Effects of Quercetin on Single Ca^{2+} Release Channel Behavior of Skeletal Muscle

Eun Hui Lee,* Gerhard Meissner,† and Do Han Kim*

*Department of Life Science, Kwangju Institute of Science and Technology (K-JIST), Kwangju 500-712, Korea; and
†Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599-7260, USA

ABSTRACT Quercetin, a bioflavonoid, is known to affect Ca^{2+} fluxes in sarcoplasmic reticulum, although its direct effect on Ca^{2+} release channel (CRC) in sarcoplasmic reticulum has remained to be elucidated. The present study examined the effect of quercetin on the behavior of single skeletal CRC in planar lipid bilayer. The effect of caffeine was also studied for comparison. At very low $[Ca^{2+}]_{cis}$ (80 pM), quercetin activated CRC marginally, whereas at elevated $[Ca^{2+}]_{cis}$ (10 μM), both open probability (P_o) and sensitivity to the drug increased markedly. Caffeine showed a similar tendency. Analysis of lifetimes for single CRC showed that quercetin and caffeine led to different mean open-time and closed-time constants and their proportions. Addition of 10 μM ryanodine to CRC activated by quercetin or caffeine led to the typical subconductance state ($\sim$54%) and a subsequent addition of 5 μM ruthenium red completely blocked CRC activity. When 6 μM quercetin and 3 mM caffeine were added together to the *cis* side of CRC, a time-dependent increase of P_o was observed (from mode 1 (0.376 $\pm$ 0.043, $n = 5$) to mode 2 (0.854 $\pm$ 0.062, $n = 5$)). On the other hand, no further activation was observed when quercetin was added after caffeine. Quercetin affected only the ascending phase of the bell-shaped Ca^{2+} activation/inactivation curve, whereas caffeine affected both ascending and descending phases. [^{3}H]ryanodine binding to sarcoplasmic reticulum showed that channel activity increased more by both quercetin and caffeine than by caffeine alone. These characteristic differences in the modes of activation of CRC by quercetin and caffeine suggest that the channel activation mechanisms and presumably the binding sites on CRC are different for the two drugs.

INTRODUCTION

The generally accepted view concerning the mechanism of excitation-contraction coupling of mammalian skeletal muscle is that excitation, initiated at the cell surface by an action potential, is propagated into the cell through transverse tubule (Ebashi and Endo, 1968; Bers, 1991). Consecutively, the L-type Ca^{2+} channel/dihydropyridine receptor located in the transverse tubule activates by mechanical coupling the sarcoplasmic reticulum (SR) Ca^{2+} release channel (CRC)/ryanodine receptor (Endo et al., 1977; Rios and Pizarro, 1991; Meissner, 1994). The native ryanodine receptor complex is composed of homotetramers, which are associated with FKBP12 in 1:1 molar ratio.

Caffeine, a 1,3,7-trimethylxanthine, is one of the most widely used exogenous activators of the CRC (Rousseau et al., 1988, 1989; Sitsapesan et al., 1990; Hernandez-Cruz et al., 1995; Meissner et al., 1997). In both skeletal and cardiac muscles, caffeine increases channel open probability (P_o) without a change of channel conductance. Caffeine (0.5–2 mM) increases the apparent affinity of the channel activator Ca^{2+} and increases P_o due to a reduced lifetime of the closed state (Rousseau et al., 1988; Sitsapesan et al., 1990). Channel activation by low millimolar caffeine requires the presence of submicromolar Ca^{2+}. However, at higher than 5 mM, caffeine can activate the channel also at picomolar Ca^{2+} by increasing the lifetime of the open channel, which is associated with the appearance of an additional long-lived open state (Sitsapesan et al., 1990). Channels activated by caffeine are characteristically modified by ryanodine, ATP, Mg^{2+}, and ruthenium red (Rousseau et al., 1989; Sitsapesan et al., 1990).

Flavonoids are common constituents of higher plants, with extensive medical uses. According to the review by Formica and Regelson (1995), flavonoids are broad modulators of antioxidants and inhibitors of ubiquitous enzymes (e.g., ornithine carboxylase and protein kinase) (Gschwendt et al., 1983; Nishino et al., 1984; Bindoli et al., 1985; Robak and Gryglewski, 1988; Fewtrell and Gomperts, 1977). They also promote vasodilatation and platelet disaggregation (Harborne, 1967; Beretz et al., 1982). Quercetin (3,3′,4′,5,7-pentahydroxyflavone), a bioflavonoid, is widely distributed in rinds, barks, clover blossoms, and ragweed pollen. Quercetin inhibits the activities of various ATPases such as Ca^{2+}-ATPase in SR (Deters et al., 1975; Kuriki and Racker, 1976; Berton et al., 1980; Shoshan et al., 1980, 1981; Havsteen, 1983; Bull et al., 1989; Fewtrell and Gomperts, 1977). Shoshan et al. (1980) reported that quercetin reversibly inhibited Ca^{2+}-ATPase activity and Ca^{2+} uptake of skeletal SR and caused a slow increase in tension in a skinned fiber loaded with Ca^{2+}.

On the other hand, Kirino and Shimizu (1982) reported that quercetin stimulated Ca^{2+} release from fragmented SR in the absence of extravesicular Ca^{2+}, although they did not provide any direct evidence for a specific Ca^{2+} efflux

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Address reprint requests to Do Han Kim, Ph.D., Kwangju Institute of Science and Technology (K-JIST), Department of Life Science, 1 Oryong-dong, Puk-gu, Kwangju, 500-712, Korea. Tel: 82-62-970-2485; Fax: 82-62-970-3411 or 2484; E-mail: dhkim@kjist.ac.kr

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pathway. Watras et al. (1983) demonstrated that quercetin increased Ca^{2+} release from skeletal SR in the presence of 50 mM inorganic phosphate. Palade et al. (1983) also showed an enhancement of spontaneous Ca^{2+} release from SR by quercetin when the SR was loaded in the presence of 100 mM inorganic phosphate. Kim et al. (1983) first reported that quercetin caused caffeine-like Ca^{2+} release from fragmented rabbit skeletal SR. However, the effect of quercetin on single CRC remains to be investigated.

In this work, we have studied the effects of quercetin on the single channel behavior of the skeletal muscle CRC using the planar lipid bilayer method. For comparison, the effects of caffeine on single CRCs were also determined. The results show that both quercetin and caffeine activate CRC at the single channel level. However, major differences in the modes of channel activation suggest that quercetin and caffeine activate the skeletal muscle CRC by two different mechanisms.

MATERIALS AND METHODS

Isolation of SR vesicles

A heavy fraction of fragmented SR vesicles representing junctional SR was prepared from rabbit fast twitch back and leg muscles using the procedure of Kim et al. (1983).

Planar lipid bilayer method

Single channel recordings of rabbit skeletal CRC incorporated into planar lipid bilayers were carried out as described previously (Miller et al., 1976; Smith et al., 1986; Oba et al., 1996). Lipid bilayers consisting of brain phosphatidylethanolamine and phosphatidylserine (1:1) in decane (20 mg/ml) were formed across a hole of ~200-μm diameter. Thinning of the bilayer film was monitored by bilayer capacitance. The basic composition of *cis*/*trans* solution was 1 M KCl, 109 μM $CaCl_2$, 100 μM EGTA ($[Ca^{2+}]_{free}$ = 10 μM), and 10 mM K-HEPES, pH 7.3 (Meissner et al., 1997). After bilayer formation, SR vesicles (1–10 μg/ml) were added to the *cis* side and gently stirred. Incorporation of ion channels was achieved as described by Miller and Racker (1976). The incorporation of CRCs into the bilayer was confirmed by recording the characteristically high single channel-conductance of the CRCs (Meissner, 1994; see also Fig. 3 *D*). More than 7 of 10 experiments showed the incorporation of a single or multiple CRCs. Multichannel recordings with SR potassium and chloride channels distinguishable in terms of their much smaller conductance than CRCs were disregarded.

The channel was incorporated in a fixed orientation into the bilayer, as checked by its sensitivity to *cis* ATP (Meissner, 1994). The *cis* side of the bilayer corresponded to the cytoplasmic side of the SR membrane, whereas the luminal side of the SR membrane corresponded to the *trans* side. Accordingly all effectors were applied to the *cis* side. The *trans* chamber was held at ground and the *cis* chamber was clamped at 30 mV relative to the ground using a bridge made of 2% agar in 200 mM KCl and Ag/AgCl electrodes. The effects of quercetin, caffeine, ryanodine, ruthenium red, K-ATP, GSSG, and GSH on channel activity were tested by adding aliquots of each stock solution (100 mM caffeine, 10 mM ryanodine, 1 mM ruthenium red, 0.5 M K-ATP, 0.5 M GSSG, and 1 M GSH in water, and 20 or 50 mM quercetin in ethanol) to the *cis* chamber. For quercetin experiments, a freshly prepared quercetin stock solution was used each time, because precipitation could occur during storage period. Solubility of quercetin in aqueous solutions was confirmed by observation of the apparent molar extinction coefficient (ϵ = 562.341 cm^2/mol at λ_{258}). The experiments were carried out at 18 to 23°C.

Solutions with different free Ca^{2+} concentrations were prepared by varying the ratio of [EGTA] and [$CaCl_2$] using the stability constant according to Martell and Smith (1974). Free Ca^{2+} concentrations below 10 μM were obtained by adding 50 mM EGTA aliquots to a solution that contained 109 μM $CaCl_2$. Free Ca^{2+} concentrations above 10 μM Ca^{2+} were obtained by adding 50 mM or 1 M $CaCl_2$ aliquots to a solution that contained 100 μM EGTA. Free $[Ca^{2+}] > 10$ μM were confirmed with a Ca^{2+} electrode (Orion Research Inc.) and using serial dilutions of an Orion Ca^{2+} standard solution.

EC_{50} values for the CRC agonists were calculated using Hill equation: $P_o = P_{o\ ini} + ((P_{o\ max} - P_{o\ ini})/(1 + (EC_{50}/X)^{nH}))$, in which $P_{o\ ini}$ and $P_{o\ max}$ are the initial and maximal P_o values; X is concentration of drug; EC_{50} is X for one-half maximal activation; nH is the Hill coefficient for the activation. EC_{50} value for the bell-shaped Ca^{2+} dependent curve was calculated using the same Hill equation, in which $P_{o\ ini}$ and $P_{o\ max}$ are the initial and maximal P_o (P_o at the peak point of the Ca^{2+} dependent curve) values. For IC_{50} value of the Ca^{2+} dependent curve, we used the equation: $P_o = P_{o\ resi} + ((P_{o\ max} - P_{o\ res})/(1 + (X/IC_{50})^{nH}))$, in which $P_{o\ res}$ and $P_{o\ max}$ are the residual and maximal P_o (P_o at the peak point of the Ca^{2+} dependent curve) values; IC_{50} is Ca^{2+} concentration for one-half maximal inhibition; nH is the Hill coefficient for the inhibition. The graph fittings were carried out using the "Origin" computer software.

Single channel data acquisition and analysis

Single channel currents were displayed on an oscilloscope through a patch-clamp amplifier (Axopatch 200 B amplifier), filtered at 1 kHz using a 4-pole low pass Bessel filter, recorded with a 16-bit VCR-based acquisition and digital tape recorder (Biologic DTR-1205), and digitized at 2 kHz for analysis. Mean open probability (P_o) of channels and the lifetime of open and closed events were determined by 50% threshold analysis using Axon Instruments software and hardware (pClamp v6.0.3, Digidata 1200 AD/DA interface).

Single channel P_o was obtained from 120-s continuous recordings. Lifetime analysis was carried out only when a single channel was incorporated into the bilayer. Individual life times were fitted to a probability density function by the method of maximal likelihood according to the equation: $F(t) = \Sigma P_n \times (1/\tau_n) \times \exp(-t/\tau_n)$, in which P_n and τ_n are the relative areas of the distribution and time constants of the n^{th} state, respectively (pClamp6 v6.0.3, pSTAT).

[^{3}H]ryanodine binding assay

Equilibrium ryanodine binding to SR was performed by incubation of 0.05 mg of SR in 250 μl of reaction mixture containing 0.2 M KCl, 20 mM MOPS, 10 nM [^{3}H]ryanodine, and 10 μM-free Ca^{2+} (pH 7.3) for 2 h at 37°C (Kim et al., 1994). Caffeine- and/or quercetin-activated ryanodine binding was measured in the presence of caffeine in various concentrations with or without 10 μM quercetin. One-hundred microliters of polyethyleneglycol solution (30% polyethyleneglycol, 1 mM EDTA, and 50 mM Tris, pH 7.3) was added to each vial, and incubation was continued for 5 min at room temperature. Precipitated protein was sedimented for 5 min at 14,000 rpm in an Eppendorf microcentrifuge, and the pellets were rinsed twice with 0.4 ml of the relevant ryanodine binding buffer without radioactive ryanodine. The pellets were then solubilized in 100 μl of Soluene 350 (Packard) at 70°C for 30 min, and the solution was counted in 4 ml of Picofluor (Packard) by liquid scintillation. For nonspecific binding, a 100-fold amount of nonradioactive ryanodine (Calbiochem) was included.

Statistical analysis

Results are given as mean ± SE with the number of experiments in parentheses. The mean ± SE is included within the figure symbol or indicated by error bars if it is larger. Significance levels of the differences were analyzed by paired or unpaired *t*-test (GraphPad InStat, v 2.04). Differences were considered to be significant when $p < 0.05$.

RESULTS

Quercetin is a potent activator of CRC in the presence of optimal cytosolic Ca^{2+}

The effect of quercetin on behavior of single CRC was tested by the planar lipid bilayer method in the presence of two different Ca^{2+} concentrations (80 pM and 10 μM) (Fig. 1), and compared with that of caffeine (Fig. 2), a well-known CRC activating drug (Endo, 1977). At 80 pM *cis* Ca^{2+}, the open probability (P_o) of CRC increased gradually with increasing *cis* quercetin concentration, however, no apparent saturation was attained even at 0.7 mM quercetin (Fig. 1 *C*). On the other hand, at 10 μM Ca^{2+}, a much higher maximal P_o (0.89 ± 0.01) was reached at ~250 μM quercetin (EC_{50} = 91.0 ± 15.3 μM) (Fig. 1 *C* and Table 1). In the presence of 10 μM Ca^{2+}, the Hill coefficient (nH) for activation of CRC was close to 1, suggesting that there is no cooperative binding of quercetin on single CRC. The effects of various quercetin concentrations on CRC were attained within 10 s of stirring time, and there was no time-dependent change of P_o afterwards.

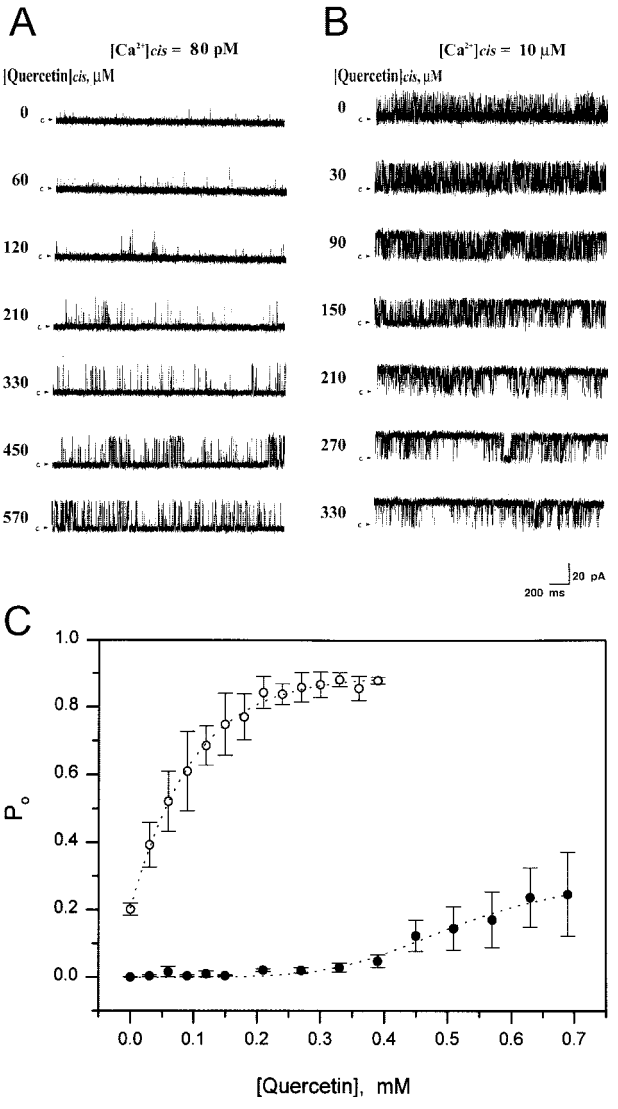

FIGURE 1 Single skeletal CRC activated by quercetin. Single channel activity during the application of quercetin in the presence of 80 pM (*A*) or 10 μM (*B*) free Ca^{2+} was measured after incorporation into planar lipid bilayer. Single channel currents shown as upward deflections from the closed level (marked *c*), were recorded in symmetrical 1 M KCl, 10 mM K-HEPES, pH 7.3 and 109 μM $CaCl_2$ plus 100 μM EGTA (free [Ca^{2+}] = 10 μM) (*B*) or plus 125 mM EGTA (free [Ca^{2+}] = 80 pM) (*A*). Quercetin was added cumulatively to the *cis* side to obtain the indicated concentrations (*left panel*). Current recordings were obtained at +30 mV. (*C*) P_o versus [quercetin] in the presence of 80 pM (●) or 10 μM (○) Ca^{2+}. The data are the mean ± SE of five experiments.

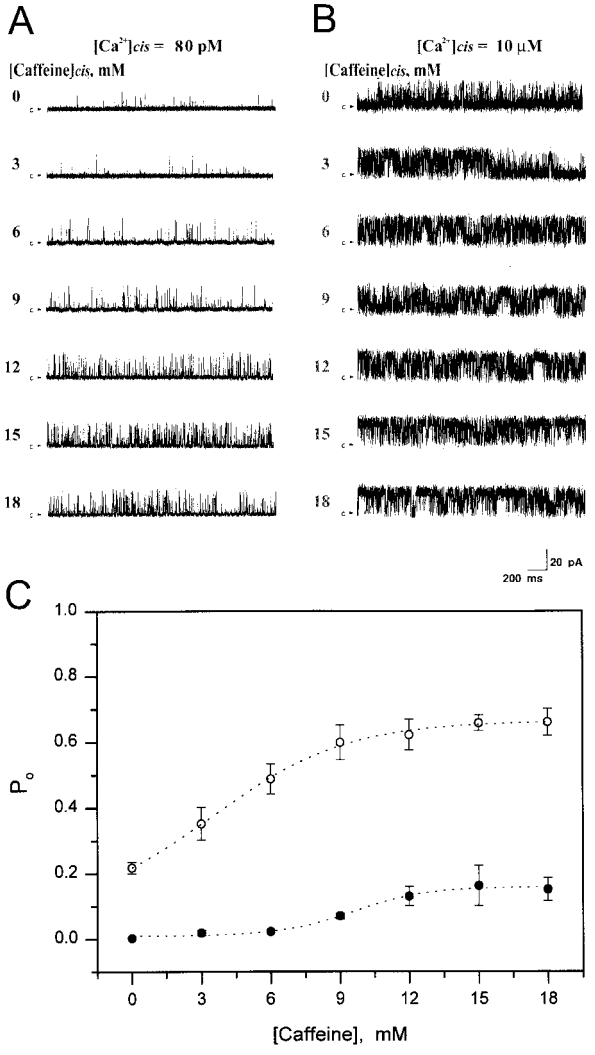

FIGURE 2 Single skeletal CRC activated by caffeine. Single channel activity during the application of caffeine in the presence of 80 pM (*A*) or 10 μM (*B*) Ca^{2+} was measured after incorporation into planar lipid bilayer. Single channel currents shown as upward deflections from the closed level (marked *c*), were recorded in symmetrical 1 M KCl, 10 mM K-HEPES, pH 7.3 and 109 μM $CaCl_2$ plus 100 μM EGTA (free $[Ca^{2+}] = 10$ μM) (*B*) or plus 125 mM EGTA (free $[Ca^{2+}] = 80$ pM) (*A*). Caffeine was added cumulatively to the *cis* side. Current recordings were obtained at +30 mV. (*C*) P_o versus [caffeine] in the presence of 80 pM (●) or 10 μM (○) Ca^{2+}. The data are the mean ± SE of five experiments.

TABLE 1 Ca^{2+}-dependent activation of single CRCs by quercetin or caffeine

Drug	$[Ca^{2+}]_{free}$	Maximal P_o	EC_{50}
Quercetin	10 μM	0.89 ± 0.01*	91.0 ± 15.3 μM
Caffeine	10 μM	0.66 ± 0.01	2.93 ± 0.42 mM

The experiments were carried out, as described in the legend to Fig. 1. Values are the mean ± SE for five experiments.
*Significantly different between caffeine and quercetin groups (unpaired student *t*-test, $p < 0.05$).

Millimolar concentrations of caffeine were required for the maximal activation of the CRC

At 80 pM *cis* Ca^{2+}, the maximal P_o (0.17 ± 0.02) was reached at ~12 mM caffeine (EC_{50} = 9.38 ± 0.72 mM), whereas at 10 μM Ca^{2+}, a much higher maximal P_o (0.66 ± 0.01) was attained at ~9 mM caffeine (EC_{50} = 2.93 ± 0.42 mM) (Fig. 2 *C* and Table 1). However, the maximal P_o attained at 10 μM Ca^{2+} was significantly less than that of quercetin (0.66 ± 0.01 vs. 0.89 ± 0.01). These results indicate that both drugs require an elevated Ca^{2+} concentration for full activation of CRC, and quercetin is a more potent drug than caffeine for activation of CRC (Figs. 1 and 2). In the presence of 10 μM Ca^{2+}, the Hill coefficient (nH) for activation of CRC was close to 1, suggesting that there is no cooperative binding of caffeine on single CRC. On the other hand, at 80 pM Ca^{2+}, the nH value for activation of CRC was close to 5. Thus, it appears that caffeine is bound with a high cooperativity to CRC in the presence of very low cytosolic Ca^{2+} concentrations. However, relatively high caffeine concentrations (>5 mM) were required for activation of CRC in the presence of 80 pM Ca^{2+} (Fig. 2). Similar results were previously obtained in sheep cardiac CRC (Sitsapesan and Williams, 1990). It is important to note that the highest amount of ethanol (~1%) used for solubilization of quercetin did not show a significant effect on the recordings of CRC activities.

Characterization of behavior of single CRC activated by quercetin and caffeine

When quercetin and caffeine were added together to the *cis* side, time-dependent changes in CRC gating mode were observed (Fig. 3 *A*). During the initial 2 min after the addition of both drugs, the apparent gating mode (mode 1: short openings with τ_o = 0.3–0.5 ms (80–90%)) was similar to the one activated by quercetin or caffeine alone (Fig. 3 *A* and Table 2). As time passed (2 min), P_o increased, which appeared to be due to a change in the mode of channel gating (P_o = 0.854 ± 0.062, n = 5) (Fig. 3 *A* and Table 2; mode 2, longer openings with $\tau_o > 5$ ms (~50%)). The period between mode 1 and mode 2 (intermode) was short (<10 s) and during the intermode period, both mode 1 and mode 2 appeared (data not shown). As we shall describe below, mode 1 and mode 2 displayed clearly distinguishable gating properties.

To examine the nature of mode 2, serial treatments of the two drugs were conducted. When 6 μM quercetin and 3 mM caffeine were added sequentially to the *cis* side at 5-min intervals in the presence of 10 μM Ca^{2+}, mode 2 appeared and did not return intermittently to mode 1 (Fig. 3 *B*) (14 times in 15 trials, ~93%). On the other hand, when first 3 mM caffeine and, then after 5 min, 6 μM quercetin were added, we observed no increase in P_o within ~25 min before the spontaneous breakage of the lipid bilayer in 9 of

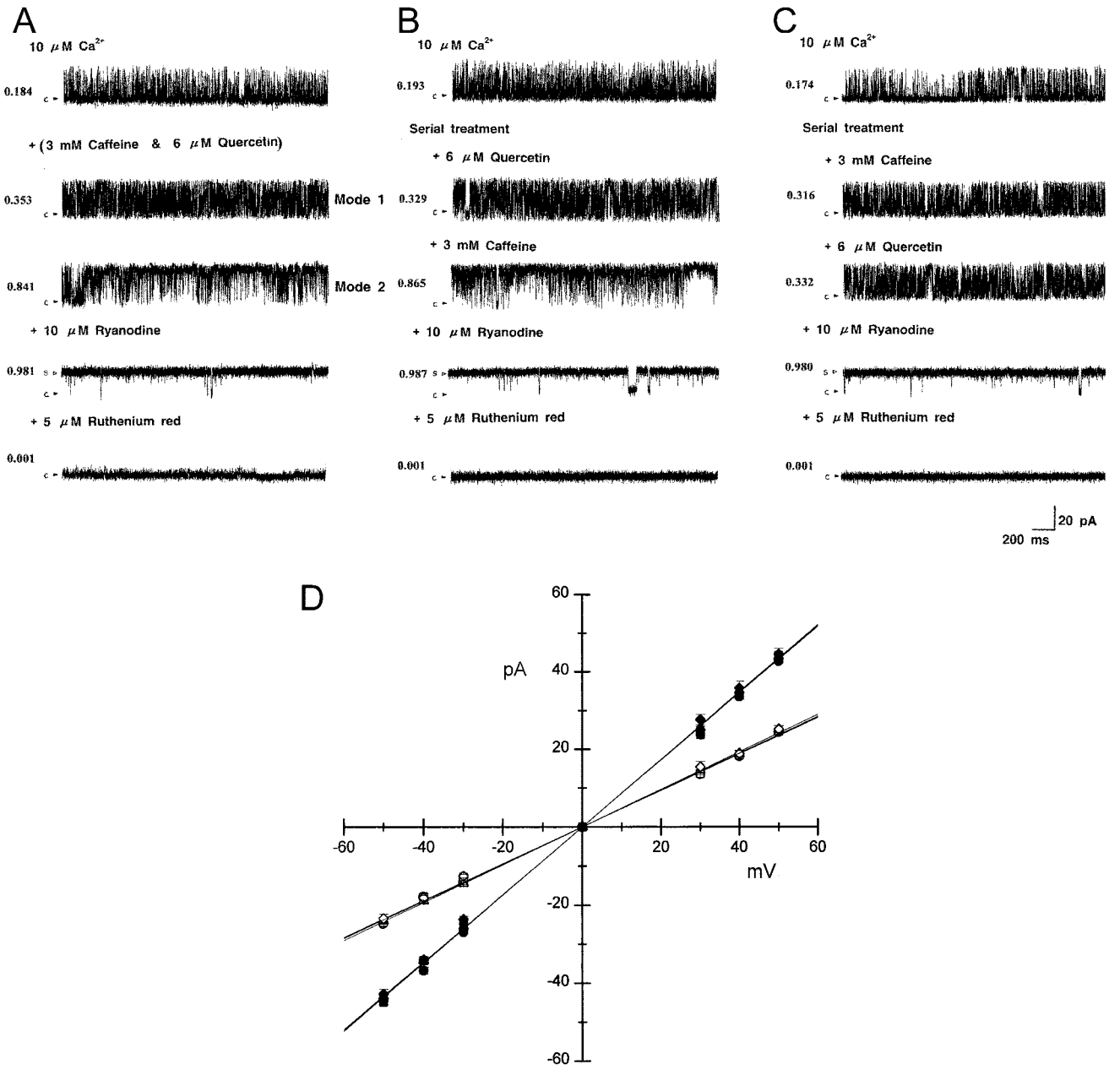

FIGURE 3 Single CRC activated by co-treatment, serial additions of quercetin and caffeine, or caffeine and quercetin. Activation of single CRC by 10 μM Ca^{2+}, 3 mM caffeine, and 6 μM quercetin (*A*), serial addition of 6 μM quercetin and 3 mM caffeine (*B*) or 3 mM caffeine and 6 μM quercetin (*C*), and modification of the activities by 10 μM ryanodine and 5 μM ruthenium red were measured in the presence of 10 μM of *cis* Ca^{2+} by the planar lipid bilayer method. (*D*) I-V curves for each experimental condition. Sub- and full-conductance states are represented by open and closed symbols, respectively. The symbols are: 10 μM Ca^{2+} (○, ●), 10 μM Ca^{2+} and 6 μM quercetin (△, ▲), 10 μM Ca^{2+} and 3 mM caffeine (□, ■), and 10 μM Ca^{2+} and co-treatment (◇, ◆).

11 experiments (Fig. 3 *C*). Two of the 11 experiments showed a smaller increase in P_o compared with that of mode 2 (the increment of P_o was ~18% of the difference between mode 1 and mode 2). Single CRC currents activated by 10 μM Ca^{2+} were locked in a subconductance state by addition of 10 μM ryanodine to the *cis* side (data not shown). Similarly, single CRC currents activated by 6 μM quercetin or 3 mM caffeine in the presence of 10 μM Ca^{2+} were also locked in the subconductance state (data not shown). According to the I-V curves shown in Fig. 3 *D*, regardless of the type of addition, 10 μM ryanodine led to the formation of a subconductance state (~54%), indicating that drug binding to the CRC did not interfere with the ryanodine-induced conductance change. Additional treatments with 5

TABLE 2 Mean open and closed times of single CRCs in various experimental conditions

Types of Experiment	p_o	Open Events, (S^{-1})	Open		Closed	
			τ_o (ms)	Proportion (%)	τ_c (ms)	Proportion (%)
10 μM Ca^{2+} (26)	0.174 ± 0.069	580 ± 103	0.292 ± 0.064	92.1 ± 3.8	0.701 ± 0.088	80.9 ± 3.4
			1.817 ± 0.098	7.9 ± 3.8	8.420 ± 0.201	19.1 ± 3.4
10 μM Ca^{2+} + 6 μM quercetin (5)	0.316 ± 0.032*	704 ± 91	0.520 ± 0.012	91.2 ± 4.3	0.354 ± 0.032	24.4 ± 3.5
			2.679 ± 0.045	8.8 ± 4.3	1.257 ± 0.201	75.6 ± 3.5
10 μM Ca^{2+} + 3 mM caffeine (5)	0.312 ± 0.021*	844 ± 108	0.302 ± 0.032†	79.9 ± 3.1	0.435 ± 0.055	95.6 ± 4.7†
			1.886 ± 0.131†	20.1 ± 3.1	5.827 ± 0.131†	4.4 ± 4.7†
10 μM Ca^{2+} + co-treatment (5)						
Mode 1	0.376 ± 0.043*	790 ± 121*	0.478 ± 0.045	91.3 ± 8.1	0.679 ± 0.042	87.5 ± 7.3
			2.187 ± 0.212	8.7 ± 8.1	2.589 ± 0.119	12.5 ± 7.3
Mode 2	0.854 ± 0.062**	134 ± 137**	0.745 ± 0.019**	53.7 ± 12.1**	0.316 ± 0.020**	90.7 ± 8.9
			5.432 ± 0.261**	46.3 ± 12.1**	2.499 ± 0.107	9.3 ± 8.9
10 μM Ca^{2+} + 0.3 mM quercetin (5)	0.826 ± 0.049	951 ± 109‡	1.343 ± 0.067‡	22.1 ± 11.4‡	0.537 ± 0.046‡	89.3 ± 8.9
			2.579 ± 0.241‡	77.9 ± 11.4‡	2.427 ± 0.114	10.7 ± 8.9
{10 μM Ca^{2+} + both drugs} − quercetin (3)	0.327 ± 0.055	863 ± 188	0.320 ± 0.044	89.2 ± 3.7	0.504 ± 0.052	79.4 ± 4.8
			1.690 ± 0.112	10.8 ± 3.7	4.470 ± 0.132	20.6 ± 4.8
{10 μM Ca^{2+} + both drugs} − caffeine (3)	0.332 ± 0.054	785 ± 101	0.491 ± 0.035	89.0 ± 6.9	0.304 ± 0.079	39.3 ± 10.1
			2.993 ± 0.061	11.0 ± 6.9	1.099 ± 0.081	60.7 ± 10.1

Values are the means ± SE for N number of channels shown in parenthesis. Each drug experiment was paired with 10 μM Ca^{2+} and the paired data of 10 μM Ca^{2+} were used for paired student t-test. The overall combined data for 10 μM Ca^{2+} are shown in the table for reference.

*Significantly different between 10 μM Ca^{2+} and 10 μM Ca^{2+} + 6 μM quercetin, 10 μM Ca^{2+} + 3 mM caffeine or 10 μM Ca^{2+} + co-treatment for P_o (paired t-test, $p < 0.05$).

**Significantly different between mode 1 and mode 2 for P_o, open events, mean open-time, and closed-time constants (paired t-test, $p < 0.05$).

†Significantly different between 10 μM Ca^{2+} + 6 μM quercetin and 10 μM Ca^{2+} + 3 mM caffeine for mean open-time and closed-time constants (unpaired t-test, $p < 0.05$).

‡Significantly different between 10 μM Ca^{2+} + co-treatment (mode 2) and 10 μM Ca^{2+} + 0.3 mM quercetin for open events, mean open-time, and closed-time constants (unpaired t-test, $p < 0.05$).

μM ruthenium red led to complete channel closing in all three cases (Fig. 3, *A–C, n* = 5).

To examine whether the high P_o shown in mode 2 was due to an irreversible modification of CRC, we first removed quercetin by perfusion of the *cis* chamber with a solution containing 3 mM caffeine, followed by removal of caffeine using a caffeine-free perfusion solution (Fig. 4 *A*, *n* = 3). P_o was reduced from 0.898 ± 0.083 to 0.327 ± 0.055 (which was not significantly different from that of 3 mM caffeine in the presence of 10 μM Ca^{2+}) after removal of quercetin, and to 0.192 ± 0.011 (which was not significantly different from that of 10 μM Ca^{2+}) after further removal of caffeine (Fig. 4 *A*). In addition, sequential removal of both drugs in reverse order (caffeine first and quercetin second), showed similar results (Fig. 4 *B*, *n* = 3). According to the open-time and closed-time constants determined below (Table 2), overall patterns of their gating modes were also regained in each step by the sequential perfusions of both drugs. These results suggest that under the experimental conditions of this study, the appearance of mode 2 was not due to an irreversible modification of CRC.

Analyses of P_o and lifetime of single CRC activated by quercetin, caffeine, or both

To obtain a clearer picture of the single channel events leading to the appearance of mode 2, we analyzed the effects of caffeine, quercetin, or both on P_o and the lifetimes of single CRCs (Table 2). Addition of 6 μM quercetin or 3 mM caffeine in the presence of 10 μM Ca^{2+} changed the number of open events per second from 525 ± 59 to 704 ± 91 or from 571 ± 88 to 844 ± 108. The major mean open-time (τ_{o1}) and closed-time (τ_{c2}) constants in the presence of quercetin were 0.520 ± 0.012 ms (91.2 ± 4.3%) and 1.257 ± 0.201 ms (75.6 ± 3.5%), respectively. However, the major respective τ_{o1} and τ_{c1} in the presence of caffeine were 0.302 ± 0.032 ms (79.9 ± 3.1%) and 0.435 ± 0.055 ms (95.6 ± 4.7%), which are significantly different from those of quercetin. It is important to note that 6 μM quercetin (only 7% of EC_{50}) leads to a larger open time constant than 3 mM caffeine (EC_{50}). These data indicate that the two drugs activated the CRC differently, even though their P_o values were not significantly different (quercetin, 0.316 ± 0.032; caffeine, 0.312 ± 0.021) (Table 2). Co-treatment of caffeine and quercetin changed the number of open events per second from 551 ± 71 to 790 ± 121 in mode 1. However, a switch from mode 1 to mode 2 decreased significantly the number of open events per second from 790 ± 121 to 134 ± 137, which was due to the formation of long open events (τ_{o2} = 5.432 ± 0.261 ms, 46.3 ± 12.1% and τ_{c1} = 0.316 ± 0.020 ms, 90.7 ± 8.9%)) (Table 2, mode 2).

Fig. 1 and Table 2 show that in the presence of 10 μM Ca^{2+}, 0.3 mM quercetin led to an almost maximal P_o

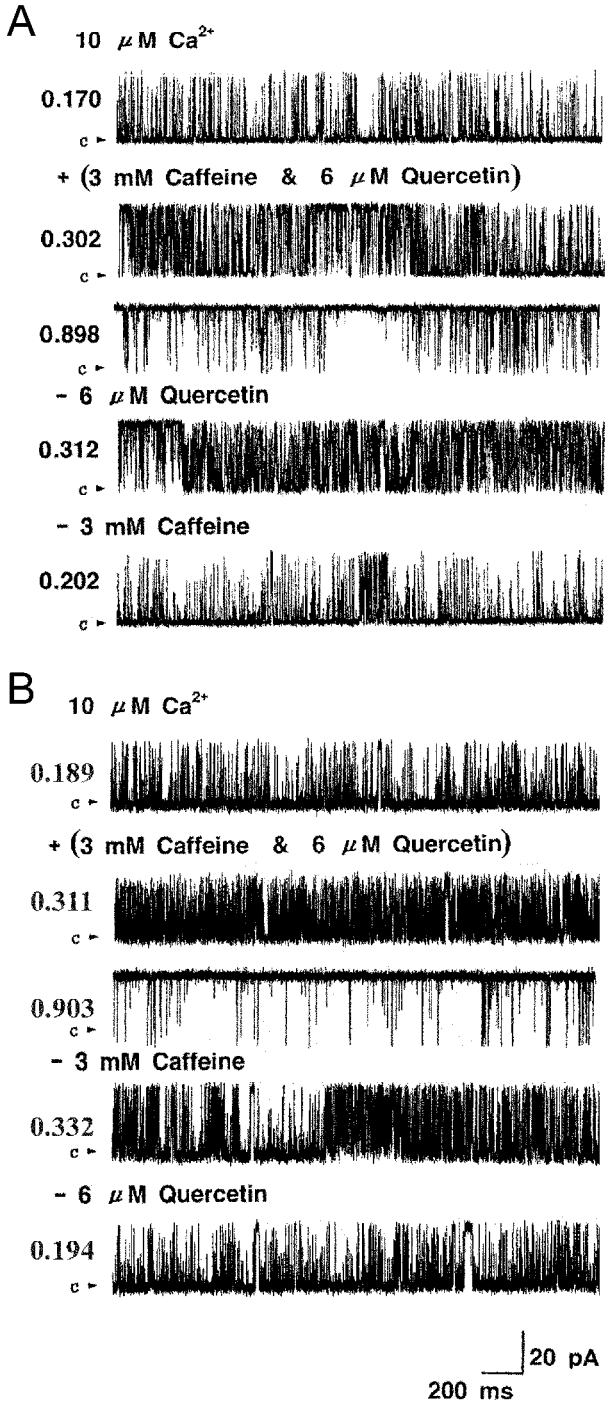

FIGURE 4 Effects of sequential removal of quercetin and caffeine (*A*) or caffeine and quercetin (*B*) after co-treatment of both drugs on CRC activity by perfusion of *cis* solutions. P_o of each state is given on the left side. Single channel currents shown as upward deflections from the closed level (marked *c*) were recorded in symmetrical 1 M KCl, 10 mM K-HEPES, pH 7.3, and 10 μM *cis* Ca^{2+} (*top trace*), 30 s (*second trace*), and 3 min (*third trace*) after the addition of 3 mM caffeine and 6 μM quercetin, and after sequential removal of quercetin and caffeine (*A*) or caffeine and quercetin (*B*) by perfusion (*fourth* and *fifth trace*). Current recordings were obtained at +30 mV. The data are the mean ± SE of three experiments.

(0.826 ± 0.049) that was not significantly different from that of mode 2 (0.854 ± 0.062). However, the major open times for the two conditions were significantly different (0.3 mM quercetin: τ_{o2} = 2.579 ± 0.241 ms, 77.9 ± 11.4% vs. mode 2: τ_{o2} = 5.432 ± 0.261 ms, 46.3 ± 12.1%), suggesting that the mechanism for the apparent maximal activation of single CRCs by quercetin plus caffeine is different from that by quercetin alone.

Quercetin and caffeine affect Ca^{2+} dependence of CRC differently

Fig. 5 compares the Ca^{2+}-dependence of CRC activity in the absence and presence of quercetin or caffeine. Fig. 5, *A* and *D* show the typical bell-shaped Ca^{2+}-dependence of channel activity (Kim et al., 1983; Meissner et al., 1986). When 10 μM quercetin was added to the *cis* side, P_o increased at the ascending phase of the Ca^{2+} activation/inactivation curve but was without effect on the descending phase (Fig. 5, *B* and *D*). The maximal P_o value increased significantly (0.386 ± 0.020 to 0.510 ± 0.011, n = 5), however, the [Ca^{2+}] concentration required to obtain the maximal P_o (Fig. 5 *D*) did not change significantly. Table 3 shows that [Ca^{2+}] for one-half maximal activation (EC_{50}) and one-half maximal inhibition (IC_{50}) of CRC were not significantly altered by quercetin.

On the other hand, when 3 mM caffeine was added to the *cis* side, P_o increased at both the ascending and descending phases (Fig. 5, *C* and *D*). Caffeine did not change significantly the [Ca^{2+}] required to obtain the maximal P_o value (Fig. 5 *D*). Table 3 shows that caffeine did not significantly change EC_{50} for Ca^{2+}, but increased the IC_{50} for Ca^{2+} significantly (530 ± 73 vs. 186 ± 34 μM, n = 5). These results suggest that the low affinity Ca^{2+} binding site(s) of CRC is affected by caffeine but not by quercetin.

[^{3}H]Ryanodine binding in the presence of caffeine and quercetin

The mechanisms of CRC activation by quercetin and caffeine were also assessed by [^{3}H]ryanodine binding, using rabbit skeletal SR in the presence of 10 μM Ca^{2+} and various concentrations of caffeine with (open circle) or without (filled circle) 10 μM quercetin (Fig. 6). Caffeine and quercetin were added at the same time, duplicating the conditions of Fig. 3 *A*. Caffeine increased [^{3}H]ryanodine binding, yielding a B_{max} value of 6.62 ± 0.01 pmol/mg SR protein (n = 5). In the presence of 10 μM quercetin, caffeine increased [^{3}H]ryanodine binding additionally to a maximal value (8.64 ± 0.08, n = 5) without a change in EC_{50} (Table 4). These results indicate that the effects of the two drugs are additive.

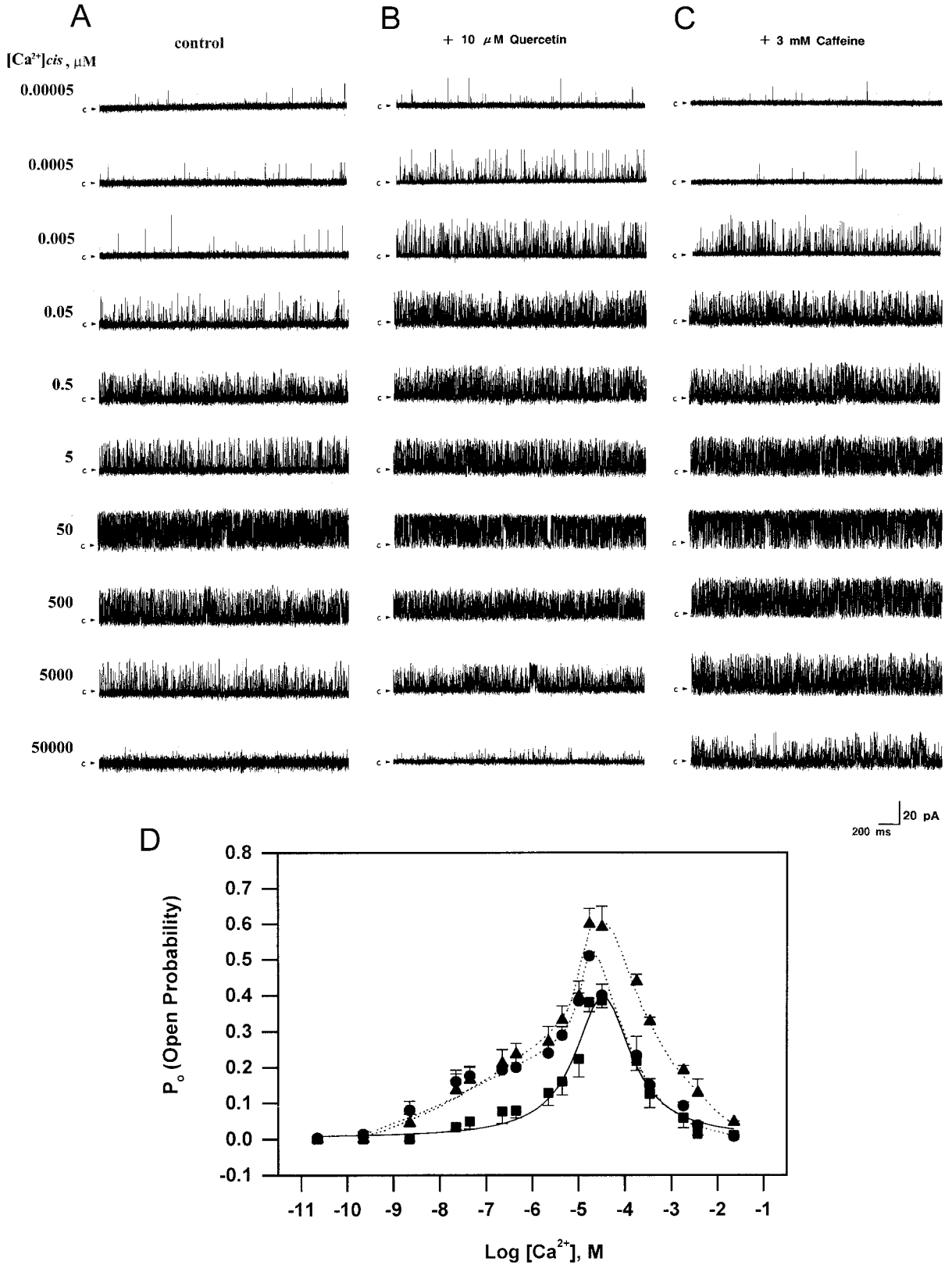

FIGURE 5 Ca^{2+}-dependence of single CRC activity in the presence of quercetin or caffeine. Recordings were made at various [Ca^{2+}] alone (*A*, control) or in the presence 10 μM quercetin (*B*) and 3 mM caffeine (*C*). (*D*) The plots of P_o versus [Ca^{2+}] in the absence (■, control) or presence of 10 μM quercetin (●) and 3 mM caffeine (▲). Single channel currents shown as upward deflections from the closed level (marked *c*) were recorded in symmetrical 1 M KCl and 10 mM K-HEPES, pH 7.3 at indicated [Ca^{2+}]. Current recordings were obtained at +30 mV. The values are the mean ± SE of five independent experiments.

TABLE 3 Ca^{2+} concentrations for one-half maximal activation (EC_{50}) and one-half maximal inhibition (IC_{50}) of P_o in the absence or presence of quercetin and caffeine

Addition	EC_{50} (μM)	IC_{50} (μM)
10 μM Ca^{2+}	5.31 ± 1.81	186 ± 34
10 μM Ca^{2+} + 10 μM quercetin	3.29 ± 1.26	158 ± 29
10 μM Ca^{2+} + 3 mM caffeine	3.17 ± 1.05	530 ± 73*

Values are the mean ± SE of five experiments.
*Significantly different between 10 μM Ca^{2+} and 10 μM Ca^{2+} + 10 μM quercetin or 10 μM Ca^{2+} + 3 mM caffeine groups (unpaired student *t*-test, $p < 0.05$).

TABLE 4 Effects of caffeine on [^{3}H]ryanodine binding in the absence and presence of quercetin

Assay Medium	B_{max}, pmol/mg Protein	EC_{50}, mM
10 μM Ca^{2+}	6.62 ± 0.01	1.24 ± 0.01
10 μM Ca^{2+} + 10 μM quercetin	8.64 ± 0.08*	1.38 ± 0.08

The experiments were carried out, as described in Fig. 6. Values are the mean ± SE of five experiments.
*Significantly different between 10 μM Ca^{2+} and 10 μM Ca^{2+} + 10 μM quercetin groups (unpaired student *t*-test, $p < 0.05$).

Effects of quercetin on single CRC in the presence of glutathione

Glutathione, a low molecular weight peptide, sets the redox potential in cells (Deneke and Fanburg, 1989). To examine whether the effects of quercetin on CRC shown above were related to redox states, partially Ca^{2+}-activated CRCs (1 μM *cis* Ca^{2+}) were exposed to glutathione. In agreement with a previous report (Zable et al., 1997), addition of 5 mM *cis* GSSG, an oxidized form of glutathione, increased P_o from 0.072 ± 0.012 to 0.171 ± 0.022 ($n = 3$) (Fig. 7 *A*). Subsequent addition of 0.3 mM quercetin further increased the P_o to 0.795 ± 0.065 ($n = 3$). This P_o value was similar to that measured for Ca^{2+} and quercetin-activated channels not pretreated with 5 mM GSSG (P_o, 0.782 ± 0.061, $n = 3$). When 5 mM GSH, a reduced form of glutathion, was added to the activated channel, the P_o did not change significantly (0.784 ± 0.073, $n = 3$). The results suggest that the mechanisms for quercetin activation of CRC are likely not directly related to the redox states of CRC.

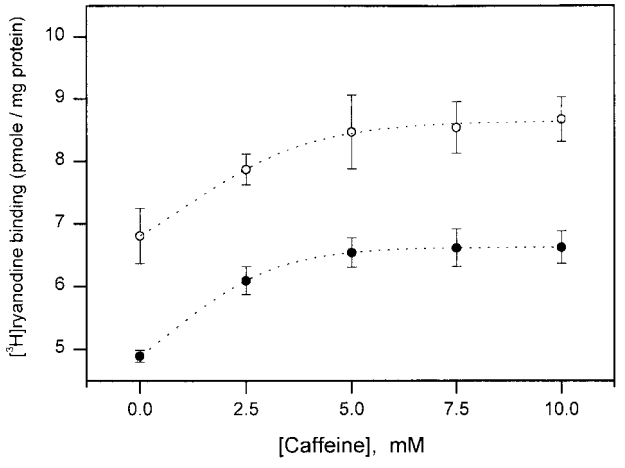

FIGURE 6 [^{3}H]ryanodine binding as function of caffeine concentration in the presence or absence of quercetin. Equilibrium ryanodine binding to SR was performed, as described in Materials and Methods, in 250 μl of a reaction mixture containing 0.2 M KCl, 20 mM MOPS (pH 7.3), 10 nM [^{3}H]ryanodine, 10 μM free Ca^{2+}, 0 (●) or 10 μM quercetin (○), and the indicated concentrations of caffeine for 2 h at 37°C. The values are the mean ± SE of five independent experiments.

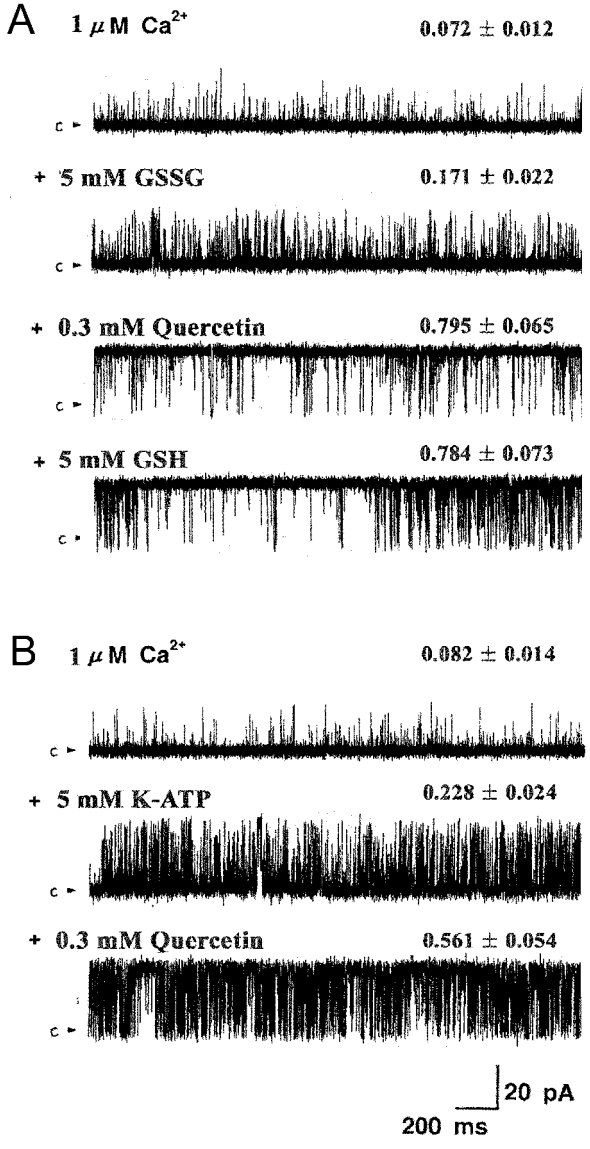

FIGURE 7 Modulation of quercetin-activated single CRC by glutathione or ATP. CRC activated at 1 μM Ca^{2+} was recorded following the sequential addition of 5 mM GSSG, 0.3 mM quercetin, and 5 mM GSH (*A*) or 5 mM ATP and 0.3 mM quercetin (*B*). Single channel currents shown as upward deflections from the closed level (marked *c*) were recorded in symmetrical 1 M KCl and 10 mM K-HEPES, pH 7.3. Current recordings were obtained at +30 mV. P_o are given on the right side. The values are the mean ± SE of three independent experiments.

The hypothesis that quercetin shares the same binding site(s) on CRC with ATP was tested in Fig. 7 *B*. Partially Ca^{2+}-activated single CRCs were first activated by 5 mM K-ATP (P_o, 0.228 ± 0.024, $n = 3$) (Fig. 7 *B*). The subsequent addition of 0.3 mM quercetin led to a further increase of P_o to 0.561 ± 0.054 ($n = 3$). However, this value was significantly lower than the one measured for channels recorded in the presence of 1 μM Ca^{2+} and 0.3 mM quercetin but absence of ATP (P_o, 0.782 ± 0.061, $n = 3$). Thus, it appears that ATP can partially inhibit the action of quercetin on the CRC. This result could be explained if ATP and quercetin share a binding site on the CRC. Alternatively, ATP binding could allosterically influence the effects of quercetin. More extensive biochemical and biophysical studies will be required to elucidate the relationship of the action of ATP and quercetin on CRC activity.

DISCUSSION

Quercetin, a bioflavonoid, is known to increase Ca^{2+} release from SR (Kirino and Shimizu, 1982; Watras et al., 1983; Kim et al., 1983; Palade et al., 1983). Increased Ca^{2+} release by quercetin could be associated with its inhibitory effect on the Ca^{2+} ATPase (Shoshan and MacLennan, 1981; Shoshan et al., 1980) as well as its stimulatory effect on CRC (Kim et al., 1983). However, a direct effect of quercetin on CRC was not established in earlier work. In the present study, the effect of quercetin on CRC was examined at single channel level and compared with that of caffeine, a well-characterized CRC agonist (Endo, 1977).

Although the CRC was activated by both quercetin and caffeine, there were some remarkable differences. Differences in the effects of the two drugs included: 1) quercetin was a considerably stronger agonist than caffeine in terms of EC_{50} (32×) and higher maximal P_o (0.89 ± 0.01 vs. 0.66 ± 0.01) of the dose response curve (Figs. 1 and 2); 2) gating mode of the CRC activated by quercetin was different from that by caffeine (Table 2); 3) sequential addition of quercetin and caffeine, but not caffeine and quercetin, induced time-dependent changes in CRC gating behavior (Fig. 3); 4) quercetin affected only the ascending phase of the Ca^{2+} activation/inactivation curve, whereas caffeine affected both the ascending and descending phase (Fig. 5). These results indicate that quercetin and caffeine are CRC agonists that may have different activation mechanisms, due presumably to different binding sites on CRC or to inducing different conformational changes.

Characterization of single CRC behavior affected by quercetin or caffeine

The apparent affinity of CRC for both quercetin (Fig. 1) and caffeine (Fig. 2) considerably increased by elevating [Ca^{2+}] from 80 pM to 10 μM (EC_{50} = 91.0 ± 15.3 μM in the presence of quercetin and 2.93 ± 0.42 mM in the presence of caffeine, both at 10 μM Ca^{2+} (Table 1). The result suggests that a conformational change of CRC upon binding of Ca^{2+} to its high affinity binding site could lead to an increased drug binding affinity. Similar results for caffeine were reported previously (Endo et al., 1977; Kim et al., 1983; Rousseau et al., 1988; Rousseau and Meissner, 1989; Meissner et al., 1997). The effective concentration for the activation of CRC by quercetin was ~32 times lower than for caffeine (Figs. 1 and 2). The different drug efficacy for activation of CRC is not likely due to lipid solubility of the drugs, because the caffeine-binding site is present on the cytosolic side (Meissner, 1994). An analysis of P_o and the gating mode of single CRC showed that the mean open-time and closed-time constants (τ_o and τ_c) and their proportions were significantly different for the caffeine- and quercetin-activated CRCs, even though P_o values were similar (Table 2).

Ryanodine binding to its high-affinity site(s) stabilizes the open state of CRC, however, open channel conductance is subnormal (Rousseau et al., 1987; Carroll et al., 1991; Pessah and Zimanyi, 1991; Buck et al., 1992). The I-V relations in Fig. 3 *D* show that the conductances and levels of subconductance state formed by 10 μM ryanodine were similar in all cases (Fig. 3), suggesting that quercetin does not modify the ion selectivity of the CRC.

Time-dependent synergistic effect of quercetin and caffeine on single CRC

The simultaneous addition of caffeine and a relatively low concentration of quercetin (6 μM) to the *cis* side of the bilayer induced time-dependent changes in CRC gating (Fig. 3). Two modes (mode 1 and mode 2) having clearly distinguishable gating behaviors were seen (Fig. 3). Mode 2 required the presence of both drugs and had a significantly higher P_o than mode 1 (0.854 ± 0.062 vs. 0.376 ± 0.043). This high P_o was not a simple summation of the effects of caffeine and quercetin, suggesting that their effects on CRC are synergistic. [^{3}H]ryanodine binding (Fig. 6) also suggests that caffeine and quercetin have additive effects on CRC activity. On the other hand, remarkably, the initial addition of caffeine to single CRC rendered the channels insensitive to subsequent modification by quercetin. We propose that caffeine induces conformational changes that perhaps may result in the occlusion of a site for quercetin binding (Fig. 3).

Fig. 4 shows that the sequential removal of quercetin and caffeine or caffeine and quercetin reduced P_o close to the levels measured before the addition of the drugs. This result suggests that the effects of quercetin and caffeine on single CRC are reversible and are not due to an irreversible modification of CRC. Single channel data obtained in the presence of oxidized/reduced glutathione suggest that the effects

of quercetin were largely independent on CRC redox state. It is also important to note that channel activation by high quercetin concentration (Fig. 1 and Table 2) was fast (<10 s) and therefore appeared to occur by a mechanism different from that in the presence of caffeine and 6 μM quercetin.

Quercetin and caffeine showed clearly distinguishable effects on Ca^{2+} concentration-dependence of CRC activation

The mechanisms of quercetin and caffeine action were further studied by examining their effects on the bell-shaped CRC Ca^{2+}-activation/inactivation curve (Fig. 5). Both quercetin and caffeine increased P_o of the ascending phase of the Ca^{2+}-activation/inactivation curve (Fig. 5), whereas only caffeine increased P_o of the descending phase (Fig. 5 *D*). The IC_{50} of Ca^{2+} in the presence of caffeine was significantly higher than the control (530 $\pm$ 73 μM vs. 186 $\pm$ 34 μM) (Table 3), indicating that more Ca^{2+} is required for the same degree of inhibition, if caffeine is present. On the other hand, it appears that when the low affinity Ca^{2+} binding site(s) is (are) occupied by Ca^{2+}, quercetin was without effect (Fig. 5).

CRCs have been reported to switch between different modes of activity: an inactivated mode with no channel openings, a low-activity mode with single channel openings, and a high activity mode with bursts of openings (Zahradnikova and Zahradnik, 1995; Armisen et al., 1996). This study shows that the combined presence of quercetin and caffeine transforms the skeletal muscle CRC from a low to a high activity mode. Pharmacological stabilization of the high-activity mode should help studies aimed at a better understanding of the modal gating behavior of the CRCs.

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