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Reversible Thiol-Dependent Activation of Ryanodine-Sensitive Ca^{2+} Release Channel by Etoposide (VP-16) Phenoxyl Radical

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ABSTRACT

Many phenolic compounds can act as antioxidants by donating a proton to peroxyl radicals and quenching lipid peroxidation. Phenoxyl radicals produced this way or from metabolism by peroxidases, tyrosinase, or mixed-function oxidases, however, may react with sulfhydryl groups of proteins and other endogenous thiols. In this regard, phenolic compounds may have cytotoxic potential instead of antioxidant effects. We employed the anticancer drug, etoposide (VP-16), as a model phenolic compound to study the sensitivity of ryanodine-sensitive Ca^{2+} channel (RyR) to VP-16 phenoxyl radicals. The combination of VP-16 and tyrosinase, used to generate the etoposide phenoxyl radical, produced marked Ca^{2+} release from Ca^{2+}-loaded RyR-rich vesicles prepared from terminal cisternae fraction of sarcoplasmic reticulum (SR). This effect was reversed by the SH-reagent, dithiothreitol (DTT), suggesting that cysteines within the RyR–protein complex were targets for modification by VP-16 phenoxyl radicals. VP-16/tyrosinase-induced release of Ca^{2+} was attenuated in vesicles prepared from longitudinal SR, which contain relatively little RyR. The effects of the VP-16 phenoxyl radical on Ca^{2+}-ATPase in SR vesicles resembled those observed with caffeine or 4,4′-dithiodipyridine, both of which activate RyR Ca^{2+} release and lead to activation of Ca^{2+}-ATPase via prolonged Ca^{2+} cycling. The addition of ruthenium red returned Ca^{2+}-ATPase to its original level. Thus, under these conditions Ca^{2+}-ATPase was not directly affected by VP-16 phenoxyl radical. The hypersensitive SH-groups on RyR are shown to be targets for oxidation of VP-16 phenoxyl radical, and suggest that other phenolic compounds could similarly disrupt Ca^{2+} homeostasis. Antiox. Redox Signal. 2, 73–82.

INTRODUCTION

THE SARCOPLASMIC RETICULUM (SR) controls muscle contraction and relaxation via two Ca^{2+} transport structures, Ca^{2+}-ATPase (Ca^{2+} pump) and the ryanodine-sensitive Ca^{2+} release channel (RyR), responsible for the uptake and release of Ca^{2+} ions, respectively (Zucchi and Ronca-Testoni, 1997). Both of these structures contain oxidizable free thiol groups, which are critical for their functional activity (Abramson and Salama, 1989; Zaidi *et al.*, 1989; Prabhu and Salama, 1990; Zucchi and Ronca-Testoni, 1997). Vesicular fractions of SR enriched in RyR contain approximately 5–10 pmol of RyR thiols per milligram of protein compared to 40–60 nmol thiols for Ca^{2+}-ATPase (Xu *et al.*, 1998). The RyR contains a single hypersensitive thiol whose oxidation leads to channel opening upon reaction with reactive disulfides (Zaidi *et al.*, 1989; Prabhu and Salama, 1990; Salama *et al.*, 1992) or Ag^{1+}

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(Abramson *et al.*, 1983; Salama and Abramson, 1984; Prabhu and Salama, 1990) despite the significant excess of Ca^{2+} pumps in the same membrane. RyR channel activation can be reversed by SH-reducing agents (glutathione and dithiothreitol) to promote channel closure and Ca^{2+} reuptake by the Ca^{2+} pump (Abramson and Salama, 1989; Zaidi *et al.*, 1989; Prabhu and Salama, 1990).

It has long been known that oxidative stress can perturb intracellular Ca^{2+} homeostasis within the cell. The specific molecular events responsible for this phenomenon are unresolved but may include nonspecific peroxidative damage to membrane lipids or oxidation of proteins responsible for Ca^{2+} transport and release. For this reason, we sought a model that would allow selective oxidation of protein SH groups in the absence of appreciable lipid oxidation to assess the relative contribution of protein thiol oxidation to alterations in Ca^{2+} metabolism following oxidative stress.

Etoposide (VP-16) is a semisynthetic podophyllotoxin derivative with significant clinical activity against a wide variety of neoplasms, including germ cell malignancies, small-cell lung carcinoma, non-Hodgkin's lymphomas, leukemias, Kaposi's sarcoma, neuroblastoma, and soft-tissue sarcomas (Slevin, 1991). Depsite its high degree of efficacy in a large number of cancers, the current therapeutic use of VP-16 is limited by a high incidence of toxicity, including myelosuppression, peripheral neuropathy, irreversible testicular lesions, cardiac arrythmias and failure, and induction of secondary acute myeloid leukemia. Etoposide exerts its tumoricidal effects via the formation of stable complexes with DNA and topoisomerase IIα that subsequently leads to the formation of double-stranded DNA breaks (Corbett and Osheroff, 1993). This resulting DNA damage leads to cell cycle arrest and apoptosis in the targeted tumor cells (Dubrez *et al.*, 1995). This, however, cannot fully explain the toxic effects observed on mitotically inactive tissue such as heart and peripheral neurons.

We hypothesize that the ability of etoposide to induce oxidative stress could also be a key factor in its cytotoxcity, especially in nonneoplastic tissue. The cytotoxicity of VP-16 toward cultured EMT6 mouse mammary tumor cells was found to be significantly greater under normoxic conditions as compared to cells grown under hypoxic conditions (Teicher *et al.*, 1985). Antioxidants and chelators of redox-active metals, such as copper, inhibited VP-16-induced apoptosis in thymocytes (Wolfe *et al.*, 1994). The VP-16 molecule contains an oxidizable hindered phenolic group and VP-16 phenoxyl radical is an essential intermediate in etoposide oxidative activation by cytochrome P_{450}-dependent monoxygenases, peroxidases, prostaglandin synthetase, and tyrosinase (Haim *et al.*, 1987; Kalyanaraman *et al.*, 1987; Usui and Sinha, 1990). Previously, we have shown that the reactivity of VP-16 phenoxyl radical is sufficient to oxidize protein thiols (Tyurina *et al.*, 1995), and, thus, VP-16-induced site-specific protein thiol oxidation could adversely alter cell function independent of topoisomerase II inhibition. At the same time, this hindered phenolic compound acts as an effective lipoprotective antioxidant analogous to vitamin E (Tyurina *et al.*, 1995). Hence, it provides a suitable tool with which to specifically assess protein SH-group oxidation in the absence of significant lipid peroxidation.

We have chosen the SR membrane of skeletal muscle as a model system to study the interaction of VP-16 phenoxyl radical with hyperreactive proteins thiols. Our previous studies demonstrated that VP-16 phenoxyl radicals can inhibit Ca^{2+}-ATPase activity due to random oxidation of protein thiols (Ritov *et al.*, 1995). Additionally, it was noted that VP-16-dependent inhibition of Ca^{2+}-ATPase was not reversible by the disulfide reducing agent, dithiothreitol (DTT). These experiments, however, were performed under conditions that precluded the formation of a Ca^{2+} gradient and, hence, no conclusions could be drawn regarding the function of RyR. Here we studied the effects of VP-16 phenoxyl radical on Ca^{2+} release from SR vesicles. Oxidative effects on RyR were discriminated from effects on Ca^{2+}-ATPase by assessing their reversibility by DTT and comparing the effects between vesicles prepared from RyR-rich terminal cisternae (TC) and RyR-poor longitudinal sarcoplasmic reticulum (LSR). Separate analyses of Ca^{2+}-ATPase activity revealed that RyR was selectively targeted by VP-16 phenoxyl radical.

MATERIALS AND METHODS

Membrane preparations

Unfractionated skeletal muscle SR vesicles were isolated from rabbit hind limb skeletal muscle essentially as previously described (Salama and Scarpa, 1983). The vesicle preparations were kept in liquid nitrogen until use. Crude vesicle fractions were also further fractionated into LSR and TC fractions as previously described (Ritov *et al.*, 1985) and stored frozen at −77°C in buffered glycerol medium (0.1 m*M* EDTA, 25% glycerol, 10 m*M* histidine, pH 7.2).

Measurement of Ca^{2+} transport

Ca^{2+} uptake and release from SR vesicles were measured spectrophotometrically through the differential changes in absorption of the metallochromic indicator, antipyrylazo III (AP III), an indicator of extravesicular free Ca^{2+} (Scarpa *et al.*, 1978). Differential absorption was measured at 720–790 nm with a time-sharing dual wavelength spectrophotometer (SDB-3A, University of Pennsylvania Biomedical Instrumentation, Philadelphia, PA). The measurements of Ca^{2+} uptake and release were performed in 2-ml volumes of assay medium containing 100 m*M* KCl, 1 m*M* $MgCl_2$, 0.1 m*M* AP III, 50 μM $CaCl_2$, 0.7–0.75 mg/ml of SR protein, and 10 m*M* HEPES (pH 6.8 at 28°C) (Zaidi *et al.*, 1989). The reactions were initiated by addition of ATP (100 μM, final concentration), and an ATP-regenerating system (5 m*M* creatine phosphate and 2.5–5 units/ml creatine kinase). Upon completion of Ca^{2+} uptake, the ionophore, alamethicin, (10 μg/ml) was added to the reaction mixture to determine the total amount of releasable intravesicular Ca^{2+} (Ritov *et al.*, 1993). Prior to the addition of the ATP system, the AP III response was calibrated by the addition of 25 nmol of Ca^{2+} to the incubation medium.

Ca^{2+}-ATPase activity

ATP hydrolysis during process of Ca^{2+} uptake was measured pH-metrically by monitoring the time course of acidification of a weakly buffered incubation medium (Ritov and Menshikova, 1991). The maximal shift of pH value of the incubation medium during the reaction did not exceed 0.025 units. The measurements were performed in a temperature-controlled cell under continuous stirring. The incubation mixture (4 ml) contained 100 m*M* NaCl, 4 m*M* $MgCl_2$, 5 m*M* oxalate, 2 m*M* ATP, 25 μM $CaCl_2$, 12–25 μg/ml SR protein in 2.5 m*M* HEPES (pH 7.05).

RESULTS

Generation of VP-16 phenoxyl radical

To generate VP-16 phenoxyl radicals we incubated VP-16 with tyrosinase essentially as described in our previous studies (Ritov *et al.*, 1995). In this earlier work, we showed that incubation of tryosinase with VP-16 led to the formation of the characteristic EPR spectrum of VP-16 phenoxyl radical. No such signal was observed in the presence of either VP-16 or tyrosinase alone. The magnitude of the EPR signal of VP-16 phenoxyl radical generated by tyrosinase-dependent oxidation of VP-16 decreased upon addition of SR membranes to the incubation mixture. As previously shown (Ritov *et al.*, 1995), the quenching of the EPR signal for VP-16 phenoxyl radical was accompanied by oxidation of sulfhydryls in SR.

VP-16 phenoxyl radical stimulates Ca^{2+} release from TC vesicles

To assess potential effects of VP-16 phenoxyl radical on RyR, we measured Ca^{2+} release from SR vesicles using the Ca indicator, AP III. Vesicles containing functional RyR and Ca^{2+}-ATPase were prepared from the TC of the SR. As a control, we also prepared vesicles from the LSR fraction of SR that possess little RyR, yet contain abundant Ca^{2+}-ATPase. Vesicles were incubated as described in Materials and Methods. Two sequential aliquots of $CaCl_2$ were added in the absence of an ATP-generating system to calibrate the absorbance response of AP III. Vesicles were then allowed to accumulate Ca^{2+} by the addition of ATP along with an ATP-regenerating system (creatine phosphate/creatine kinase). Figure 1 shows representative tracings of the AP III absorbance obtained after various stimuli. Time courses begin at the

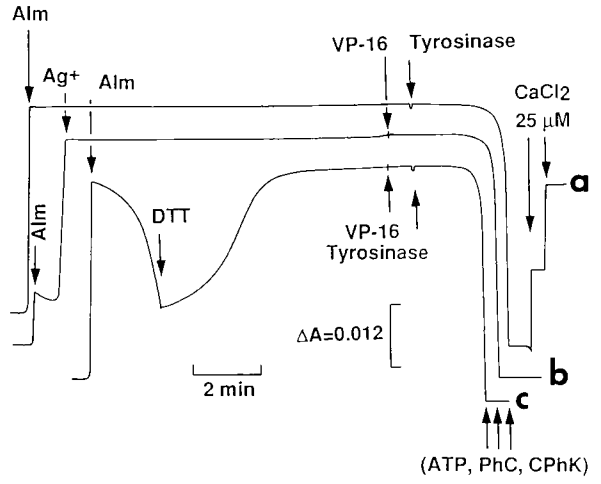

FIG. 1. Calcium release from TC fraction of SR vesicles following exposure to VP-16/tyrosinase combination. A representative tracing of AP III absorbance measured at 720–790 nm in using a dual wavelength spectrophotometer as described in Materials and Methods is shown. Time course proceeds from right to left of the figure, and Ca^{2+}-dependent changes in AP III absorbance are shown in the negative direction. The AP III Ca^{2+}-response was calibrated by addition of two sequential aliquots of $CaCl_2$ (25 μM). Calcium uptake into vesicles was then initiated by addition of the ATP-generating system (ATP, creatine phosphate (PhC), and creatine kinase (CPhK). Tracing a shows the effects of tyrosinase (1.0 U/μl) alone, as well as the amount of total releasable Ca^{2+} using 10 μg/ml alamethicin (Alm). Tracing b shows no effect of VP-16 (0.5 mM) alone followed by the AgCl-dependent (1 μM) release of Ca^{2+} from the vesicles. Tracing c shows the Ca^{2+} release response following exposure of vesicles to VP-16/tyrosinase combination and its reversibility by DTT (0.5 mM).

right of each figure and increases in Ca-dependent absorbance of AP III are shown in the negative direction. Figure 1 (trace a) shows that the membrane-pore-forming agent, alamethicin, leads to rapid appearance of extravesicular Ca^{2+}. Alamethicin-dependent release corresponded to total releasable Ca^{2+} within TC vesicles and was equal to approximately 100 nmol Ca^{2+}/mg SR protein. Figure 1 (trace b) shows that 80% of the total releasable Ca^{2+} was released by Ag^{1+} (1 μM). Because it is known that Ag^{1+} is a powerful activator of RyR-sensitive Ca^{2+} release (Salama and Abramson, 1984), this result suggests that about 80% of Ca^{2+} accumulated by vesicles of the TC fractions was under control of Ca^{2+}-release channels.

Also shown in Fig. 1, the addition of tyrosinase (trace a) or VP-16 (trace b) alone after the phase of Ca^{2+} uptake did not alter the steady-state of Ca^{2+} in TC vesicles during the period of measurements. In contrast, the addition of both VP-16 and tyrosinase produced significant Ca^{2+} efflux as detected by changes in AP III absorbance (Fig. 1, trace c). The onset of Ca^{2+} efflux was delayed by 3–5 min after addition of VP-16 in the presence of tyrosinase and proceeded with an initial fast phase, followed by a slower time course of release. During the fast phase, tyrosinase plus VP-16 released approximately 50–60% of the total Ca^{2+} available for release. This effect of tyrosinase/VP-16 combination was completely prevented in the presence of 1 mM ascorbate (data not shown). Similarly, the addition of the thiol-reducing agent, DTT, after termination of the fast phase, abrogated the release response and induced reuptake of the released Ca^{2+} (Fig. 1, trace c). The effect of DTT suggests that VP-16-induced inhibition of Ca^{2+}-ATPase is not responsible for the effects observed here. First, we have shown previously that VP-16 phenoxyl radical-mediated inhibition of Ca^{2+}-ATPase was essentially irreversible with respect to DTT (Ritov *et al.*, 1995). In addition, the fact that vesicles rapidly reincorporated Ca^{2+} upon the addition of DTT indicates that Ca^{2+}-pumping ATPase is still functional under these conditions. Thus, tyrosinase-dependent formation of VP-16 phenoxyl radical most likely directly activates Ca^{2+} release from the TC vesicles.

To ascertain whether these effects of tyrosinase and VP-16 reflect direct activation of Ca^{2+}-release channels or nonspecific increases in membrane permeability, we performed comparative experiments using vesicles prepared from the LSR fraction of SR that are relatively deficient in their content of RyR while still expressing high Ca^{2+}-ATPase activity. In these experiments, we found that alamethicin released approximately the same amount of Ca^{2+} (100 nmol Ca^{2+}) as seen in TC vesicles; however, the Ag^{1+} ion could release only approximately 20% of the available Ca^{2+} within the vesicle. Similarly, the combination of tyrosinase and VP-16 released 20% of the available Ca^{2+} comparable to the effect of Ag^{1+} and was once again reversible by DTT. Thus, the dependence of the VP-16 phenoxyl radical-induced Ca^{2+} release on the number of Ca^{2+}

channels suggests VP-16 phenoxyl radical mediates Ca^{2+} release primarily by activation of the RyR Ca^{2+} channel.

Effects of VP-16 phenoxyl radical on ATP hydrolysis

To compare the effects VP-16 phenoxyl radical on Ca^{2+} channel activation (described above) to potential effects on Ca^{2+}-ATPase, we measured the effect of VP-16/tyrosinase combination on ATP hydrolysis by SR vesicles. In these experiments, Ca^{2+} uptake was measured in the presence of oxalate to allow the continuous recording of ATP hydrolysis pH-metrically (Ritov and Menshikova, 1991) and allowed us to monitor Ca^{2+}-ATPase activity and

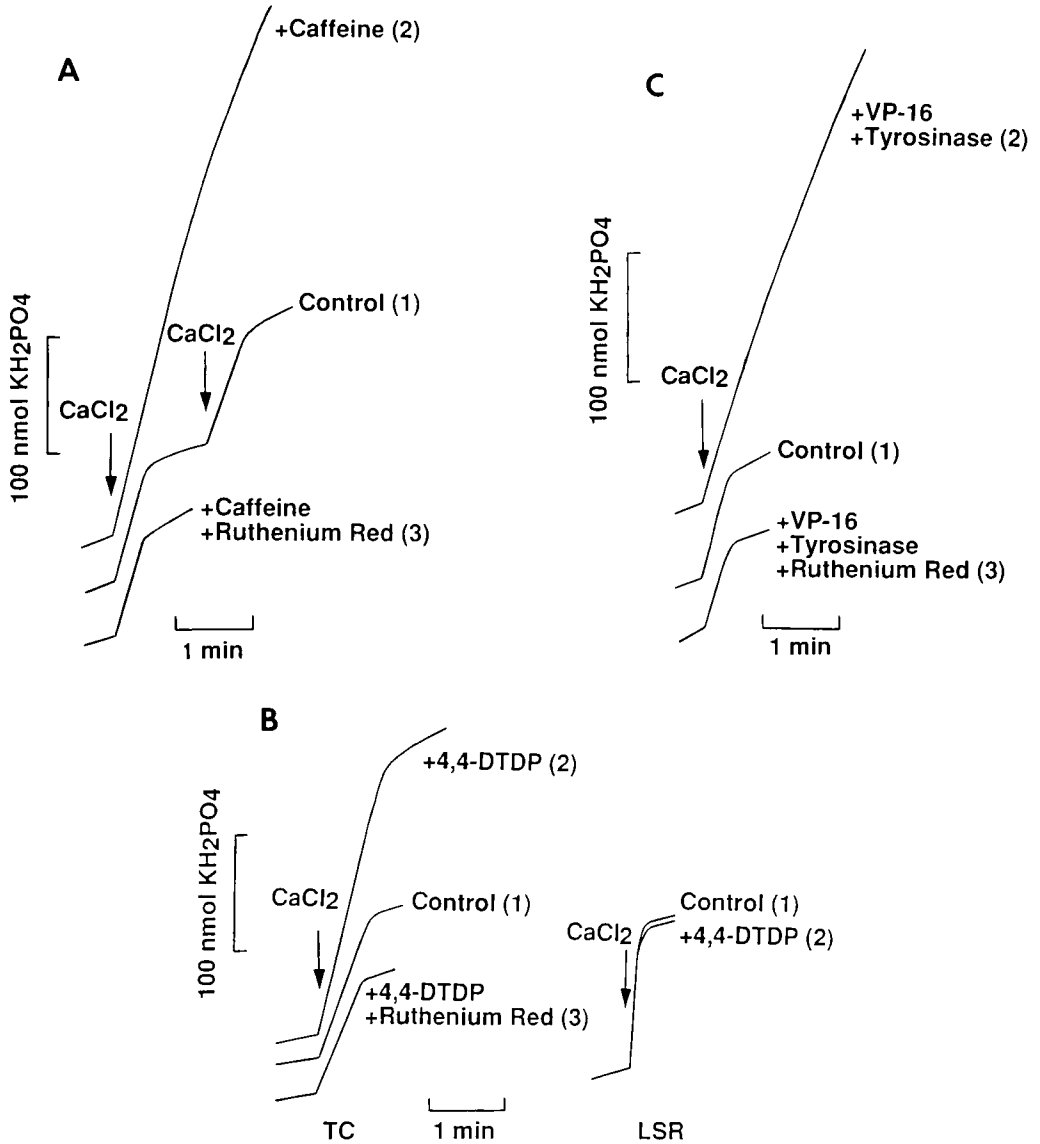

FIG. 2. Effects of VP-16/tyrosinase on Ca^{2+}-ATPase activity in SR vesicles: Comparison to caffeine, ruthenium red, and 4,4′-DTDP. Ca^{2+}-dependent ATPase activity in SR vesicles was measured pH-metrically as described in Materials and Methods. SR vesicles (12–25 μg protein/ml) were preincubated in 100 m*M* NaCl, 4 m*M* $MgCl_2$, 5 m*M* oxalate, 2 m*M* ATP, 2.5 m*M* HEPES (pH 7.05 at 37°C) containing the indicated agonists and antagonists prior to the addition of 25 m*M* $CaCl_2$ to initiate ATPase activity. **A.** Effect of $CaCl_2$ in TC vesicles in the absence (trace 1) and presence of 5 m*M* caffeine (trace 2) or caffeine plus 2.5 μ*M* ruthenium red. **B.** Compares the stimulatory effect of $CaCl_2$ (trace 1) and its sensitivity of 50 μ*M* 4,4′-DTDP (trace 2) between TC and LSR vesicles. Trace 3 in TC vesicles shows that the potentiation of ATPase activity by 4,4′-DTDP (50 μ*M*) in TC vesicles was blocked by the RyR antagonist, ruthenium red (2.5 μ*M*). **C.** Trace 2 shows the effect of VP-16/tyrosinase combination (1.5 m*M* VP-16, 1 U/μl tyrosinase) on Ca^{2+}-ATPase activity compared to untreated control (trace 1). Trace 3 was recorded with the VP-16/tryosinase combination in the presence of ruthenium red (2.5 μ*M*).

activation of Ca^{2+}-release channel under similar conditions. The kinetics of ATP hydrolysis under various experimental conditions are shown in Fig. 2. Panel A shows that the addition of $CaCl_2$ (tracing 1) to the incubation medium containing vesicles prepared from the TC fraction sharply increased the rate of ATP hydrolysis, demonstrating the Ca-dependence of the ATPase activity measured under these conditions. After a finite time interval, the rate of hydrolysis returned to its initial level. This decrease in rate reflects the intravesicular accumulation of the external Ca^{2+} because a second addition of $CaCl_2$ results in activation of ATPase activity identical to the first. The addition of caffeine, a well-known activator of SR Ca^{2+}-release channels, induced a several-fold increase in the rate of ATP hydrolysis (Fig. 2A, tracing 2). The continuation of ATP hydrolysis over time (a continuous decrease in the Ca^{2+}/ATP ratio) reflects the persistent activation of Ca^{2+} pumping produced by continuous recycling of Ca^{2+} across the membrane. The addition of ruthenium red (Fig. 2A, tracing 3), a specific blocker of RyR Ca^{2+} channels, blocked the caffeine-induced continuous fall in the Ca^{2+}/ATP ratio and ATP hydrolysis resembled that seen in control conditions with $CaCl_2$ alone.

We then checked the ability of our system to respond to oxidation of RyR thiols using the selective thiol reagent, 4,4′-dithiodipyridine (4,4′-DTDP), which induces the formation of protein disulfide bonds in thiol-exchange reactions with vicinal thiols. It has been previously shown that 4,4′-DTDP induced the release of Ca^{2+} from SR vesicles (Abramson and Salama, 1989; Zaidi *et al.*, 1989; Prabhu and Salama, 1990). Figure 2B compares the effect of 4,4′-DTDP on ATP hydrolysis in vesicles prepared from the TC fraction (RyR-rich) and LSR (RyR-poor) fractions. In TC vesicles, the effect of the thiol reagent, 4,4′-DTDP resembled that observed with caffeine with a significant increase in ATP hydrolyis upon the addition of $CaCl_2$ to the system. The sensitivity of this effect to blockade by the RyR antagonist, ruthenium red, and, the fact that 4,4′-DTDP failed to alter ATPase activity in RyR-poor LSR vesicles, indicates that 4,4′-DTDP-augmented Ca^{2+}-ATPase activity resulted from direct activation of Ca^{2+} release through activation of RyR.

We next assessed the effects of VP-16/tyrosinase combination on Ca^{2+}-ATPase in SR vesicles. Figure 2C shows that the effect of VP-16 plus tyrosinase on TC vesicles resembles that observed with caffeine or 4,4′-DTDP. Incubation of TC vesicles with VP-16 plus tyrosinase (Fig. 2C, tracing 2) leads to persistent activation of ATPase activity (decrease in Ca^{2+}/ATP ratio) compared to when $CaCl_2$ is added alone (Fig. 2C, tracing 1). Addition of ruthenium red to the VP-16/tyrosinase combination (Fig. 2C, tracing 3) ameliorates this effect and indicates that VP-16 phenoxyl radical-augmented ATPase activity depends upon enhanced flux through the activated RyR channel. The total restoration of Ca^{2+}/ATP of oxidized TC vesicles by ruthenium red under these conditions suggests that the activity of the Ca^{2+}-ATPase pump was not directly affected by the concentrations of VP-16 and tyrosinase used here. These results clearly show that incubation of SR vesicles with VP-16 plus tyrosinase primarily activated Ca^{2+} channel by oxidation of RyR thiols in spite of the more than 100-fold excess of Ca^{2+}-ATPase pump thiols in the SR membrane. Thus, the hypersensitive thiols of RyR may represent preferential targets for oxidative modification by the VP-16 phenoxyl radical.

DISCUSSION

It has been known for some time that oxidative stress can produce severe alterations in Ca^{2+} homeostasis within cells. In 1973, we discovered that reactive oxygen species (ROS) formed as a result of redox-cycling of iron by ascorbate inhibit net Ca^{2+} accumulation by SR vesicles isolated from rabbit skeletal muscles (Kozlov *et al.*, 1973). Subsequent studies demonstrated that lipid peroxidation of the membrane bilayer (lipid peroxidation) and oxidative modification of Ca^{2+}-ATPase could be involved in the inhibition of Ca^{2+} transport by SR membranes (Kagan, 1988). Because oxygen radicals and other ROS generated by standard oxidation systems are highly reactive and indiscriminatively attack various biomolecules (proteins, lipids, sugars, nucleic acids), the elucidation of the contribution of protein modifi-

cation versus membrane lipid peroxidation into the overall effect on Ca^{2+} transport could not be performed. Our recent studies demonstrated that phenoxyl radicals generated from phenolic compounds, such as the antitumor drug, VP-16, selectively oxidize low-molecular-weight and protein thiols without affecting membrane lipids (Kagan *et al.*, 1994). Using this approach, we showed that Ca^{2+} transport in SR membranes from rabbit skeletal muscles could be inhibited by a specific oxidation of protein SH groups in the Ca^{2+}-dependent ATPase pump (Ritov *et al.*, 1995). Here we show a similar effect of thiol oxidation in modulating Ca^{2+} release via activation of RyR. Notably, the effect of the VP-16 phenoxyl radical was observed independently of changes in Ca^{2+}-ATPase activity, suggesting that RyR is more sensitive to activation by thiol oxidation than is inhibition of Ca^{2+}-ATPase.

The RyR is a 565-kDa protein expressed in skeletal and cardiac muscle, as well as nervous tissue, and is responsible for the physiological release of Ca^{2+} from SR and other internal Ca storage depots. The functional channel itself exists as a large tetrameric structure in complex with other proteins such as triadin and calsequestrin (Shoshan-Barmatz and Ashley, 1998; MacKrill, 1999). Besides the large number of agents that impart pharmacological effects at SR Ca^{2+}-release channels, a variety of chemical oxidants and, in particular, SH-oxidizing reagents, have been shown to activate Ca^{2+} release. Heavy metals, copper ions (Cu^{2+}) plus cysteine, and 4,4′-DTDP have been shown to activate Ca^{2+} release by acting at a critical sulfhydryl site (SH) on the release channel protein (Abramson *et al.*, 1983; Salama and Abramson, 1984; Zaidi *et al.*, 1989; Salama *et al.*, 1992). Agents known to stimulate (*e.g.*, ATP, caffeine) or inhibit (*e.g.*, ruthenium red, Mg^{2+}, tetracaine, procaine) SR Ca^{2+} release likewise stimulated or inhibited SH-dependent activation of release. The activation or opening of Ca^{2+} release channels by sulfhydryl-oxidizing reagents could be reversed by sulfhydryl-reducing agents (*e.g.*, cysteine, DTT). Although sulfhydryl oxidation-reduction has been shown to modify SR Ca^{2+} release, the role of this mechanism in physiological excitation contraction coupling and in pathological states of the muscle remains unknown.

Our data here support the concept that RyR junctional complex contains at least one hypersensitive-thiol moiety easily oxidized by low levels of phenoxyl radicals. RyR SH groups, despite their low abundance relative to Ca-ATPase and total SR protein, must be more readily oxidizable than other SR proteins because inhibition of Ca^{2+}-ATPase was not observed under these conditions. Higher levels of oxidative stress, however, could inhibit Ca^{2+}-ATPase activity (Ritov *et al.*, 1995). In addition, Liu *et al.* have utilized fluorescent techniques to label reduced SH groups on SR proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and shown the selective loss of protein thiols specifically on the RyR and triadin proteins (<6% of total SR protein) (Liu *et al.*, 1994). It is noteworthy that cysteine oxidation of these two proteins promotes their association into a high-molecular-weight protein complex, suggesting that the redox state of hypersensitive thiols determines the stability of the functional multiprotein complex and, hence, serves to modulate its physiological function (Liu and Pessah, 1994).

Our data indicate that RyR may be a selective target for damage following exposure to oxidative stress. Whereas cardiotoxicity of some antitumor drugs, such as antracyclines, has been associated with their ability to generate radicals, cardiotoxic mechanisms of podophyllotoxin derivatives have not been investigated so far. Antracyclines are well-known examples of cardiotoxic antitumor drugs that generate oxygen radicals in cardiomyocytes. Etoposide contains a hindered phenolic ring, the presence of which is a critical structural prerequisite for its antitumor activity. It has been suggested that oxidative metabolic activation of VP-16 by cytochrome P_{450}-dependent monooxygenases, peroxidases, prostaglandin synthase, or tyrosinase may contribute to its tumoricidal activity (Haim *et al.*, 1987; Kalyanaraman *et al.*, 1987; Usui and Sinha, 1990), although the parent compound itself clearly possesses anti-topoisomerase activity. More importantly, however, these same enzymatic functions may be critical to its toxicity

in mitotically inactive tissue such as heart and nerve. The phenoxyl radical is the inevitable intermediate in the oxidative activation of VP-16 by different oxidative enzymes. Significant activities of myeloperoxidase (Raschke *et al.*, 1993), cytochrome P_{450}-dependent monooxygenases (McCallum *et al.*, 1993), tyrosinase (Quiao *et al.*, 1993), and prostaglandin synthetase (Golino *et al.*, 1993) are characteristic of cardiac tissue. Our studies demonstrated that redox cycling of VP-16 phenoxyl radicals, which are highly reactive toward protein SH-groups, efficiently modifies the Ca^{2+}-release channels (data shown here) and Ca^{2+} pump (Ritov *et al.*, 1995) in SR membranes from skeletal muscles. In contrast to anthracyclines, which generate ROS via mitochondrial and microsomal electron transport-dependent mechanisms, the cardiotoxic effects of VP-16 cannot be easily ascribed to their ability to generate oxygen-derived radicals. Instead, high reactivity of VP-16 phenoxyl radicals toward protein SH-groups may be the major contributor to its cardiotoxicity. Thus, although free radicals may be involved in cardiotoxic effects for both anthracycline and VP-16, the nature of radicals and mechanisms of radical-induced damage of cardiomyocytes are likely to be remarkably different.

In summary, we have shown that that RyR-Ca^{2+}-release channel is activated following exposure to the VP-16 phenoxyl radical. Phenolic compounds, many of which, such as resveratrol, quercetin, and isoflavones have antioxidant potential, could also serve as substrates for phenoxyl radical production and detrimentally alter Ca^{2+} homeostasis through protein thiol oxidation. Critical SH-groups within the RyR channel protein complex are likely sites for etoposide-induced oxidation, which subsequently leads to release of intravescular Ca^{2+} from SR membranes. Phenoxyl radical-induced release of Ca occurred in the absence of any changes on Ca^{2+}-ATPase activity, suggesting that SH-groups on RyR and/or its associated proteins are hypersensitive to oxidation and, thus, could represent potential molecular targets for toxicity following exposure to phenolic compounds.

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ABBREVIATIONS

Alm, Alamethicin; AP III, antipyrylazo III; CPhK, creatine kinase; 4,4′-DTDP, 4,4′-dithiodipyrindine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; LSR, longitudinal cisternae; PhC, creatine phosphate; ROS, reactive oxygen species; RyR, ryanodine-senstive Ca^{2+} release channel; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SH, sulfuhydryl; SR, sarcoplasmic reticulum; TC, terminal cisternae; VP-16, etoposide.

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