Xestoquinone, Isolated from Sea Sponge, Causes Ca\(^{2+}\) Release through Sulfhydryl Modification from Skeletal Muscle Sarcoplasmic Reticulum\(^1\)

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ABSTRACT

Xestoquinone (XQN) (3 × 10\(^{-7}\) to 3 × 10\(^{-3}\) M), isolated from the sea sponge Xestospongia sapra, induced a concentration-dependent Ca\(^{2+}\) release from the heavy fraction of fragmented sarcoplasmic reticulum (HSR) of rabbit skeletal muscle with an EC\(_{50}\) value of ~30 \(\mu\)M. On the basis of the EC\(_{50}\), XQN is 10 times more potent than caffeine. Dithiothreitol completely blocked XQN-induced Ca\(^{2+}\) release from HSR without affecting that induced by caffeine. Caffeine-induced Ca\(^{2+}\) release was reduced markedly by Mg\(^2+\), procaine, and ruthenium red, agents that are known to block release of Ca\(^{2+}\) from sarcoplasmic reticulum, whereas that induced by XQN was not inhibited. The bell-shaped profile of Ca\(^{2+}\) dependence for XQN was significantly shifted upward in a wider range of pCa (between 7 and 3), whereas that for caffeine was shifted to the left in a narrower range of pCa (between 8 and 7). The maximum response to caffeine in 

\[\text{Ca}^{2+}\text{release}\]

was not affected by 9-methyl-7-bromoeudistomin D, whereas the response was further increased by XQN. XQN caused a concentration-dependent decrease in \[^{3}H\]ryanodine binding to HSR. This effect of XQN also was abolished in the presence of dithiothreitol. Scatchard analysis revealed that the mode of inhibition by XQN was noncompetitive in \[^{3}H\]ryanodine binding to HSR. These results indicate that sulfhydryl groups are involved in both the XQN effect on ryanodine binding and on Ca\(^{2+}\) release.

The ryanodine receptor (RyR), which functions as a Ca\(^{2+}\) release channel of sarcoplasmic reticulum (SR), is postulated to play a key role in excitation-contraction (EC) coupling in skeletal muscle (Shoshan and Ashley, 1998). Depolarization of the sarcolemma leads to stimulation of Ca\(^{2+}\) release from SR by being transmitted down to the RyR from the transverse tubules through dihydropyridine receptors. Following excitation, released Ca\(^{2+}\) is taken up into the lumen of SR by Ca\(^{2+}\)-ATPase and stored mainly by binding to calsequestrin (Wang et al., 1998). Different species and different excitable cells express various RyR isoforms. In mammals, RyR-1 and RyR-2 appear to be expressed predominantly in skeletal muscle and heart, respectively, whereas RyR-3 is expressed in brain, smooth muscle, and epithelial cells (McPherson and Campbell, 1993; Furuichi et al., 1994; Giannini and Sorrentino, 1995). Furthermore, RyR-1 is expressed in sea urchin eggs and may be responsible for Ca\(^{2+}\) signaling in fertilization (McPherson et al., 1992). These reports suggest that RyRs play important roles not only in skeletal muscle but also in nonmuscle cells. In spite of the significance, the detailed regulatory mechanisms of the RyR remain unclear.

A number of thiol reagents, the reactive oxygen species, H\(_2\)O\(_2\) (Oba et al., 1998), emodin (Cheng and Kang, 1998), S-nitrosglutathione (Xu et al., 1998), N-ethylmaleimide (Aghdasi et al., 1997), and glutathione disulfide (Zable et al., 1987) act as stimulators of Ca\(^{2+}\) release channel of SR. These reports suggested that oxidation, alkylation, or nitrosylation of sulfhydryl groups on Ca\(^{2+}\) release channel of SR regulate the channel activation. It was reported that oxidation of sulfhydryl groups to disulfide caused stimulation of Ca\(^{2+}\) release from SR, contraction of skinned muscle fibers (Abramson and Salama, 1989), and alteration in high-affinity \[^{3}H\]ryanodine binding to its receptor (Stoyanovsky et al., 1997). It is still unclear whether oxidation-reduction or disulfide interchange of sulfhydryls on the Ca\(^{2+}\) release channel plays a important role in EC coupling. But an increasing body of evidence suggests that such a mechanism could be an

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ABBREVIATIONS: RyR, ryanodine receptor; EC, excitation-contraction; SR, sarcoplasmic reticulum; XQN, xestoquinone; HSR, heavy fraction of fragmented sarcoplasmic reticulum; LSR, light fraction of fragmented sarcoplasmic reticulum; MOPS, 3-(N-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(\(\beta\)-aminoethyl ether)-N,N,N\(^{'},\)N\(^{'}\)-tetraacetic acid; DTT, dithiothreitol; MBED, 9-methyl-7-bromoeudistomin D.
important element. Although there are a few articles that indicate the relationship between Ca\(^{2+}\) release from SR and sulfhydryl groups, the detailed pharmacological properties of Ca\(^{2+}\) release from SR through sulfhydryl modification have not been revealed.

It has been shown that xestoquinone (XQN) (Fig. 1), having a unique pentacyclic quinone structure, is a novel leading compound for valuable cardiotonic agents because of its unique mechanism of a positive inotropic effect on cardiac muscle (Kobayashi et al., 1991a,b). It also has been reported that XQN activated skeletal muscle actomyosin ATPase by modification of the specific sulfhydryl group in the myosin head (Sakamoto et al., 1995). Herein, we present the first report indicating that XQN powerfully induces Ca\(^{2+}\) release from the heavy fraction of fragmented sarcoplasmic reticulum (HSR) through modification of the crucial sulfhydryls.

**Experimental Procedures**

**Materials.** XQN was isolated from the Okinawan sea sponge *Xestospongia apsa* as described previously (Nakamura et al., 1985). Briefly, the fresh sea sponge was extracted with methanol, and the methanol extract was chromatographed on silica gel columns to yield pure XQN. This compound was dissolved in diethyl sulfoxide and was stored at \(-80^\circ\)C. The substances we used were purchased from the sources indicated: ryanodine (S.B. Penick, New York, NY), \(\text{[3H]}\)ryanodine \((60 \text{ Ci/mmol}; \text{NEN Life Science Products})\). All other chemicals were of analytical grade.

**Preparation of SR Vesicles from Skeletal Muscle.** For all studies, HSR was prepared from skeletal muscle as previously reported (Seino et al., 1991) with slight modification. White skeletal muscle was homogenized in 5 volumes of 5 mM Tris-maleate (pH 7.0) and centrifuged at 5000 \(g\) for 15 min. The supernatant was further centrifuged at 12,000 \(g\) for 30 min. The pellet fraction was suspended in a solution containing 90 mM KCl and 5 mM Tris-maleate (pH 7.0) and centrifuged at 70,000 \(g\) for 40 min. The obtained HSR was suspended in the same solution containing 0.3 M sucrose at \(-80^\circ\)C until use. The protein concentration was determined by the method of Bradford (1976) with BSA as a standard. The light fraction of fragmented sarcoplasmic reticulum (LSR) was prepared from rabbit white skeletal muscle as described by (Seino et al., 1991) with some modification.

**\(45\text{Ca}^{2+}\) Release Experiments.** The \(45\text{Ca}^{2+}\) release from the vesicular HSR or LSR passively preloaded with \(45\text{Ca}^{2+}\) was measured at \(0^\circ\)C as described previously (Seino et al., 1991) with slight modification. After a 12-h preincubation of 20 mg/ml HSR or LSR suspension (5 mM \(\text{CaCl}_2\) at \(0^\circ\)C, HSR or LSR suspension (5 \(g\)) was incubated with 1 nM \(\text{[3H]}\)ryanodine at 37°C for 1 h in a solution containing 0.3 M sucrose, 0.3 M KCl, 100 \(\mu\)M CaCl\(_2\), 20 mM Tris-HCl (pH 7.4), and (p-amidinophenyl) methanesulfonyl fluoride hydrochloride. The amount of \(\text{[3H]}\)ryanodine bound was determined by membrane filtration through Whatman filters (GF/B). Non-specific binding was determined in the presence of 10 \(\mu\)M unlabeled ryanodine.

**Free \(\text{Ca}^{2+}\) Concentration.** The free Ca\(^{2+}\) concentration was maintained with Ca\(^{2+}\)-EGTA buffer (0.55 mM CaCl\(_2\) plus 0.02–0.451 mM EGTA) and was estimated with a microcomputer program that took into account the binding constant for Ca\(^{2+}\)-EGTA; pH, and the concentrations of K\(^{+}\), Mg\(^{2+}\), and nucleotides (Sillen and Martell, 1964, 1971).

**Statistical Analysis.** Data are expressed as means \(\pm\) S.E. Statistical comparisons were made with Student’s \(t\) test for paired data. \(P < .05\) was considered significant.

**Results**

**\(45\text{Ca}^{2+}\) Release from SR Vesicles.** Because in the presence of Mg\(^{2+}\)-ATP and an ATP-regenerating system Ca\(^{2+}\) release is immediately followed by reuptake, we have examined the release of passively loaded Ca\(^{2+}\) in the absence of Mg\(^{2+}\)-ATP (Kim et al., 1983). The effect of XQN and caffeine on Ca\(^{2+}\) release from HSR or LSR were studied in \(45\text{Ca}^{2+}\) release experiments under conditions in which the Ca\(^{2+}\) pump does not work at \(0^\circ\)C. The \(45\text{Ca}^{2+}\) releasing activity of caffeine (1 mM) was significant in HSR (28.1 \(\pm\) 0.3\% of total Ca\(^{2+}\) loaded into HSR), whereas this was slightly observed in LSR (10.9 \(\pm\) 1.7\% of total Ca\(^{2+}\) loaded into LSR). However, XQN (30 \(\mu\)M) induced \(45\text{Ca}^{2+}\) release both from HSR (26.0 \(\pm\) 0.2\% of total Ca\(^{2+}\) loaded into HSR) and LSR (27.6 \(\pm\) 0.5\% of total Ca\(^{2+}\) loaded into LSR) (data not shown). Figure 2 shows the concentration-response curve for XQN and caffeine in \(45\text{Ca}^{2+}\) efflux. \(45\text{Ca}^{2+}\) release from HSR was accelerated markedly by XQN and caffeine in a concentration-dependent manner. Based on the EC\(_{50}\) value, XQN is 10 times more potent than caffeine. As shown in Fig. 3, \(45\text{Ca}^{2+}\) release induced by caffeine (1 mM) was not changed by dithiothreitol (DTT) (1 mM). However, the amount of \(45\text{Ca}^{2+}\) release induced by XQN (30 \(\mu\)M) was markedly inhibited by it (30 \(\mu\)M, 1 mM). Figure 4 shows the effects of typical inhibitors of Ca\(^{2+}\) release on XQN and caffeine-induced \(45\text{Ca}^{2+}\) release. Interestingly, \(45\text{Ca}^{2+}\) release induced by caffeine (1 mM) was inhibited by those blockers of Ca\(^{2+}\)-induced Ca\(^{2+}\) release such as Mg\(^{2+}\) (Fig. 4A), procaine (Fig. 4B), and ruthenium red (Fig. 4C), respectively, in a concentration-dependent manner, whereas \(45\text{Ca}^{2+}\) release that induced by XQN (30 \(\mu\)M) was not inhibited by them. As shown in Fig. 5, XQN (10 \(\mu\)M)
and caffeine (1 mM) potentiated 45Ca2+ release from HSR vesicles by XQN and caffeine. 45Ca2+ release was measured at pCa 7 as described in Experimental Procedures. The amount of released Ca2+ was calculated from the decrease in the 45Ca2+ content in HSR vesicles during 1 min after dilution. Each value was obtained by subtracting the amount of released 45Ca2+ measured in the absence of the test substances from that measured in its presence. Caffeine (○); XQN (●). Values are means ± S.E. (n = 3).

μM) and caffeine (1 mM) potentiated 45Ca2+ release from HSR with a bell-shaped profile of Ca2+ dependence, whereas both the patterns were different from each other. The sensitivity of Ca2+ for the channels increased in the presence of caffeine at concentrations of pCa 7 and 8, whereas it was not changed at pCa 6 to 3. However, XQN shifted that upward in a wider range of pCa 7 to 3.

The interrelations between the Ca2+-releasing activities of XQN, 9-methyl-7-bromoeudistomin D (MBED), and caffeine were examined by measuring the 45Ca2+ release from HSR. The additional application of MBED (1 μM) did not increase the maximum response of caffeine (30 mM) (Fig. 6). In contrast, 45Ca2+ release induced by caffeine was significantly increased by XQN (30 μM).

**[3H]Ryanodine Binding to HSR.** [3H]ryanodine binding to HSR was examined in the presence or absence of XQN. XQN caused a concentration-dependent decrease in [3H]ryanodine binding to HSR (Fig. 7). Figure 8 shows a typical saturation curve (A) and its corresponding Scatchard plots (B) of [3H]ryanodine binding to HSR in the presence or absence of XQN (5 μM). XQN was shown to decrease [3H]ryanodine binding noncompetitively. The B_max value was decreased from 28.3 to 19.4 pmol/mg by adding XQN, whereas the K_D value was unaffected (10.02 nM for control and 9.95 nM with XQN). XQN (30 μM) induced a marked decrease in [3H]ryanodine binding to HSR in a wider range of pCa 7 to 4 (Fig. 9). Moreover, we investigated the effect of DTT on XQN-induced [3H]ryanodine binding to HSR to demonstrate...
Xestequinone Causes Ca$^{2+}$ Release through Sulphydryl Modification

Discussion

The fundamental idea that sulphydryl-to-disulfide conversion of cysteine residues on various ion channels serves as a regulatory mechanism of their activity is becoming increasingly documented and persuasive. Regulatory sulphydryls have been demonstrated in K$^+$ channels (Lei et al., 1992), N-methyl-D-aspartate receptor-channel complex (Ruppersberg et al., 1991), the inositol 1,4,5-triphosphate receptor (Kaplin et al., 1994), and the RyR (Zaidi et al., 1989; Salama et al., 1992). The RyR generally known as a Ca$^{2+}$-releasing channel may be the physiological mechanism of EC coupling in skeletal muscle (Shoshan et al., 1998). Recently, it has been reported that sulphydryl groups have important roles in modulation of the activity of skeletal muscle Ca$^{2+}$ release channel (Aghdasi et al., 1997; Stoyanovsky et al., 1997). However, the detailed pharmacological properties of Ca$^{2+}$ release from SR through sulphydryl modifications are not known yet.

XQN, isolated from sea sponge, has been indicated to act on substances possessing sulphydryl groups (Sakamoto et al., 1995). It has been reported that bastadins, isolated from another class of sponge, convert ryanodine-insensitive leak states into ryanodine-sensitive channels that recognize $[^{3}H]$ryanodine with high affinity through their modulatory actions on the FKBP12/RyR-1 complex (Mack et al., 1994). In the present study, we found that XQN caused a concentration-dependent $^{45}$Ca$^{2+}$ release from HSR and that the Ca$^{2+}$-releasing potency of XQN was 10 times more potent than that of caffeine. XQN-induced $^{45}$Ca$^{2+}$ release from HSR was completely inhibited in the presence of DTT but that of caffeine was not affected by it. Furthermore, the effect of XQN on $[^{3}H]$ryanodine binding to HSR was abolished by treatment with DTT. Scatchard analysis of $[^{3}H]$ryanodine binding to HSR revealed that XQN decreases it by decreasing $B_{max}$ but does not affect the change in dissociation rate of bound $[^{3}H]$ryanodine, suggesting that XQN binds to sulphydryl of Ca$^{2+}$ release channels to cause a decrease in $[^{3}H]$ryanodine binding to HSR noncompetitively. In general, $[^{3}H]$ryanodine binding with high affinity to saturable and Ca$^{2+}$-dependent sites in the Ca$^{2+}$ release channel is enhanced by channel activators, whereas it is decreased by channel inhibitors (Pessah et al., 1987; Michalak et al., 1988). In this study, we found XQN markedly decreased $[^{3}H]$ryanodine binding to HSR in a Ca$^{2+}$-independent manner. Pessah et al. (1997) have shown that bastadins, another class of sponge toxins, enhance the number of high-affinity binding sites of $[^{3}H]$ryanodine to SR. However, it has been reported that thimerosal, a thiol-oxidizing reagent, stimulates Ca$^{2+}$ release from skeletal muscle SR with an EC$_{50}$ value of $\sim$200 $\mu$M but inhibits $[^{3}H]$ryanodine binding by decreasing $B_{max}$ without affecting $K_D$ (Abramson et al., 1995). These observations suggest that the pharmacological properties of XQN on HSR are similar to those of thimerosal and that XQN is approximately seven times more potent than thimerosal on the basis of EC$_{50}$. Recently, it has been reported that nanomolar naphthoquinone enhances occupancy of $[^{3}H]$ryanodine, whereas low micromolar naphthoquinone inhibits the binding of $[^{3}H]$ryanodine to SR membrane (Feng et al., 1999).

Procaine, ruthenium red, and Mg$^{2+}$ have been used extensively as the inhibitors of Ca$^{2+}$-induced Ca$^{2+}$ release (Smith et al., 1988; McPherson and Campbell, 1993; Kawano, 1998). For example, procaine has been shown to interact selectively with a closed state of the channel rather than with an open state (Zahradnikova and Palade, 1993). We examined the effects of those inhibitors on $^{45}$Ca$^{2+}$ release induced by XQN and caffeine. Interestingly, caffeine-induced $^{45}$Ca$^{2+}$ release was inhibited by these blockers in a concentration-dependent manner, whereas XQN-induced $^{45}$Ca$^{2+}$ release was not inhibited by them. Furthermore, $^{45}$Ca$^{2+}$ release from HSR was potentiated by caffeine in a narrower range of pCa, whereas it was significantly stimulated by XQN in a wider range of

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**Fig. 6.** Interrelations among the Ca$^{2+}$-releasing activities of XQN, MBED, and caffeine. $^{45}$Ca$^{2+}$ release from HSR for 1 min was measured. Experimental protocols were the same as described in Fig. 4. The concentration of XQN, caffeine, and MBED were 30 $\mu$M, 30 mM, and 1 $\mu$M, respectively. Values are means $\pm$ S.E. (n = 3). *P < .01.

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**Fig. 7.** Concentration-dependent effects of XQN on $[^{3}H]$ryanodine binding to HSR. HSR (200 $\mu$g/ml) was incubated with 1 nM $[^{3}H]$ryanodine in the presence of various concentrations of XQN for 1 h at 37°C. Specific binding was derived by subtracting nonspecific binding determined in the presence of 10 $\mu$M unlabeled ryanodine. Values are means $\pm$ S.E. (n = 3). Whether XQN affected $[^{3}H]$ryanodine binding to HSR through sulphydryl modification. $[^{3}H]$Ryanodine binding to HSR was decreased 90% by XQN (30 $\mu$M), and the effect of XQN on $[^{3}H]$ryanodine binding was abolished by treatment with DTT (1 mM) (Fig. 10).
pCa, demonstrating that the profile of Ca\textsuperscript{2+}-dependence of XQN-induced 45Ca\textsuperscript{2+} release was different from that of caffeine. Recently, it was reported that a functionally important interaction between RyR-1 and triadin exists that partially involves redox cycling of hyperreactive sulfhydryls in response to channel activation and inactivation (Liu and Pesah, 1994). Triadin has been shown to decrease [3H]ryanodine binding to HSR and openings of the RyR incorporated into the planar lipid bilayers (Ohkura et al., 1998). However, we found that XQN caused 45Ca\textsuperscript{2+} release not only from HSR but also LSR, which had far fewer RyRs than HSR. On the basis of these observations, it is suggested that there are three possibilities: XQN causes Ca\textsuperscript{2+} release through sulphydryl modification of 1) novel Ca\textsuperscript{2+} release channels; 2) the RyR or novel Ca\textsuperscript{2+} release channels having changed gating properties; or 3) regulatory protein, including triadin.

It has been reported that there are several effector-binding domains in the Ca\textsuperscript{2+} release channel (Shoshan et al., 1998). MBED, a derivative of bromo eudistomin D, induced a contraction of chemically skinned fiber from skeletal muscle, and has been shown to elicit Ca\textsuperscript{2+} release from the Ca\textsuperscript{2+} store site in isolated myocytes (Seino et al., 1990). MBED, which binds to the same site as that of caffeine, does not alter the maximal 45Ca\textsuperscript{2+} release induced by caffeine, whereas adenosine triphosphate, which binds to different site from
that of caffeine, further increases that induced by caffeine (Seino et al., 1990). In the present study, the interrelation among the stimulatory effects of XQN, caffeine, and MBED was examined. The maximum response of 45Ca2+ release to caffeine was additively increased by XQN, whereas it was not increased by MBED. These observations suggest that XQN acts on a different site from that of caffeine and/or MBED in the Ca2+ release channels or novel Ca2+ release channels as well as RyRs.

In summary, it has been demonstrated that sulphydryl groups are involved in both the XQN effect on Ca2+ release from HSR on ryanodine binding to HSR. XQN may be a useful pharmacological tool to elucidate the function of sulphydryls in Ca2+ release channels in skeletal muscle.

References


