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Membrane repolarization stops caffeine-induced Ca^{2+} release in skeletal muscle cells

(excitation–contraction coupling/sarcoplasmic reticulum/Ca^{2+}-induced Ca^{2+} release/patch clamp/fura-2)

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ABSTRACT **We have combined the patch-clamp technique with fura-2 measurements to investigate whether the Ca^{2+}-induced Ca^{2+}-release channel is under the control of membrane potential in rat skeletal myoballs. We report that Ca^{2+} release induced by 10 mM caffeine is turned off by membrane repolarization, a phenomenon that we term RISC (repolarization-induced stop of Ca^{2+} release). The RISC phenomenon is voltage- and time-dependent. It is evident only when the release channels are first transferred into a functionally "voltage-activated" state through membrane depolarization. The results demonstrate that membrane repolarization actively closes the caffeine-activated release channels and suggest that the ryanodine receptor is actually the physiological depolarization-induced Ca^{2+}-release channel. Thus, our data provide compelling evidence for a bidirectional voltage control (depolarization and repolarization) of the Ca^{2+}-release channel in the sarcoplasmic reticulum by a voltage sensor in the transverse tubule membrane.**

In skeletal muscle cells, depolarization of the transverse (T) tubular system induces Ca^{2+} release from the sarcoplasmic reticulum (SR), the internal Ca^{2+} store. The Ca^{2+} released from the SR causes muscle contraction (1). This initial step of skeletal muscle excitation–contraction coupling does not seem to require second messengers such as calcium (2–8) or inositol 1,4,5-trisphosphate (9). It is not yet clear, however, how a change in potential across the T-tubule membrane leads to the release of Ca^{2+} from the SR. It is unlikely that physiological Ca^{2+} release is activated directly by the potential change across the SR membrane (10, 11). The current notion about this process is that the dihydropyridine receptor (DHPR), located in the T-tubular membrane, senses a change in membrane voltage and undergoes a molecular rearrangement that is postulated to directly gate the Ca^{2+}-release channel in SR membrane (12–16). Consistent with this is the existence of voltage-dependent intramembrane charge movement (12, 13).

The Ca^{2+}-release channel protein has been purified as the ryanodine receptor (RyR) (17, 18) and morphologically identified with the "foot" structures (19), which span the junctional gap between the SR and T-tubule membranes. Studies in skinned fibers (5, 20–22), fragmented SR vesicles (23, 24), and reconstituted RyR in lipid bilayer membranes (17, 25, 26) have shown that the Ca^{2+}-release channel can be activated by an increase in cytoplasmic free Ca^{2+} (Ca^{2+}-induced Ca^{2+} release, CICR). However, there is no strict functional evidence that the CICR channel protein (RyR) functions as the physiological depolarization-gated Ca^{2+}-release channel. Therefore, it is possible to hypothesize that there exist two different types of Ca^{2+}-release channels in skeletal muscle cells, the depolarization-induced Ca^{2+} release (DICR) channel and the CICR channel (27). In the case of cardiac muscle cells, there seems to be no direct communication between the voltage sensors and the CICR channels (28).

Caffeine is known to potentiate CICR such that resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers Ca^{2+} release from the SR (5, 20, 22). It is well established that the site of action of caffeine is at the RyR (29). The present study shows that Ca^{2+} release induced by caffeine is curtailed by membrane repolarization, a phenomenon that we term RISC (repolarization-induced stop of Ca^{2+} release). The RISC is voltage- and time-dependent and seems to occur only when the release channels are functionally in a "voltage-activated" state, suggesting that the release channel is under bidirectional voltage control of a voltage sensor and that the RyR is indeed the physiological DICR channel.

METHODS

Primary cultures of rat skeletal myoblasts were prepared from forelimbs and hindlimbs of newborn rats (1–3 days old) by slightly modifying the procedure of Beam and Knudson (30). Two to 3 days after fusing the myoblasts to form the myotubes, 25 nM colchicine was added for 2 days to obtain round cells (20–100 μm in diameter), which were viable for electrophysiological experiments for up to 2 weeks. Combined patch-clamp (31) and fura-2 $[Ca^{2+}]_i$ measurements (32) were performed using a computer-controlled data acquisition system (EPC-9). The tight-seal whole-cell configuration was used to control membrane voltage, and liquid junction potential correction (8 mV) was made before seal formation. Experiments were performed at 20–25°C in standard saline solution (145 mM NaCl/2–5 mM $CaCl_2$/1 mM $MgCl_2$/11 mM glucose/10 mM Hepes–NaOH/10 mM tetraethylammonium chloride, pH 7.2). Sylgard-coated patch pipettes had resistances between 1.5 and 2 MΩ after filling with internal solution (solution 1; 145 mM cesium glutamate/6 mM NaCl/0.1 mM fura-2/1 mM NaATP/7 mM MgATP/10 mM Hepes–CsOH, pH 7.2). The free Mg^{2+} concentration was calculated to be ≈0.6 mM, assuming an apparent affinity of 6.9×10^3 M^{-1} for Mg^{2+} to ATP (33). In some experiments, the following solutions were used: 145 mM cesium glutamate, 6 mM NaCl, 0.1 mM fura-2, 5 mM MgATP, 1 mM $MgCl_2$, 10 mM Hepes–CsOH, pH 7.2 (solution 2) and 145 mM cesium glutamate, 6 mM NaCl, 0.1 mM fura-2, 8 mM MgATP, 10 mM Hepes–CsOH, pH 7.2 (solution 3; intracellular Mg^{2+} concentration ≈1 mM). These solutions did not alter the general observations described here. In some experiments, the con-

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Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SR, sarcoplasmic reticulum; T, transverse; CICR, Ca^{2+}-induced Ca^{2+} release; DICR, depolarization-induced Ca^{2+} release; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; RISC, repolarization-induced stop of Ca^{2+} release.

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centration of fura-2 was increased to 0.2–4.0 mM, as indicated in the text or figure legends. Caffeine application was made by local ejection from wide-tipped pipettes. These pipettes contained 10 mM caffeine in nominally calcium-free saline solution in which Ca^{2+} was replaced by an equimolar concentration of Mg^{2+}. $[Ca^{2+}]_i$ was monitored with a photomultiplier-based system where two fluorescence intensities (emission measured at 500 nm, excitation at 360 nm and 390 nm) were sampled at 2–5 Hz by a computer-driven charting program. An *in situ* calibration was made to estimate absolute values of $[Ca^{2+}]_i$ as described (34). We used bis(2-aminophenoxy)ethane-*N*,*N*,*N*^{1},*N*^{1}-tetraacetate (BAPTA) for adjusting $[Ca^{2+}]_i$ in the calibration solutions, assuming a dissociation constant of 225 nM.

RESULTS AND DISCUSSION

Ca^{2+} Release Induced by Depolarization and Caffeine. In skeletal muscle cells, membrane depolarization induces Ca^{2+} release from the SR (DICR). In Fig. 1*A*, we applied long-lasting (10 s) depolarizing pulses from a holding potential of −70 mV to four different test potentials. Upon depolarization, a rapid increase in $[Ca^{2+}]_i$ occurred, which was followed by a gradual decrease in $[Ca^{2+}]_i$. This transient increase in $[Ca^{2+}]_i$ is due predominantly to release of Ca^{2+} from the SR, because removing extracellular Ca^{2+} ($n = 7$) or blocking Ca^{2+} channels with 0.5 mM La^{3+} ($n = 3$) did not significantly affect $[Ca^{2+}]_i$ changes induced by membrane depolarization (15, 35). Furthermore, Ca^{2+} release also occurs close to the reversal potential of Ca^{2+} currents (+80 mV), where Ca^{2+} influx is negligible.

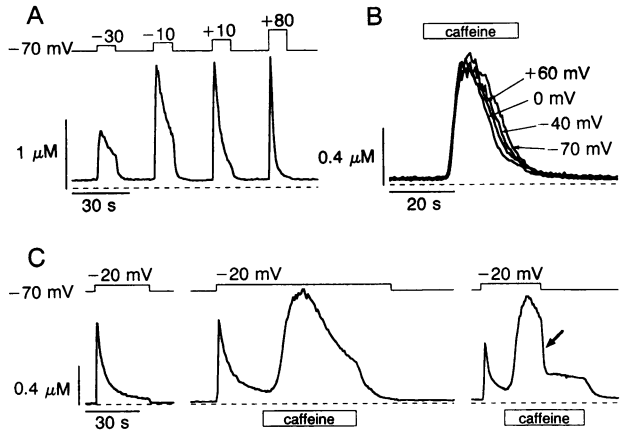

FIG. 1. Repolarization-induced stop of caffeine-induced Ca^{2+} release (RISC). Data shown are representative of a total of 12 (*A*), 4 (*B*), and 22 (*C*) experiments. In all cases, caffeine was applied in nominally Ca^{2+}-free saline (internal solution 1). (*A*) DICR at four different depolarizing pulses of 10-s duration. The upper trace indicates membrane potential change (in mV). The lower trace represents changes in $[Ca^{2+}]_i$ in response to membrane depolarization. Note that the falling phase of $[Ca^{2+}]_i$ becomes steeper at higher potentials. The cell was bathed in standard saline solution (in this case, the extracellular Ca^{2+} concentration was 3 mM). (*B*) Caffeine-induced Ca^{2+} release at different membrane potentials. Data were taken from the same cell and were superimposed by aligning the initial rising phase of $[Ca^{2+}]_i$. Caffeine (10 mM) was applied during the time indicated. (*C Left*) Depolarization-induced Ca^{2+} release at −20 mV. (*C Center*) Caffeine-induced Ca^{2+} release during long-lasting depolarization. The cell was first depolarized to −20 mV, and then 10 mM caffeine was applied while keeping the membrane depolarized. Note that $[Ca^{2+}]_i$ remained at a higher level in the continuous presence of caffeine. After stopping the application of caffeine, $[Ca^{2+}]_i$ rapidly returned to the resting level. (*C Right*) Repolarization-induced stop of caffeine-induced Ca^{2+} release. The same protocol as in *C Center* was used except that repolarization was performed 30 s after depolarization. Note that $[Ca^{2+}]_i$ abruptly dropped upon membrane repolarization despite the continuous presence of caffeine (indicated by the arrow).

The shape and amplitude of the $[Ca^{2+}]_i$ changes varied somewhat from cell to cell, possibly due to differences in the expression level of RyR during cell culture (35), different levels of fura-2 concentration during intracellular perfusion (36, 37), and/or the filling state of the SR (5). Essentially, however, $[Ca^{2+}]_i$ transients were clearly dependent on voltage and became steeper in both rising and falling phases at higher depolarizing potentials. The threshold potential for inducing Ca^{2+} release was invariably between −40 and −50 mV. Even during maintained depolarizations, $[Ca^{2+}]_i$ decayed to resting levels, possibly due to inactivation of DHPRs (13), Ca^{2+}-dependent inactivation of Ca^{2+} release (7, 8, 38), increased Ca^{2+} removal (acceleration of SR Ca^{2+} pumping activity) (5), decreased levels of Ca^{2+} in the SR (5), etc. However, some Ca^{2+}-release activity appears to be maintained during depolarization, since repolarization accelerated the decay of $[Ca^{2+}]_i$ (39), as seen, for example, in Fig. 1*A* (−30 to +10 mV depolarizations).

Ca^{2+} release from the SR can also be activated by the CICR mechanism, and caffeine is a well-known potentiator of this process (5, 20, 22). When the holding potential was increased from −70 mV to different values slowly enough to inactivate voltage sensors, there was no DICR, but caffeine could still induce Ca^{2+} release at any potential. Fig. 1*B* shows that with this protocol, the shape and the amplitude of $[Ca^{2+}]_i$ changes induced by 10 mM caffeine were independent of the membrane potential. The threshold concentration for caffeine-induced Ca^{2+} release was ≈2 mM. The onset of caffeine responses varied from cell to cell, possibly depending on factors such as different expression levels or sensitivities of RyR to caffeine, the filling-state of the SR, and/or different $[Ca^{2+}]_i$ levels.

Repolarization Stops Caffeine-Induced Ca^{2+} Release (RISC). To investigate interactions between the voltage sensor and the CICR channel, we designed experiments in which membrane potential was changed during the caffeine transient (Fig. 1*C*). Fig. 1*C Left* shows a control $[Ca^{2+}]_i$ change induced by −20 mV depolarization for 30 s from a holding potential of −70 mV. In Fig. 1*C Center*, the cell was first depolarized and then 10 mM caffeine was applied during the depolarization, causing a reopening of release channels presumably by the CICR mechanism. In Fig. 1*C Right*, the same protocol as used in Fig. 1*C Center* was applied except that the membrane potential was repolarized to −70 mV right after the peak of the caffeine-induced transient. Surprisingly, $[Ca^{2+}]_i$ abruptly dropped toward resting levels despite the continuous presence of caffeine. Similar results were obtained in 22 cells, some of which showed a complete abrogation of Ca^{2+} release where $[Ca^{2+}]_i$ fully returned to pre-caffeine levels. The onset of this reduction in $[Ca^{2+}]_i$ was extremely fast and occurred within 30 ms as measured in high time-resolution recordings using single-wavelength excitation (data not shown).

This repolarization-induced stop of caffeine-activated Ca^{2+} release (RISC) is not associated with Ca^{2+} influx through Ca^{2+} channels, because in most cases (including Fig. 1*C*), the caffeine solution did not contain Ca^{2+} and the RISC phenomenon was also observed when cells were immersed in nominally calcium-free solution before application of caffeine ($n = 2$). RISC was also observed in the presence of external Ca^{2+} ($n = 5$). It is unlikely that the RISC phenomenon is due to Na^+/Ca^{2+} exchange since similar results were obtained in the presence of 0.5 mM La^{3+}, a blocker of Na^+/Ca^{2+} exchange (40) ($n = 3$), or in the absence of external Na^+ (by replacing the NaCl in standard solution with equimolar tetraethylammonium chloride; $n = 4$).

A simple explanation for these results is that even when DICR is reduced 10 or 20 s after depolarization, caffeine still

enhances Ca^{2+} release from DICR channels presumably by the CICR mechanism and that these channels are suddenly closed upon repolarization. This would suggest that voltage sensors directly regulate the caffeine-gated channels and that the caffeine-activated channel is identical with the DICR channel.

RISC Is Voltage- and Time-Dependent. Fig. 2*A* shows the voltage dependence of the RISC phenomenon using three different long-lasting depolarizing pulses (−40, 0, and +20 mV). In this experiment, RISC was clearly observed at 0 mV but was not very prominent at −40 and +20 mV (Fig. 2*A*). Similar results were obtained in 11 other cells. The most effective depolarizing potential for RISC varied from cell to cell, ranging from −30 to 0 mV. This suggests that the release channels respond to repolarization only when the voltage sensors have transferred them into a functionally voltage-activated state and the voltage sensors are still responsive to repolarization at the time when the cell is repolarized. That is, the release channels may be effectively activated above −30 mV and still be sensitive to membrane repolarization after long-lasting depolarization, whereas they might not be sufficiently activated below −40 mV. With stronger depolarizations, release channels might again be unresponsive to voltage at the time when the cell is repolarized, presumably due to a faster and more complete inactivation of the voltage sensors during long-lasting depolarizations.

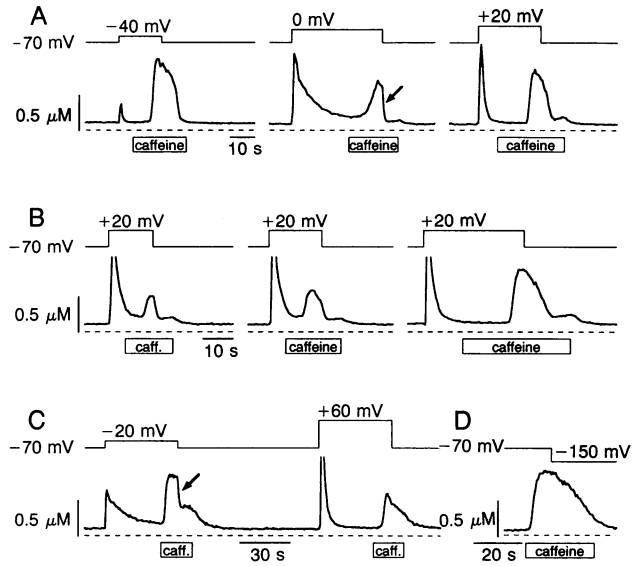

FIG. 2. Voltage and time dependence of RISC after long-lasting depolarizations. (*A*) Voltage dependence of RISC. The upper traces correspond to membrane potential, and the lower traces correspond to $[Ca^{2+}]_i$ changes obtained from a single cell representative of 12 cells. The cell was first depolarized to three different potentials (−40, 0, +20 mV), and then 10 mM caffeine was applied during the time indicated to match the time of repolarization with that of the peak of caffeine response. Note that RISC is clearly observed at 0 mV (indicated by the arrow), while it is not prominent at −40 and +20 mV (internal solution 1). (*B*) Time-dependent inactivation of RISC. The same cell as used in *A* was used, which is representative of 11 cells. The cell was depolarized to the same potential (+20 mV) but with different durations. Note that the time course of the decay of $[Ca^{2+}]_i$ upon repolarization is slowed as the duration of the depolarizing pulse is prolonged. The half-decay times of $[Ca^{2+}]_i$ after repolarization were 0.8 s (*Left*), 1.2 s (*Center*), and 5.5 s (*Right*). (*C*) RISC is not dependent on the size of caffeine transient but exclusively on voltage. The same protocol as used in *B* was used. RISC is observed at −20 mV (indicated by the arrow), while it is not pronounced at +60 mV, despite the smaller caffeine transient (internal solution 1). (*D*) Simple hyperpolarization does not curtail caffeine transient. Ten millimolar caffeine was applied at a holding potential of −70 mV, and then the cell was hyperpolarized to −150 mV (internal solution 1).

To further investigate the time dependence of the RISC phenomenon, we applied caffeine after +20 mV depolarizations of different duration (Fig. 2*B*) and repolarized the membrane at the peak of caffeine-induced Ca^{2+} release. Consistent with our expectations, RISC became less prominent with longer depolarizations (n = 11), suggesting that long-lasting depolarization indeed causes functional decoupling between the voltage sensor and the release channel. An analogous behavior was observed in experiments in which the time interval between a prepulse (−20 or −30 mV) and a test pulse (0 mV) was changed. In this type of experiment, the amplitudes of Ca^{2+} transients at the test pulse decreased faster at prepulse potentials of −20 mV than at potentials of −30 mV (data not shown). These findings seem consistent with the immobilization of charge movement with long-lasting depolarizations in frog skeletal muscle cells (13). Functional inactivation of the release channel by long-lasting depolarizations has also been observed for potassium contractures (41).

RISC showed no apparent dependence on the size of the caffeine-induced Ca^{2+} transients (compare *B* and *C* in Fig. 2), suggesting that the most important determinant of the RISC phenomenon is the sudden change in membrane voltage. However, RISC requires both depolarization and subsequent repolarization; a simple hyperpolarization does not curtail caffeine-induced Ca^{2+} release, as there was no significant change in caffeine-induced Ca^{2+} transients upon hyperpolarization to −100, −120, or −150 mV from a holding potential of −70 mV (n = 6; Fig. 2*D*).

To confirm that caffeine induces Ca^{2+} release by potentiating CICR, we dialyzed cells with 1–2 mM fura-2 (n = 6). Under these strong Ca^{2+}-buffering conditions, the regenerative rising phase of the caffeine response was considerably slowed, while DICR, which happens faster than the temporal resolution of our recordings (2–5 Hz), was not noticeably affected. However, dialyzing cells with 4 mM fura-2 prevented caffeine-induced Ca^{2+} release and also slowed and reduced the magnitude of DICR (n = 2). Furthermore, in the presence of 20 mM procaine, an inhibitor of CICR (5), 10 mM caffeine hardly induced Ca^{2+} release in any state of the release channel (n = 3).

Time-Dependent Activation of RISC. Quantitative assessment of the development of the temporal coupling between voltage sensors and Ca^{2+}-release channels was obtained from experiments in which a strong depolarization (+20 mV) of increasing duration was applied right after the peak of caffeine-induced Ca^{2+} release. The effectiveness of RISC was measured as the required time for reduction of the peak $[Ca^{2+}]_i$ (before depolarization) to 50% after repolarization (half-decay time). Fig. 3*A* shows that RISC is considerably more effective with a 1-s pulse than with a 100-ms depolarization. Fig. 3*C* shows the average half-decay times of caffeine responses upon repolarization as a function of the duration of the depolarizing pulses. Depolarizing pulses of +20 mV require at least 0.5–1 s to maximally curtail caffeine-induced SR Ca^{2+} release. Similar results were also obtained for depolarizing pulses to 0 and +60 mV. These results further support the idea that the release channels in the functionally voltage-activated state are under the control of T-tubule membrane voltage sensors.

The abrogation of the caffeine-induced Ca^{2+} release described above is not caused by the preceding depolarization-induced Ca^{2+} release, since the caffeine-induced Ca^{2+} transient was maintained during longer depolarizing pulses (5–10 s) until the cell was repolarized to −70 mV (Fig. 3*B*). Furthermore, the magnitude of the decay in $[Ca^{2+}]_i$ was strongly reduced, and its time course slowed when the repolarizing potential was changed from −70 mV to −20 mV (Fig. 3*D*). Also, SR Ca^{2+} release induced by flash photolysis of "caged calcium" did not curtail caffeine-induced Ca^{2+}

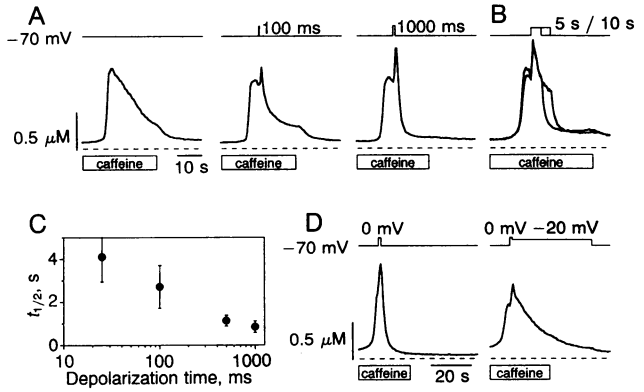

FIG. 3. (*A*) Time-dependent activation of RISC. Data were obtained from the same cell (representative of seven experiments). The upper traces show membrane potential and the lower traces show $[Ca^{2+}]_i$. The cell was first stimulated with 10 mM caffeine during the time indicated to induce SR Ca^{2+} release. Immediately after the peak $[Ca^{2+}]_i$, depolarizing pulses (+20 mV) with increasing durations were applied. (*Left*) Control caffeine response. Note that RISC becomes more obvious with longer duration of the depolarizing pulses (internal solution 1, 0.2 mM fura-2). (*B*) RISC is not due to SR Ca^{2+} release but due to repolarization. Depolarizing pulses (0 mV) with long durations were applied (5 and 10 s). The resulting $[Ca^{2+}]_i$ changes were superimposed to match the onset of depolarization. Note that the longer pulse (10 s) sustained higher $[Ca^{2+}]_i$ levels during depolarization than the shorter pulse (5 s) (internal solution 2, 0.2 mM fura-2). (*C*) Activation curve of the time dependence of RISC. Data are given as mean ± SEM (n = 7) obtained by the protocol illustrated in *A* and plotted as half-decay time of $[Ca^{2+}]_i$ after repolarization as a function of pulse duration (internal solution 1). (*D*) RISC is not observed when the repolarizing potential is set to −20 mV. (*Left*) The cell was depolarized to 0 mV for 1 s after the peak of caffeine transient and then repolarized to −70 mV. (*Right*) The same protocol as used in *Left* was used except that the repolarizing potential was −20 mV (internal solution 3, 0.2 mM fura-2).

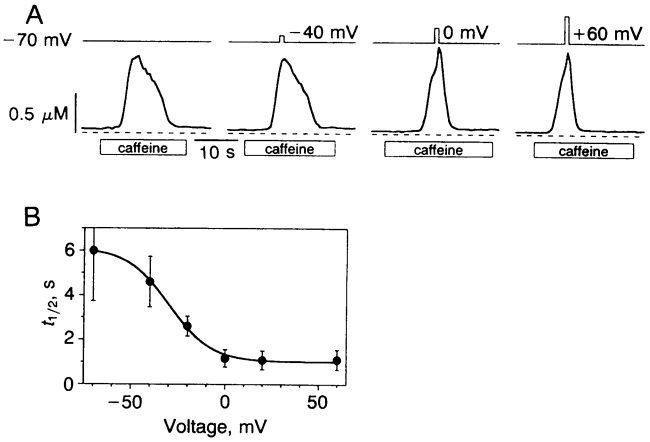

FIG. 4. (*A*) Voltage-dependent activation of RISC. Data are taken from a single cell representative of five experiments (internal solution 1). The protocol was similar to that used in Fig. 3*A*, except that depolarizing pulses of 1-s duration to different potentials were applied (the leftmost panel shows the control caffeine response). Note that RISC became prominent at higher potentials (0 and +60 mV). (*B*) Activation curve for the voltage dependence of RISC. The data points represent the voltage dependence of RISC obtained by the protocol illustrated in *A*. Data are given as the mean ± SEM (n = 5) and plotted as half-decay time of $[Ca^{2+}]_i$ after repolarization as a function of the depolarizing pulse potential. The data were fitted according to $T = \{(T_{max} - T_{min})/[1 + \exp(V_m - V_{1/2})/k]\} + T_{min}$, where T_{max} and T_{min} are the maximum and minimum half-decay times, respectively, V_m is the test potential, $V_{1/2}$ is the potential at which RISC is half-maximal, and k is a slope factor. The fitted values of $V_{1/2}$ and k are −30 mV and 11 mV, respectively.

transients; it was simply superimposed on caffeine-induced Ca^{2+} release (data not shown). Together, these results make it unlikely that RISC is caused by a sudden acceleration of SR Ca^{2+}-pump activity or depletion of Ca^{2+} stores. However, we cannot entirely rule out the possibility that the filling state of the SR may to some extent influence the RISC phenomenon.

Voltage-Dependent Activation of RISC. We quantified the voltage-dependent activation of the RISC phenomenon by experiments in which the membrane was depolarized to different potentials (from −70 to +60 mV) of optimal duration (1 s) after the peak of caffeine-induced Ca^{2+} release. Again, the effectiveness of RISC was measured as the half-decay time. Fig. 4*A* shows that the more positive the depolarizing pulse, the more effective the RISC. Fig. 4*B* shows the average half-decay times of caffeine responses upon repolarization following 1-s depolarizing pulses to different potentials. The data could be described by a Boltzmann function with a midpoint of −30 mV and a slope of 11 mV. It should be stressed that for depolarizing pulse to +60 mV, RISC is very prominent when the pulse duration is 1 s (rightmost panel in Fig. 4*A*) while with long-lasting depolarizations RISC is not observed (compare with Fig. 2*C Right*). This further supports the interpretation that during long-lasting stronger depolarizations, the voltage sensors and/or the release channels may be functionally inactivated.

The voltage dependence of activation of RISC described here (midpoint of activation = −30 mV) is compatible with the existing data derived from charge movement experiments. The midpoints of the voltage dependence of charge movement in mammalian skeletal muscle preparations vary from −49 to −6 mV (42, 43). Since it takes at least 0.5–1.0 s of depolarization to maximally curtail the caffeine-induced $[Ca^{2+}]_i$ transient (see Fig. 3*C*) it seems that full voltage-activation of the SR Ca^{2+}-release channels requires a considerably longer time than that of voltage-dependent charge movement in T-tubular membranes, which is normally considered to cease within 10 ms at most in rat skeletal muscle cells (42) and is not affected by caffeine (44). While short depolarizations may be sufficient to attain maximal rates of Ca^{2+} release, longer depolarizations may still be able to transfer more release channels into a functionally voltage-activated state, producing a more effective manifestation of the RISC phenomenon.

Fig. 5*A* shows that the repolarization-induced closing of release channels in the presence of caffeine is transient. The cell was first stimulated with 10 mM caffeine during the time indicated to induce SR Ca^{2+} release. Immediately after the peak $[Ca^{2+}]_i$, a depolarizing pulse (+20 mV, 1 s) was applied to transfer the RyR into the functionally voltage-activated state. The repolarization abrogated the caffeine effect, but in the continuous presence of caffeine the RyR returned into an activatable state to enter a new round of Ca^{2+} release. Note that $[Ca^{2+}]_i$ abruptly returned to the resting level when removing caffeine, suggesting that the second release phase was due to caffeine and not some other process. Thus, release channels appear to be able to return into a caffeine-activatable state. The dotted line represents a control caffeine transient without depolarizing pulse.

In summary, our results strongly suggest that there exists a bidirectional voltage control of the release channel by voltage sensors in the T-tubule membrane and that the CICR channel (RyR) is indeed the physiological depolarization-induced Ca^{2+}-release channel. Although the detailed mechanism of the RISC phenomenon was not investigated in the present study, the phenomenon itself is very important to further understand the closing mechanism of the release channel as well as the initial step of skeletal muscle excitation–contraction coupling. A scheme that illustrates the observed phenomena is depicted in Fig. 5*B*.

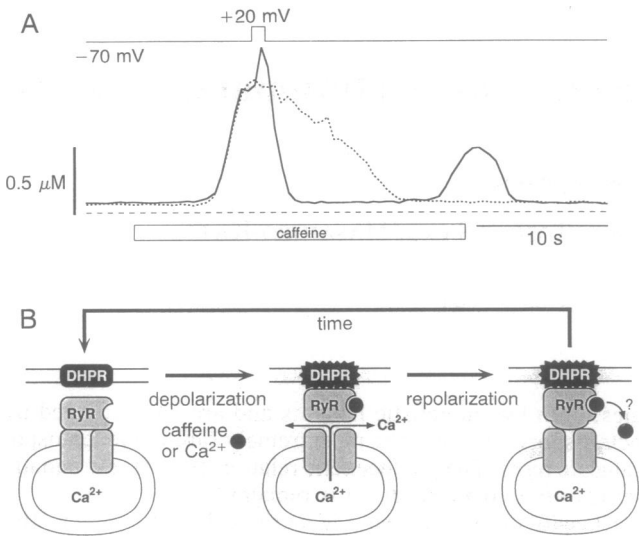

FIG. 5. (*A*) Reactivation of Ca^{2+} release by caffeine after RISC. RISC was induced by a 1-s depolarizing pulse during the sustained application of caffeine. The upper trace shows membrane potential, and the lower trace shows $[Ca^{2+}]_i$. The dotted line represents a control caffeine transient without depolarizing pulse (internal solution 1). (*B*) Schematic diagram of DICR, CICR, and RISC. The conceptual model assumes the involvement of a voltage sensor (presumably the DHPR) in the T-tubule membrane and the Ca^{2+}-release channel (RyR) in the SR membrane. The RyR may be activated to release Ca^{2+} by depolarization or Ca^{2+}/caffeine. A depolarization transfers the RyR into a functionally voltage-activated state. If caffeine is bound to the voltage-activated state, a repolarization renders the RyR functionally insensitive to caffeine (RISC). Whether this is due to unbinding of Ca^{2+}/caffeine or to some other mechanism that closes the release channels is presently unknown. The voltage-activated state in which caffeine-induced Ca^{2+} release is curtailed by repolarization appears to be transient, as prolonged exposure to caffeine can reopen the RyR (*A*), suggesting that with time, the system returns to the resting state. For simplicity, we have omitted the state where RISC is disabled by long depolarizations, presumably through inactivation of the DHPR.

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