgCl₂, 10 HEPES, and 3 Na₂ATP, and pH was adjusted to 7.2 using KOH. When Ca²⁺ fluorescence was to be measured, 50 μM fluo-3 (pentafluorobenzoyl) was included in the pipette solution. For some experiments, 10 mM 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid (BAPTA) was included in the pipette solution so that multiple channel openings would not cause a significant increase in cytosolic Ca²⁺ and cell contraction. In addition, with the BAPTA pipette solution, the LCC open time was usually reduced, which facilitated examination of the effectiveness of an agent in activating the channel. When we present data obtained using other solutions, the contents are listed in the figure captions. All chemicals, except fluo-3 (Molecular Probes), were purchased from Sigma. The stock solutions for thapsigargin (10 mM) and ryanodine (100 mM) were in DMSO and stored at −20°C.

Caffeine and other agents were usually applied to the cell by pressure ejection from a glass “puffer” pipette via a Picospritzer (General Valve). Cells bathed in the standard bath solution (containing 1 mM Ca²⁺) might contract if multiple channel openings are induced. Therefore, to prevent cells from contracting, the duration of the caffeine application and/or the distance between the puffer pipette and the cell was sometimes adjusted so that only a few channels were open simultaneously. As such, although 20 mM caffeine was usually included in the puffer pipette solution, this concentration was not necessarily reached at the cell membrane. This practice presumably also contributes to the variations and delays sometimes seen in the response to caffeine application. Longer openings are usually related to the prolonged presence or higher concentration of caffeine. Brief applications from a distance tend to induce brief openings, even with the standard pipette solution (without BAPTA).

When an agent was applied to the cell to activate LCCs, caffeine was also applied to the same cell sequentially (see Fig. 3). This caffeine application served two purposes. 1) Since some cells did not appear to have LCCs judging from the response to caffeine, caffeine application controlled for the “false negative” if no LCC openings were observed after application of a test agent. 2) We could compare the openings caused by caffeine with those caused by the other agent.

For most of the experiments described here, we wished to remove any possible contributions from intracellular Ca²⁺ stores. Therefore, thapsigargin (1 μM) was added to the bath solutions. Caffeine was also applied to the cells before the beginning of experiments using the Picospritzer. These treatments have been previously employed to successfully eliminate effects of the intracellular Ca²⁺ stores (54). In the cardiac cells, we saw no evidence of Ca²⁺ release from stores when we later applied a methylexanthine. However, in the absence of this procedure, we could record Ca²⁺ sparks, and caffeine generally induced Ca²⁺ waves in addition to opening LCCs. Figure 11 is the only figure that shows results obtained from an experiment carried out in the absence of thapsigargin.

Wide-Field Digital Imaging and Fluorescence Measurements

Methods for two-dimensional Ca²⁺ fluorescence imaging and data processing were similar to those we used previously (54–56). Fluorescence images were acquired using a custom-built, high-speed, wide-field, digital imaging microscope with fluo-3 as the Ca²⁺ indicator. The standard system setup used an oil immersion objective lens (×40 magnification, 1.3 numerical aperture). Each image was composed of 128 × 128 pixels, each usually a 0.8-μm square. We generally focused at the middle of the cell. At each pixel, the fluorescence at rest (F₀) was subtracted from the fluorescence (F) for each image in the image set, and the difference was used to construct the images [ΔF = F − F₀ or ΔF/F₀ = (F − F₀)/F₀], which were then smoothed (with a 3 × 3 kernel approximating a Gaussian, with σ = 1 pixel) before display (56). The resting fluorescence, F₀, was usually obtained by averaging the fluorescence intensity of 10 consecutive images when there was no fluorescence transient. The pixel with the maximum fluorescence increase for the transient was taken as the location of the channel, and its fluorescence time course was plotted. Without three-dimensional imaging with a plasma membrane marker, we could not be sure of the proximity of the plasma membrane to a Ca²⁺ fluorescence event. However, since Ca²⁺ store release was blocked, the only source for the Ca²⁺ increase would have been the extracellular medium. Simultaneous patch-clamp recordings confirmed that these localized Ca²⁺ fluorescence transients were due to LCC openings (see below).

Several image sets were generally obtained from the same cell. To facilitate capture of the desired transients/channel openings, we sometimes used a circular image buffer protocol (54) (software provided by Dr. Karl D. Bellve, Biomedical Imaging Group, University of Massachusetts Medical School). The outline of the cell in the fluorescence ratio images was usually determined by application of a fluorescence intensity threshold. When the cell outline could not be completely determined by the threshold, we generated a mask by manually tracing the edge of the cell from the images.

Simultaneous Recording of Ca²⁺ Fluorescence and Membrane Electrical Activity

Simultaneous Ca²⁺ imaging and voltage-clamp recording enable us to correlate a single LCC opening with a localized Ca²⁺ fluorescence transient, i.e., a SCCaFT. Moreover, under current-clamp conditions (when the membrane potential, not the current, is recorded) we can identify the opening of a single LCC by the time course of the associated SCCaFT. For the latter, if the recorded SCCaFT is from the same location at which we record the LCC current under voltage clamp, it is likely that the same channel opened under both recording conditions. Therefore, the SCCaFT obtained under current clamp can help us determine whether and where an LCC opening caused a transient cell membrane depolarization.

To analyze these simultaneous recordings, temporal alignment of the patch-clamp recording with the fluorescence recording was required. The “read-camera” signal, which occurred at the end of each exposure and triggered the reading of the charge-coupled device camera, was simultaneously sampled (at 1 kHz) with the current or membrane potential with use of a multichannel, analog-to-digital data acquisition card (National Instruments, Woburn, MA). The measured fluorescence, which was the accumulated photon flux during the entire exposure time, was aligned to the midpoint of the exposure time as if it were a point sample at that time (and the value was plotted as described above).

RESULTS

Caffeine Activates an LCC in Atrial and Ventricular Myocytes Isolated From Rats and Mice

When caffeine was applied to freshly isolated cardiac myocytes from mice and rats, LCC openings were observed in a majority of the cells. Although most of our experiments were carried out with rat atrial cells (>150 cells), the LCC unitary current was also recorded in cells from rat ventricles (7 cells), mouse atria (6 cells), and mouse ventricles (45 cells; Fig. 1). Unless otherwise stated, the results presented here were obtained from rat atrial cells. Although the caffeine application was controlled to limit the number of simultaneous LCC openings (see MATERIALS AND METHODS), for many cells, there were only a couple of channels open simultaneously, even with 20 mM caffeine. For some other cells, more channels could be open simultaneously (Fig. 1C); occasionally, they even exhibited synchronized openings and closings (see below).

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This shows that there was no release of Ca\textsuperscript{2+} transients were observed in the absence of channel openings. The fluorescent Ca\textsuperscript{2+} clamp technique. discern single-channel openings using the whole-cell patch-clamp technique. long as several seconds. The sufficiently large unitary current, together with the discrete long openings, made it possible to discern single-channel openings using the whole-cell patch-clamp technique.

**Relationship of Caffeine-Activated LCCs to RyRs**

The caffeine-activated LCC is a Ca\textsuperscript{2+}-permeable, nonselective cation channel. When cytosolic Ca\textsuperscript{2+} was monitored using the fluorescent Ca\textsuperscript{2+} indicator fluo-3, localized fluorescence increases were observed in response to caffeine application. When these localized fluorescence changes were temporally aligned with the simultaneously recorded whole-cell current, we found that each fluorescence transient correlated with a channel opening [Fig. 2A (mouse ventricular myocyte, 7 cells) and B (rat atrial myocyte, >30 cells)]. No fluorescence transients were observed in the absence of channel openings. This shows that there was no release of Ca\textsuperscript{2+} from intracellular stores under our experimental conditions. As such, the localized fluorescence transients that accompanied channel openings were due to Ca\textsuperscript{2+} entry through the LCCs and, therefore, were SCCaFTs (10, 54). This observation suggests that, even in the mixture of different extracellular cations as occurs under physiological conditions, a significant amount of Ca\textsuperscript{2+} can pass through the LCCs.

Caffeine-induced LCC current was also recorded when half (4 cells) or all (3 cells) of the Na\textsuperscript{+} was replaced by Cs\textsuperscript{+} in the bath solution. Shown in Fig. 2C is an example where Cs\textsuperscript{+} was the only monovalent cation in the bath solution. The unitary current is slightly smaller (~21 pA, instead of ~26.5 pA, at ~70 mV) than in the standard bath solution. In the standard bath solution, the unitary conductance of the LCC in rat atrial cells is ~370 pS at negative membrane potentials (Fig. 2D). The reversal potential extrapolates to ~0 mV. The steady-state membrane potential does not appear to have an effect on gating or caffeine activation of the LCCs (Fig. 2D, insets). These results suggest that the LCC is a Ca\textsuperscript{2+}-permeable, large-conductance, nonselective cation channel.

LCCs can be activated by other methylxanthines. We tested several methylxanthines that had been shown to enhance the release of Ca\textsuperscript{2+} from sarcoplasmic reticulum (SR) vesicles (41) and/or enhance the binding of ryanodine to the RyR (25) and found that they also activate LCCs in rat atrial cells. As shown in Fig. 3, theophylline (1,3-dimethylxanthine, 5 cells), 7-(β-hydroxyethyl)theophylline (HET, 5 cells), theobromine (3,7-dimethylxanthine, 3 cells), and paraxanthine (1,7-dimethylxanthine, 3 cells) activated the LCCs, as did caffeine (1,3,7-trimethylxanthine), when applied to the same cells (see MATERIALS AND METHODS). Theophylline, theobromine, and paraxanthine are natural metabolites of caffeine. A current-voltage relationship for the LCC activated by theophylline obtained from one of these cells was found to be almost identical to that of the caffeine-activated LCC. On the other hand, application of isocaffeine (1,3,9-trimethylxanthine),
which neither enhances ryanodine binding to the RyR (25) nor releases Ca\(^{2+}\) from SR vesicles (41), did not open the LCCs (4 cells). In another preparation, mouse ventricular cells, both theophylline and caffeine activated LCCs when applied to the same cell (12 cells). These results demonstrate that the caffeine-activated LCCs can also be activated by other methylxanthines that can activate RyRs based on their ability to affect ryanodine binding and/or Ca\(^{2+}\) flux. The ineffectiveness of 20 mM isocaffeine on opening LCCs also indicates that the effect of higher doses of caffeine was not due to changes in osmolarity.

Ryanodine can cause the channel to open to a subconductance state. In contrast to results reported for the LCCs in guinea pig atria (39) and rabbit ventricles (22), where subconductance levels were often observed, it was very rare for the LCC in rat atrial cells to open to subconductance levels. However, when the cells were treated with ryanodine (20, 50, or 100 \(\mu\)M), the LCCs could open to subconductance levels in response to caffeine application (10 cells, Fig. 4). However, not every cell treated with ryanodine showed openings to subconductance states. The 10 cells that showed subconductance states were among the total of 17 cells where we saw LCC openings; the other 7 cells showed only normal openings. Since there has been inconsistency in the literature regarding the response of LCCs to ryanodine (22, 46), we carried out these experiments with ryanodine applied to the cell in different ways: added to the bath solution (4 cells), added to the whole-cell patch pipette solution (5 cells), or added to both pipette and bath solutions (8 cells). Subconductance states were observed under all three of these experimental conditions. Usually, the channel opened to the full-conductance state before dwelling at the subconductance state (Fig. 4B, inset).
inhibit the channels [at 100 μM, ryanodine would be expected to completely inhibit the RyR channel (13, 57)]. Moreover, in current recordings showing openings of multiple channels, there was a mixture of openings to the full-conductance and subconductance levels, even with 100 μM ryanodine (Fig. 4B).

Tetracaine diminishes LCC activity. To examine the effect of tetracaine [a RyR channel inhibitor (47, 49)] on LCCs, we used two puffer pipettes to deliver the agents: one contained caffeine (20 mM) alone and the other contained a mixture of caffeine (20 mM) and tetracaine (200 or 500 μM). LCC activity induced by caffeine in the presence of tetracaine was significantly reduced in all three cells tested compared with applying caffeine alone. For two of these cells, the mixture of tetracaine and caffeine was applied while caffeine was also being applied from the other puffer (Fig. 5). For the other cell, the mixture of tetracaine and caffeine was applied with the other puffer stopped. Although tetracaine increased the channel closed time, it did not appear to change the unitary current amplitude. The effect of tetracaine on channel activity was readily reversed on its removal.

Ruthenium decreases LCC current amplitude. When 10 μM ruthenium red, another RyR inhibitor (27, 51), was applied to the cell, together with caffeine, many openings showed smaller unitary currents (4 cells, Fig. 6). This effect could be at least partially reversed after ruthenium red was washed out. Such a reduction in unitary current was also observed when 1 μM ruthenium red was applied (2 cells). This amplitude reduction could be due to a blocking effect of ruthenium red on the channel (27) or a gating effect, causing the channel to open to a subconductance level (51).

Fig. 3. Effects of various methylxanthines on caffeine-activated LCCs in rat atrial cells. Application of 7-(8-hydroxyethyl) theophylline (HET, B), theophylline (D), theobromine (E), or paraxanthine (F) activated LCCs, which were also activated by caffeine. Application of isocaffeine, however, did not activate caffeine-activated LCCs (C). Structure of each methylxanthine is shown to the right of the trace. For all traces, whole-cell recordings were carried out in the standard bath solution (which contained thapsigargin), with the membrane potential held at about 70 mV. Standard pipette solution was used in A and B; the 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) pipette solution was used in the other recordings. The concentrations of caffeine and its analogs used in the experiments were: 20 mM in A, B, and C, 10 mM in D and F, and 5 mM in E. Different concentrations were used because of the solubility differences of the methylxanthines.

Fig. 4. LCCs can open to subconductance levels in the presence of ryanodine. Ryano
dine (20 μM in A and 100 μM in B) was included in both the pipette and bath solutions. The subconductance level is about one-third of the full opening in A and about one-half in B. Channel opened to full conductance state before locking to a subconductance level (inset in B). Duration of the trace in the inset is 2.5 s. As one channel was locked into a subconductance level, openings from other channels were to the full-conductance state. Toward the end of the recording in B, 5 channels opened synchronously. Membrane potential was held at about 70 mV for these whole-cell recordings. Caffeine (20 mM) application was stopped before the beginning of the trace in B. Standard bath solution (which contained thapsigargin) and standard pipette solution were used.
The membrane-permeant agent 4-chloro-m-cresol could activate the channel at a concentration as low as 200 μM. However, 500 μM cresol did not activate the LCCs either when applied to the outside of the cell (3 cells) or when included in the patch pipette (2 cells). It did induce a slow outward current when applied to the cell from the extracellular side. For the caffeine-activated LCC in toad stomach smooth muscle cells (16, 54), cresol could activate the channel at a concentration as low as 200 μM (unpublished results).

The same LCC can open repeatedly with both brief and long openings. One of the advantages of imaging SCCaFTs is that we can follow the openings and closings of the (same) channel at a particular location. This provides unique information regarding individual channels that cannot be obtained from patch-clamp recordings alone, especially in the whole-cell recording configuration. Because long (i.e., seconds) and brief (i.e., milliseconds) openings were shown in the current records, it is possible that there are two groups of channels, each with distinct open times. Our experimental observations do not support this possibility. As shown in Fig. 2 for recordings from a rat atrial myocyte and a mouse ventricular myocyte, brief and long SCCaFTs were observed from the same location corresponding to the openings of what appeared to be the same channel with different open times. Another example can be found in Fig. 10B. These observations suggest that the brief and long openings can come from the same channel, not different groups of channels. One other conclusion that can be drawn from these observations is that the channel can have a high open probability when activated by caffeine. Such an example showing that a single channel opened frequently in response to caffeine application is shown in Fig. 7. While this could suggest a low density of the channel in the plasma membrane, there are other possibilities (see DISCUSSION).

Synchronized openings and closings of LCCs were observed. From Ca²⁺ imaging studies (15, 47), it appears that Ca²⁺ sparks are usually not due to the opening of a single RyR but, rather, near-synchronous openings of more than one RyR. Consistent with these results, synchronized activity of LCCs was sometimes observed in rat atrial cells. These synchronized openings were observed with different pipette and bath solutions, including the standard bath and pipette solutions. Several such examples are shown in Fig. 8. Channels that opened synchronously did not always close synchronously, and vice versa. An example of brief synchronous openings of five channels can also be found in Fig. 4 with the standard solutions.

Other Properties of the Caffeine-Activated LCC

LCC activation appears to be a direct effect of caffeine on the channel. Experiments were carried out to examine the possibility that caffeine activates LCCs via inhibition of adenosine receptors. Although 200 μM caffeine could open LCCs (see below), the activity induced by higher concentrations of caffeine was usually much greater. For example, an increase in or the appearance of LCC activity was found when 1 or 2 mM caffeine was applied to a cell that was bathed in 200 μM caffeine (5 cells). Since the affinity of caffeine for adenosine receptors is in the micromolar range (17), 200 μM caffeine should have caused a maximum response. Therefore, it is unlikely that the effect of caffeine was via antagonism of adenosine receptors. These experiments were carried out in the absence of thapsigargin to mimic more physiological conditions.

Fig. 5. Tetracaine inhibits caffeine-activated LCC activity. Two puffer pipettes were used: one contained 20 mM caffeine alone and the other, 20 mM caffeine + 200 μM tetracaine. When tetracaine was applied, the two puffers were both on; therefore, tetracaine concentration around the cell should be <200 μM. Standard bath solution (which contained thapsigargin) and BAPTA pipette solution were used. The whole-cell membrane potential was held at −70 mV.

Fig. 6. Ruthenium red decreases caffeine-activated LCC current amplitude. Application of 10 μM ruthenium red together with 20 mM caffeine (middle trace) decreased channel current amplitude [compared with 20 mM caffeine (top trace)]. This effect is at least partially reversible on washout of ruthenium red (bottom trace). Bottom trace was recorded -5 min after application of ruthenium red was stopped and caffeine-containing bath solution had been applied by puffer pipette to wash out ruthenium red. These whole-cell currents were recorded with the membrane potential held at −70 mV. Standard bath solution (which contained thapsigargin) and standard pipette solution were used.

Fig. 7. Effect of 500 μM cresol on caffeine activation of LCCs. While 200 μM caffeine could open LCCs synchronously, cresol did not activate the channel. This effect is at least partially reversible on washout of ruthenium red.
The activation of LCCs by caffeine does not appear to be via increasing cAMP levels by phosphodiesterase inhibition. When 200 μM cAMP was included in the pipette solution and 1 mM 8-bromo-cAMP (a membrane-permeable hydrolysis-resistant cAMP analog) was added to the bath solution, no LCC openings were observed until caffeine was applied (2 cells).

For most of our experiments, 1 μM thapsigargin was included in the bath solution to block Ca^{2+} uptake into intracellular stores and, thus, eliminate Ca^{2+} release. Therefore, the activation of LCCs by caffeine could not be due to an increase in cytosolic Ca^{2+} caused by caffeine-induced Ca^{2+} store release. In addition, caffeine or other xanthine analogs could open LCCs, even when 10 mM BAPTA was included in the patch pipette (>50 cells) to buffer the cytosolic Ca^{2+} to a minimal level. As such, LCC activation appears to be a direct effect of caffeine (or other xanthine analog) itself, rather than an indirect effect through Ca^{2+} release from intracellular Ca^{2+} stores.

Effects of cation channel inhibitors. We also examined the effect of some nonselective cation channel blockers on LCCs. When Gd^{3+} [100 μM, a concentration sufficient to block several other cation channels (e.g., see Refs. 2 and 52)] was applied to the cell, together with caffeine, it did not inhibit caffeine-induced LCC activity in any of the five cells tested. However, when 1 mM Gd^{3+} was used, the LCCs were opened only initially and then could no longer be activated, even when caffeine was applied alone later (4 cells, including 1 cell in which 100 μM Gd^{3+} did not show an effect). These findings are consistent with those reported for LCCs from rat ventric-
ular cells (46). We also found that 5 mM Ni²⁺ decreased the amplitude of the unitary current in both of the two cells tested.

Effects of opening LCCs on cell membrane potential. Although the existence of caffeine-activated LCCs appears to be a common finding in cardiac cells from different animals, the significance of the channel might not be appreciated because of its apparent low density. However, because of its large conductance, even single openings of these LCCs could have a significant impact on cell membrane potential. To determine whether this was the case, we monitored the membrane potential while applying caffeine to rat atrial cells.

We observed two types of rapid membrane potential changes in response to caffeine: transient or more sustained membrane depolarizations (9 cells; Fig. 9, A and B). The initial rates of membrane depolarization were similar between the two, with an average of 192 ± 9 (SE) mV/s (n = 57). Whole-cell current was recorded in seven of these nine cells, and the presence of LCCs was confirmed. Taking advantage of our ability to image SCCaFTs, we simultaneously monitored the membrane potential and cytosolic Ca²⁺ and used localized fluorescence changes as an indication of the underlying LCC activity. Localized fluorescence increases were observed during the membrane depolarizations (7 cells; Fig. 9, A and B). In five of these cells, channel current was also recorded with simultaneous Ca²⁺ imaging. On caffeine application, SCCaFTs were observed from the same locations (Fig. 9, C and D) at which fluorescence transients were observed when membrane depolarizations occurred. Therefore, it is most likely that the localized fluorescence transients associated with membrane depolarizations were also SCCaFTs due to single LCC openings with brief or long open times. To our knowledge, this is the first report directly linking cell membrane potential changes to an opening of a single ion channel.

In four cells, action potentials, in addition to the above-mentioned membrane depolarizations, were observed with caffeine application. Simultaneous Ca²⁺ fluorescence imaging and membrane potential recording captured such a series of events in one of these cells: a SCCaFT-like fluorescence transient associated with an initial depolarization preceded the action potential that was associated with a global fluorescence increase (Fig. 10A). This finding is consistent with the idea that openings of LCCs, although resulting only in localized Ca²⁺ increases, can depolarize the cell membrane sufficiently to induce action potentials. The action potentials, in turn, can cause global Ca²⁺ increases and cell contraction.

Although up to 20 mM caffeine was usually used in these studies to focus our study on the channel characteristics, LCC openings, albeit mostly brief and much less frequent, were also observed when 200 μM caffeine was present in the bath (8 cells). LCCs in three cells appeared to be more sensitive to this concentration and showed some spontaneous activity. In six cells, LCC openings were seen, or the spontaneous activity increased, after the membrane potential was briefly changed to a positive level. These experiments were conducted in rat atrial cells without thapsigargin, mimicking a more physiological condition (Fig. 11).

**DISCUSSION**

This paper reports our studies of a caffeine-activated, large-conductance, nonselective cation channel in cardiac myocytes freshly isolated from rats and mice. This is the first report of such channels in mouse cardiac cells and rat atrial cells. With a focus on the LCC in rat atrial cells, we investigated the properties of the channel and compared them with those of cardiac RyRs. Despite the uncertainty of its molecular identity, LCC activation was shown to impose a significant impact on the membrane potential and cytosolic Ca²⁺ and used localized fluorescence changes as an indication of the underlying LCC activity. Localized fluorescence increases were observed during the membrane depolarizations (7 cells; Fig. 9, A and B). In five of these cells, channel current was also recorded with simultaneous Ca²⁺ imaging. On caffeine application, SCCaFTs were observed from the same locations (Fig. 9, C and D) at which fluorescence transients were observed when membrane depolarizations occurred. Therefore, it is most likely that the localized fluorescence transients associated with membrane depolarizations were also SCCaFTs due to single LCC openings with brief or long open times. To our knowledge, this is the first report directly linking cell membrane potential changes to an opening of a single ion channel.

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cell membrane potential and, therefore, could have an effect on cardiac function.

**Relationship Between Caffeine-Activated LCCs in Cardiac Myocytes and the RyR**

There is a precedent for Ca\(^{2+}\) store release channels in the plasma membrane. Several reports provide evidence for the existence of inositol 1,4,5-trisphosphate receptors (IP\(_3\)R), usually found in the membrane of the endoplasmic reticulum (ER), in the plasma membrane (20, 23, 32, 40). Moreover, there is also evidence for a RyR-like molecule in the plasma membrane of osteoclasts, where it may act both as an extracellular Ca\(^{2+}\) sensor and a Ca\(^{2+}\) influx channel (35, 53). The LCC in rabbit ventricular cells is sensitive to ryanodine and, therefore, is also claimed to be a RyR (22). These observations suggest that, although RyRs and IP\(_3\)Rs are mainly found on the SR/ER membrane as Ca\(^{2+}\) release channels, it is also possible that RyRs and IP\(_3\)Rs play a role in Ca\(^{2+}\) signaling in the plasma membrane.

Results from the present studies provide evidence that the cardiac LCCs are similar to RyRs. 1) They are large-conductance, Ca\(^{2+}\)-permeable, nonselective cation channels. 2) Methylxanthines (including theophylline, theobromine, paraxanthine, and HET) that activate LCCs are agonists for RyRs (25, 41). Since HET is not likely to be membrane permeable (36), the binding site for xanthine activation could be located on the outer surface of the plasma membrane. 3) LCCs can open to a subconductance level in the presence of ryanodine. 4) The RyR...
inhibitors tetracaine and ruthenium red also inhibit LCCs.

5) Repeated openings of the same channel and synchronized openings and closings of multiple LCCs were observed. These observations are consistent with results obtained from studies of Ca\(^{2+}\) sparks and RyRs in cardiac myocytes. For example, Mackenzie et al. (29) found that Ca\(^{2+}\) sparks in rat atrial cells occur frequently at predetermined, discrete locations, denoted “eager sites”. It has also been suggested that (synchro-
nous) openings of multiple RyRs underlie Ca\(^{2+}\) sparks with different amplitudes or initial rates of rise (15, 47). Synchronous (coupled) openings of two to three RyRs have been shown in current recordings in lipid bilayers infused with RyR2-containing cardiac microsomes (30).

On the other hand, some results from the present studies do not appear to support the hypothesis that cardiac LCCs are RyRs in the plasma membrane. 1) Cresol, which activates both RyR1 and RyR2 at <200 \(\mu\)M [based on contraction or Ca\(^{2+}\) imaging assays reported by Choisy et al. (5, 6) and Fessenden et al. (12)], appeared to have no effect on LCCs at 500 \(\mu\)M. However, the absence of sensitivity to cresol could be explained if the LCC in rat atrial cells has more in common with RyR3 than with RyR2. RyR3 has been reported to be much less sensitive to cresol (11, 12). In astrocytes, where the intracellular Ca\(^{2+}\) increase was used as a measure of the RyR3 response to cresol, an EC\(_{50}\) of 1.5 mM was reported, and there was a small increase in Ca\(^{2+}\) at 500 \(\mu\)M (31). 2) It has been reported that Gd\(^{3+}\) inhibits skeletal muscle RyR single-channel activity with an IC\(_{50}\) of ∼5.5 \(\mu\)M (42) from either side of the membrane. However, 100 \(\mu\)M Gd\(^{3+}\) had no obvious effect on LCCs, although 1 mM Gd\(^{3+}\) completely blocked LCC activity. 3) The sensitivity of LCCs to different concentrations of ryanodine appeared to be different from that for RyRs. Subconductance states, as well as full openings, were observed with both 20 and 100 \(\mu\)M ryanodine. Ryanodine at 100 \(\mu\)M should have completely inhibited the activity of the RyRs (13, 57). Furthermore, for current traces with openings from multiple channels, openings to full conductance levels were still observed with the occurrence of subconductance states in the presence of ryanodine. It is possible that some of the variation comes from an environmental difference—the plasma membrane vs. the SR membrane, requiring higher ryanodine concentrations (in the millimolar range) to inhibit the channel (57). Therefore, further studies (especially those involving molecular biology techniques) are needed to determine whether the cardiac LCCs are, indeed, RyRs.

If LCCs are, indeed, RyRs or similar to RyRs, it is somewhat unclear how they might finally appear in the plasma mem-
brate. One possibility is that these channels are purposely targeted to the plasma membrane to carry out a function that has yet to be determined. Another possibility is that these are normal or partially modified RyRs that, instead of being inserted into the sarcoplasmic membrane, are inserted into the plasma membrane by mistake (16, 22). Although this could normally occur, the effect of such mistaken targeting may only be seen during pathophysiological events with exposure to higher concentrations of methylxanthines.

It is also possible that, during the cell isolation procedure, a few RyRs are transferred to the plasma membrane by brief fusion of SR vesicles to the plasma membrane, as can occur when SR vesicles are allowed to fuse to artificial lipid bilayers for recording RyR currents (G. Meissner, personal communication). However, it is unlikely that this transfer of RyRs to the plasma membrane is caused by formation of the patch and/or by its rupture for whole-cell current recording, a possibility raised by Kondo et al. (22). If this were the case, then all the channels would be expected to be located in the vicinity of the patch pipette. From the locations of SCCaFTs, it is clear that not all the channels are located around the pipette or even in one area. Instead, they can be located far from one another (Fig. 10).

Other Possible Candidates for the Molecular Identity of Cardiac LCCs

The caffeine-activated LCC in rat ventricle has been suggested to be a polycystin-2-like channel by Volk et al. (46). They ruled out the possibility that the LCCs are RyRs, on the basis that no change in the activation of LCCs by caffeine was observed either with 10 \(\mu\)M ryanodine in the bath for up to 3 min or with 25 \(\mu\)M ryanodine in the whole-cell patch pipette. Based on our experience, higher concentrations of ryanodine and/or a longer exposure time might be required to observe an effect of ryanodine on these channels. Many factors could contribute to this “insensitivity” to ryanodine. For example, if ryanodine is included only in the pipette, the diffusion of the ryanodine inside the cell may not be sufficient to allow ryan-
odine to reach the same concentration at the site of the channel as in the pipette solution. The suggestion that the LCCs are polycystin-2-like channels was mainly based on the presence of polycystin-2 messenger RNAs in rat ventricular cells. Additional experimental evidence linking the LCC and the poly-
cystin-2-like channel was lacking.

Another possibility is that these LCCs are, in fact, gap junctional hemichannels, as proposed by Pott and Mechmann.
(39). This possibility arises not only because these LCCs have a large conductance and are Ca\(^{2+}\) permeable, but also because RyR, shares some characteristics with gap junction channels (28). However, this possibility is unlikely, because gap junctional hemichannels in cardiac myocytes are activated by the removal of extracellular Ca\(^{2+}\) and partially blocked by 100 \(\mu\)M Gd\(^{3+}\) (21). From our preliminary studies, the LCCs could not be opened by caffeine when extracellular Ca\(^{2+}\) was removed (not shown), and 100 \(\mu\)M Gd\(^{3+}\) did not have an apparent effect on the LCC current.

**Total Number of LCCs in the Plasma Membrane of Cardiac Myocytes**

In some cells, only a couple of simultaneous channel openings were observed, even when 20 mM caffeine was applied. The lack of simultaneous multiple openings indicates that either the number of the channels in the plasma membrane or the channel open probability is low. However, as shown in Fig. 7, the opening frequency for a particular channel might not be that low, suggesting that the number of LCCs in the plasma membrane is low.

The reason some channels appear to open more frequently than others could be related to the way caffeine was applied. Since caffeine was applied by pressure ejection through a puffer pipette, the caffeine concentration may be higher in some parts of the cell than in other parts. It is possible that channels in the part of the cell exposed to higher caffeine concentrations tend to open more frequently. Delivering caffeine to the cell through bath perfusion would provide a more uniform exposure to the cell.

It is also possible that the openings we interpreted to be repeated openings of the same channel could have been openings of different channels clustered in such a small space that their different locations could not be distinguished at the optical resolution we used. This is certainly possible, given that we have observed synchronized openings of several channels that, presumably, were located in the same cluster.

In addition, the low apparent density could be due to damage resulting from the cell enzymatic isolation procedure. It has been shown that excessive enzymatic digestion could damage plasma membrane ion channels (for example, see ref. 35). The possibility that some of the channels were damaged and their activation was impaired could explain the sparse locations where frequent LCC openings were observed. This could also contribute to different sensitivities to caffeine observed in different cells: LCCs opened in response to 200 \(\mu\)M caffeine in some cells but required higher concentrations in others. The occurrence of synchronized openings of multiple channels is in agreement with the possibility that the channel density is not quite as low as it appeared.

**Effect of Opening Caffeine-Activated LCCs**

Large doses of caffeine and theophylline can cause tachycardia, arrhythmias, and extrasystoles (4, 18, 34). In severe cases, though rare, caffeine overdose was fatal (3, 19). Mechanisms proposed to underlie these effects include adenosine receptor inhibition, cytosolic Ca\(^{2+}\) increase, catecholamine release, and phosphodiesterase inhibition. A recent study further suggested a mechanism for caffeine-induced arrhythmias involving perturbation of cellular Ca\(^{2+}\) homeostasis (1). Here, our studies suggest a possible additional mechanism: opening the LCCs in the plasma membrane of cardiac myocytes. This possibility requires, first, examining what might happen in an isolated cell. In the isolated myocyte as shown in Fig. 10, the opening of an LCC can depolarize the membrane sufficiently to initiate an action potential. For a cell being driven by an external pacemaker, such an action potential could cause an extra contraction. In addition, a prolonged opening of one or a few LCCs could depolarize the membrane sufficiently to inactivate voltage-gated Na\(^{+}\) channels. Any action potential arising out of such a cell would now be generated by the Ca\(^{2+}\) current alone. Therefore, the opening of LCCs in a single cell would constitute altered electrical activity in that cell.

These effects in a single cell can be extended to the intact heart if concurrent openings of LCCs in a group of cells would produce what would resemble a “triggered response.” The triggered response from a group of cells could then be sufficient to propagate to other cells, resulting in an extrasystole. Moreover, sufficient depolarization in a group of cells could, by inactivating voltage-gated Na\(^{+}\) and Ca\(^{2+}\) channels, lead to a transient one-way block and, therefore, the arrhythmias associated with re-entry.

It is also possible that, in the absence of thapsigargin, the localized Ca\(^{2+}\) increase due to Ca\(^{2+}\) entry during LCC openings could cause Ca\(^{2+}\)-induced Ca\(^{2+}\) release and its associated transient inward current. As such, LCC openings can serve as a trigger for Ca\(^{2+}\) store release, which may lead to cell contraction.

The caffeine-activated LCC appears to be a common finding in isolated ventricular and/or atrial cells from different animals, including rats, mice, rabbits, and guinea pigs. It is important to understand their characteristics and physiologic/pathophysiologic function, because, with a very large conductance and associated Ca\(^{2+}\) influx, as shown in the present studies, single LCC openings can depolarize the cell membrane, significantly increase Ca\(^{2+}\), and lead to cell contraction (Figs. 9 and 10). The seconds-long openings caused by 10 or 20 mM caffeine can be an extreme situation, since cells are not normally exposed to caffeine in such high concentrations. However, the ability of LCCs to open in response to much lower caffeine concentrations (e.g., 200 \(\mu\)M) is pathophysiologically relevant. Heavy coffee drinkers can ingest an average of 1,000 mg of caffeine daily (14), which can bring their serum caffeine concentration close to toxic levels (>129 \(\mu\)M) (24). Such a toxic level can also be reached when someone accidentally uses an excessive amount of caffeine-containing substances (3). Perhaps some individuals are much more sensitive to caffeine than others, just as some cells are more sensitive. It is in the latter two situations that most of the severe consequences could occur. In addition, the degradation of caffeine is slow [the half-life of caffeine is ~5 h (24)], and its metabolic products (paraxanthine, theophylline, and theobromine) are all activators of the LCC (Fig. 3). Furthermore, potential caffeine-stimulated catecholamine release can have a synergic effect on LCC activation (39). All these factors would suggest that, in addition to the generally accepted mechanisms (e.g., adenosine receptor antagonism), opening LCCs may also contribute to the cardiac response to caffeine and other methylxanthines. Further studies are needed to explore the molecular identity of the LCCs and their possible involvement in cardiac function.
addition, any natural ligand or process that opens the LCCs needs to be determined.

ACKNOWLEDGMENTS

We thank Drs. Richard Fenton and John McCullough for helpful discussions and comments on earlier versions of the manuscript. This study was supported by NIH grant AR47067.

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