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Skeletal Muscle Ryanodine Receptor Channels Are Activated by the Fungal Metabolite, Gliotoxin

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Abstract. Interactions between the reactive disulfide fungal metabolite, gliotoxin (GTX), and rabbit skeletal ryanodine receptor (RyR) calcium release channels have been examined. RyRs in terminal cisternae vesicles formed a covalent complex with 100 μM ^{35}S-GTX, which was reversed by 1 mM dithiothreitol (DTT) or 1 mM glutathione. GTX (80–240 μM), added to either cytoplasmic (*cis*) or luminal (*trans*) solutions, increased the rate of Ca^{2+} release from SR vesicles and the frequency of opening of single RyR channels in lipid bilayers. Channel activation was reversed upon addition of 2 mM DTT to the *cis* solution, showing that the activation was due to an oxidation reaction (2 mM DTT added to the *cis* solution in the absence of GTX did not affect RyR activity). Furthermore, RyRs were not activated by *trans* GTX if the *cis* chamber contained DTT, suggesting that GTX oxidized a site in or near the membrane. In contrast to *cis* DTT, 2 mM DTT in the *trans* solution increased RyR activity when added either alone or with 200 μM *trans* GTX. The results suggest that (i) GTX increases RyR channel activity by oxidizing cysteine residues that are close to the membrane and located on RyR, or associated proteins, and (ii) a disulfide bridge or nitrosothiol, accessible only from the luminal solution, normally suppresses RyR channel activity. Some of the actions of GTX in altering Ca^{2+} homeostatsis might depend on its modification of RyR calcium channels.

Key words: Ryanodine receptors — Gliotoxin — Calcium regulation

Introduction

Ryanodine receptor (RyR) Ca^{2+} release channels are responsible for the increase in cytoplasmic [Ca^{2+}] which causes contraction in skeletal and cardiac muscle (Dulhunty et al., 1996). The RyR protein contains four identical subunits each with a mass of ~565 kD and containing 89 (cardiac isoform) to 101 (skeletal isoform) cysteine residues (Takeshima et al., 1989; Otsu et al., 1990). Many of these cysteine residues (21 per subunit in cardiac RyRs; Xu et al., 1998) present free sulfhydryl (-SH) groups whose covalent modification substantially alters channel activity (Aghdasi et al., 1997), first activating and then inhibiting RyRs (Boraso & Williams, 1994; Eager & Dulhunty, 1998, 1999).

Modification of -SH groups following S-Nitrosylation by nitric oxide (NO), or its derivatives, also leads to RyR activation or inhibition (Mészáros et al., 1996; Stoyanovsky et al., 1997; Xu et al., 1998; Hart & Dulhunty, 2000). Oxidizing reagents and NO modify cysteine residues, removing free -SH groups from RyRs (Xu et al., 1998), and their actions can be prevented by sulfhydryl reducing agents (Boraso & Williams, 1994; Favero, Zable & Abramson, 1995; Eager, Roden & Dulhunty, 1997; Eager & Dulhunty, 1998, 1999; Xu et al., 1998). Both S-Nitrosylation-, and oxidation-, induced activation of RyRs proceed in vivo, in the presence of glutathione (GSH) (Koshita, Miwa & Oba, 1993; Simon et al., 1996; Xu et al., 1998) and may be enhanced during ischaemia, reperfusion and inflammatory challenges where oxygen free radicals increase and [GSH] falls (Curello et al., 1985).

The use of different types of sulfhydryl oxidizing reagents, such as dithiodipyridines (DTDPs), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), thiomerosal, or S-Nitrosylating reagents (NO or nitrosothiol compounds [SNOs]) has revealed (i) that different classes of cysteine residues can modify the gating of RyR channels in different ways and (ii) that cysteine residues which modify channel activity are located in the cytoplasmic and transmembrane domains of RyRs (Xu et al., 1998; Eager & Dulhunty, 1999; Haarmann, Fink & Dulhunty, 1999).

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In this study we examine the naturally occurring reactive disulfide, gliotoxin (GTX; Fig. 1), which is chemically distinct from the DTDPs, DTNB or thimerosal, and is a member of the epithiodioxopiperazine fungal metabolites which contain a bridged polysulfide piperazine ring. The disulfide bridge in GTX is required for its biological activity, which includes immunosuppression and cytotoxicity (Waring, Eichner & Mullbacher, 1988). GTX, induces apoptosis and inhibits T and B cell proliferation after antigen stimulation (Braithwaite et al., 1986; Waring & Beaver, 1996; Waring et al., 1988).

GTX stimulates Ca^{2+} release from mitochondria via thiol modification (Schweizer & Richter, 1994) and, at high concentrations (50 μM), can increase cytoplasmic [Ca^{2+}] in thymocytes via plasmalemmal Ca^{2+} pathways (Beaver & Waring, 1994; A.M. Hurne et al., *unpublished results*). Some of the actions of GTX may depend on its interaction with RyR channels. T-cell responses to antigen stimulation depend on prolonged Ca^{2+} release from internal stores through RyRs (Guse et al., 1999; Sei, Gallagher & Basile, 1999), and apoptosis can be associated with increased cytoplasmic [Ca^{2+}] from internal stores (Waring & Beaver, 1996).

Two novel observations are reported in this communication. Firstly, GTX activates skeletal muscle RyR channels by oxidation of thiol groups in or near the transmembrane domain of the protein. Channel activation occurs mainly because of an increase in the frequency of opening and is thus similar to activation by NO (Hart & Dulhunty, 2000), but is distinctly different from activation by the DTDPs, DTNB and thimerosal which preferentially increase channel open time (Eager & Dulhunty, 1999; Haarmann et al., 1999). Secondly, we found that the reducing agent DTT (2 mM) activated skeletal RyRs when added to the *trans* solution, suggesting that a disulfide bridge or protein nitrosothiol, accessible from the luminal solution, suppresses RyR activity under control conditions. Activation of RyR channels by GTX raises the possibility that some of the biological actions of the toxin are due to modification of the Ca^{2+} release channels.

Materials and Methods

The preparation of SR vesicles, lipid bilayer techniques and recording and analysis of single channel activity are described in detail in Kourie et al. (1996).

Biological Material

Preparation of SR vesicles was based on Saito et al. (1984). Rabbit back and hindlimb muscle was dissected, homogenized and centrifuged to yield a crude microsomal fraction that was then run on a sucrose gradient. Heavy SR vesicles were collected and the crude fraction and heavy SR were stored at −70°C.

Gel Electrophoresis

Heavy SR vesicles (100 μM) were incubated with 100 μM ^{35}S-labeled GTX (in 44.5 μl of *cis* solution, see Materials and Methods below) at 37°C. Aliquots were removed after 5, 30 and 60 min and added to 50 μl of loading buffer, containing 62.5 mM Tris-HCl, 20% glycerol (w/v), 5% SDS and 0.05% bromophenol blue, pH 6.8, for 5 min at 95°C. The sample (20 μl/well) was loaded onto 6% and 8% SDS-PAGE gels (Laemmli, 1970). The gels were run at 200V and 25mA for 50 min in a mini-PROTEAN II© electrophoresis cell (Bio-RAD). Gels were then fixed (methanol, 40%; acetic acid, 10%; distilled H_2O, 50%) for 1 hr, and either exposed to autoradiographic film or stained with Coomassie© Blue (Waring et al., 1988).

In a second series of experiments, the sample was divided into 4 aliquots after incubation for 10 min at 37°C with ^{35}S-labeled GTX. Two were kept as controls, 1 mM DTT was added to one and 1 mM GSH added to the other. The four samples were incubated for a further 10 min at 37°C before denaturation and SDS-PAGE.

Lipid Bilayer Techniques

Bilayers were formed from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (5:3:2 w/w) (Avanti Polar Lipids, Alabaster, Alabama) across an aperture with a diameter of ~200 μm in the wall of a 1.0 ml Delrin cup (Cadillac Plastics, Australia). Terminal cisternae (TC) vesicles (final concentration 10 μg/ml) were added to the *cis* chamber and stirred until vesicle incorporation was observed. The cytoplasmic side of channels incorporated into the bilayer faced the *cis* solution. The bilayer potential was controlled, and single channel activity recorded, using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). For experimental purposes, the *cis* chamber was held at ground and the voltage of the *trans* chamber controlled. Bilayer potential is expressed in the conventional way as $V_{cis} - V_{trans}$, (i.e., $V_{cytoplasm} - V_{lumen}$).

Bilayers were formed and vesicles incorporated into the bilayer using a *cis* solution containing (mM): 230 cesium methanesulphonate (CsMS); 20 CsCl; 1.0 $CaCl_2$; 10 N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES). The *trans* solution contained (mM): 30 CsMS; 20 CsCl; 1.0 $CaCl_2$; 10 TES. 20 mM Cl^- was retained in the solutions because preliminary experiments suggested that skeletal RyR channel openings are better resolved if solutions contain some Cl^-. This observation has not been pursued further. Following vesicle incorporation, the *cis* chamber was perfused (to prevent further vesicle incorporation) with a recording solution containing (mM): 230 CsMS; 20 CsCl; 0.1 $CaCl_2$; 10 TES. All solutions were adjusted to pH 7.4 with CsOH.

Recording and Analysis of Single Channel Data

Channel activity was recorded at +40 mV. Drugs were added to either the *cis* or *trans* chamber with a ~10 sec stirring period, and then activity recorded for several min. Channel activity was filtered at 1 kHz (10-pole low pass Bessel, −3 dB) and digitized at 2 kHz. Analysis of single channel records (using *Channel 2*, written by P.W. Gage and M. Smith) yielded channel open probability (P_o), frequency of events (F_o), open times, closed times and mean open or closed times (T_o or T_c), as well as mean current (I', i.e., the average current in all data points, including channel open and closed periods, in a period of recording). The open and closed discriminators were set at ~25% of the maximum current, so that openings to both subconductance and maximum conductance levels were included in the analysis. Single channel parameters were measured from sections of activity lasting 20–120 sec.

Calcium Release from SR Vesicles

Extravesicular Ca^{2+} was monitored at 710 nm with the Ca^{2+} indicator, antipyrylazo III, using a Cary 3 Spectrophotometer (Dulhunty et al., 1999). Temperature was controlled at 25°C and the solution was magnetically stirred. TC vesicles (100 μg of protein) were added to the cuvette, to a final volume of 2 ml, of a solution containing (in mM): 100, KH_2PO_4 (pH = 7); 4, $MgCl_2$; 1, Na_2ATP; 0.5, antipyrylazo III. Vesicles were partially loaded with Ca^{2+} by four sequential additions of $CaCl_2$, each increasing $[Ca^{2+}]$ by ~7.5 μM (Fig. 9 below). Ca^{2+}, Mg^{2+}-ATPase activity was then suppressed with thapsigargin (500 nM), to allow extravesicular $[Ca^{2+}]$ to increase following activation of RyRs. Ethanol or the same volume ethanol containing GTX were then added, followed by the RyR blocker ruthenium red (5 μM), to determine whether Ca^{2+} release through RyR channels was responsible for Ca^{2+} release from SR. In some experiments, ruthenium red was added before GTX. The Ca^{2+} ionophore A23187 (3 μg/ml) was finally added to release the Ca^{2+} remaining in the TC vesicles. A calibration curve for OD changes in response to 4 sequential additions of 12.5 μM $CaCl_2$ was established at the start of each experiment.

Chemicals and GTX Stock Solutions

Salts were obtained from Aldrich (CsCl and CsOH), AnalaR ($CaCl_2$) and Sigma (CsMS and TES). DTT, GTX and ruthenium red were purchased from Sigma. Salts, DTT and ruthenium red were dissolved in deionized water. GTX was dissolved in ethanol at a maximum concentration of 8.5 mM. ^{35}S-GTX was prepared as previously described (Waring et al., 1994). Small aliquots of stock GTX and DTT solutions were stored at −70°C and thawed shortly before use.

Statistics

Average data are given as mean ± SEM. In all cases channel activity was measured under control conditions before addition of drugs, and drug effects on each channel compared with the control activity (*see* figure legends). The significance of the difference between control and test values was tested using a Students *t*-test, either 1- or 2-sided for paired data as appropriate. Differences were considered to be significant when $P \leq 0.05$.

Results

Gliotoxin Binds to RyRs in Skeletal SR Vesicles

Skeletal SR vesicles were exposed to 100 μM ^{35}S-GTX for 5, 30 and 60 min, then run on SDS PAGE gels and either stained with Coomassie Blue (Fig. 2*A*) or exposed to autoradiographic film (Fig. 2*B* and *C*). The gels showed the characteristic distribution of SR proteins with strong bands at >300 kDa—corresponding to the RyR, ≧97 kDa—including the SR Ca^{2+}, Mg^{2+}-ATPase (Kourie et al., 1996) and the 97 kDa triadin and several other lower molecular weight bands. The two distinct bands labeled with GTX in the radiographs corresponded to the high molecular weight RyR and to triadin (97 kDa). The density of labeling of the SR proteins was similar for each of the three exposure times, indicating that binding to the proteins was complete after 5 min exposure to GTX. No GTX binding was apparent if the vesicles were exposed to 1 mM DTT or 1 mM GSH after 10 min exposures to GTX (Fig. 2*C*), showing that the –SH reducing reagents were able to remove GTX from the RyR and the 97 kDa protein. These results suggest that GTX formed a covalent disulfide bond with the RyR and with a 97 kDa protein.

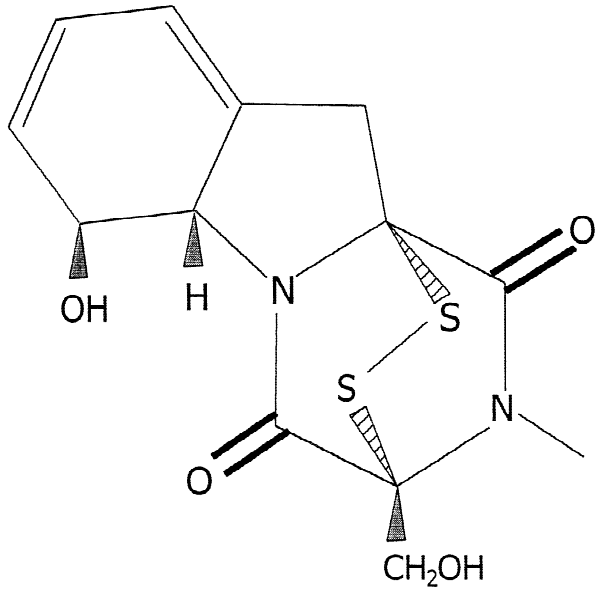

Fig. 1. The structure of gliotoxin.

Single RyR Channels are Activated by *cis* GTX

RyR channels were identified by their characteristic single channel conductance of ~420 pS and their sensitivity to 800 μM ruthenium red which reduced channel activity when added to the *cis* chamber. Channels were exposed to *cis* 800 μM ruthenium red at the end of each of 5 experiments to confirm (i) that channels were RyRs and (ii) that the cytoplasmic domain of the channel faced the *cis* solution. Activity in all 5 channels was either partially or fully blocked within 2 min of addition of the polyvalent cation. We found here and elsewhere that activity in very active channels is reduced by 90% or more by 0.8 mM ruthenium red, but is not fully blocked after 1 to 2 min.

Control RyR channel activity at +40 mV in the presence of 100 μM Ca^{2+} consisted of occasional brief, poorly resolved, channel openings (Figs. 3*A*, 4*A*, 5*A* and 7*A*). The different frequencies of control activity in the four figures reflect the normal channel to channel variability between RyRs. Addition of 200 μM GTX to the *cis* chamber was followed by an increase in the number of channel openings within 90 sec of application of the

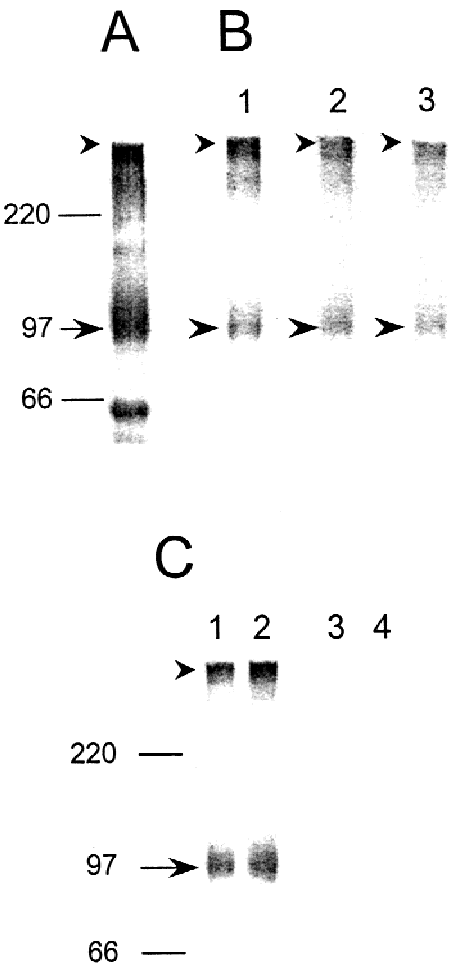

Fig. 2. ^{35}S-GTX binds covalently to proteins in the SR including RyRs. 8% SDS-PAGE gels of native SR vesicles (100 μg of protein; 20 μg/well) (*A*); radiographs of equivalent SDS-PAGE gels run after incubation of SR for 5, 30 or 60 min in 100 μM ^{35}S-GTX at 37°C (lanes 1, 2 and 3 in *B*) and after incubation of SR at 37°C for 10 min with 100 μM ^{35}S-GTX (lanes 1 and 2 in *C*) or with 100 μM ^{35}S-GTX plus 1 mM DTT (lane 3 in *C*) or 1 mM GSH (lane 4 in *C*). Positions of molecular weight markers at 220 kDa and 68 kDa are shown beside the gels in *A* and the radiographs in *B* and *C*. The arrows indicate the position of the RyR and an ~97 kDa protein.

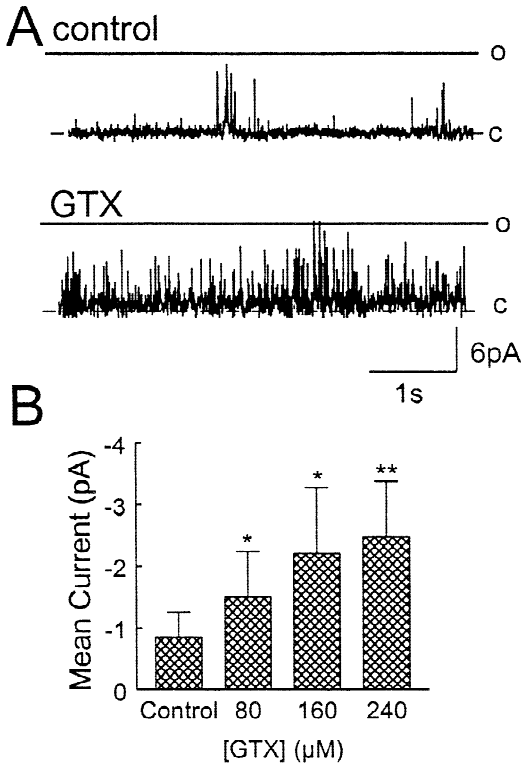

Fig. 3. Sequential addition of GTX to the cytoplasmic solution causes progressive activation of RyR channels in lipid bilayers. Activity in a single RyR channel at +40 mV with 100 μM *cis* Ca^{2+} is shown in *A* before (control) and after (GTX) addition of 200 μM GTX to the *cis* chamber. Channel opening is upward from the zero current level (broken line, labeled C) to the maximum open conductance (continuous line labeled O). (*B*) Average mean current (in pA) recorded under control conditions (control, $n = 7$), after addition of 80 μM GTX (80, $n = 6$), 160 μM GTX (160, $n = 6$) or 240 μM GTX ($n = 7$). Asterisks indicate that average mean current with each GTX concentration were significantly different from control (*$P < 0.05$, **$P \leq 0.01$).

toxin (Fig. 3*A*). Mean current (I') through RyRs with 80 μM ($n = 6$), 160 μM ($n = 6$) or 240 μM ($n = 7$) GTX was significantly greater than control I', with an ~3-fold increase in the seven channels exposed to 240 μM GTX (Fig. 3*B*). There was a progressive increase in channel activity as GTX concentration increased. I' with 240 μM GTX was significantly greater than I' with 80 μM GTX. The graded increase in single RyR channel activity with increasing GTX concentration indicated that GTX interacts with several sites on the RyR channel complex. The increase in RyR activity induced by GTX was reversed in 5–30 sec (average 17.5 sec) after adding 2 mM DTT to the *cis* chamber. DTT was added 2–3 min after adding 200 μM *cis* GTX (Fig. 4*A*). Channel openings to the maximum conductance in Fig. 4 are longer and better resolved in the presence of *cis* GTX and GTX + DTT and thus appear larger than most under control conditions.

Addition of *cis* GTX was followed by an increase in RyR channel activity and I' in 17 out of 18 bilayer experiments, with a average 2.5-fold increase in average I' (Fig. 4*B*). The effects of 200 to 240 μM GTX on single RyR channel characteristics were analyzed in 9 experiments in which one channel only was active in the bilayer (Fig. 4*B*). There were significant increases in average open probability (P_o) and frequency of opening (F_o) after adding GTX to the *cis* chamber. Although there was not a significant increase in average mean open time (T_o), T_o did increase in 7 of 9 channels and in one channel increased from 1.2 msec under control conditions to 25 msec with GTX. An example of increased duration of openings to maximal and submaximal conductances in the presence of GTX can be seen in Fig. 4*A*.

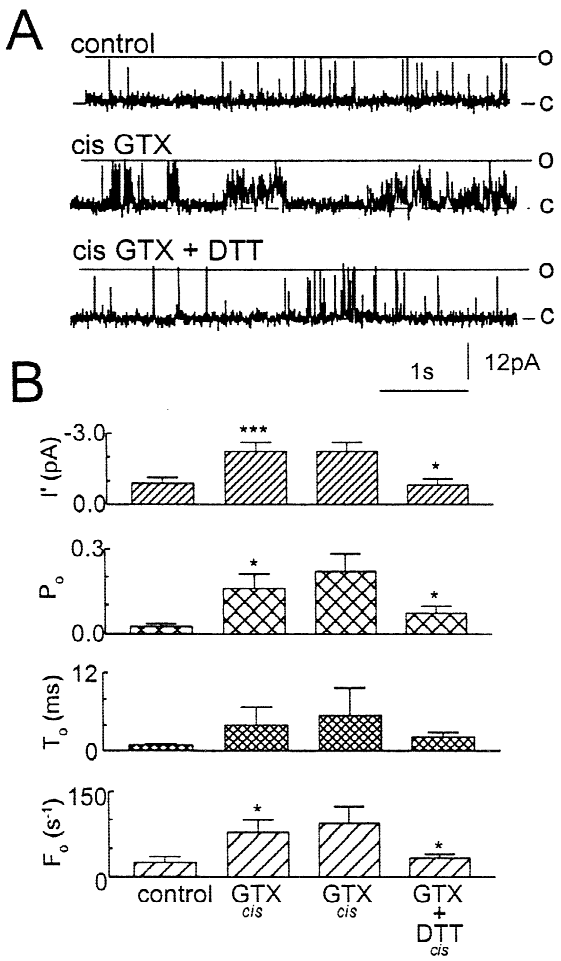

Fig. 4. Activation of RyR channels by 200 μM *cis* GTX is reversed after addition of 2 mM DTT to the *cis* chamber. (*A*) Single RyR channel activity recorded under control conditions (control), increased after addition of GTX (*cis* GTX) and then fell again after further addition of DTT (*cis* GTX + DTT). Channel opening is upward from the zero current level (broken line, labeled C) to the maximum open conductance (continuous line labeled O). (*B*) Average mean current (I'), open probability (P_o), mean open time (T_o) and open frequency (F_o) before (control, first histogram) and after (GTX *cis*, second histogram) addition of 200 μM GTX to the *cis* side of 18 single RyR channels. Average I', P_o, T_o and F_o is then shown for a subset of 8 channels exposed to 200 μM *cis* GTX (GTX *cis*, third histogram) and then exposed to GTX + 1 mM *cis* DTT. Asterisks indicate that average parameter values with GTX (second column of histograms) are significantly different from control values in the first column of histograms, or average parameter values with DTT (fourth column of histograms) are significantly different from those with GTX alone in the second column of histograms (*$P < 0.05$, ***$P < 0.0005$).

Although there appears to be an increase in duration of submaximal conductance openings in Fig. 4*A*, a preferential effect on substate opening was not generally observed. In summary, the toxin activated RyRs by increasing the frequency of opening in all channels and by increasing the open dwell time in some channels.

The effects of GTX on I', P_o and F_o were reversed when 2 mM DTT was added to the *cis* chamber (in the presence of 200 μM GTX) in 6 of 8 experiments. On average, I' in the presence of 200 μM GTX was 2.2 ± 0.4 pA and this fell to 0.84 ± 0.27 pA after adding DTT (Fig. 4). Six experiments in which one channel only was active, showed a significant 3-fold decrease in average F_o and P_o after adding DTT. T_o was not significantly reduced after adding DTT, but was 5.5 ± 4.2 msec respectively with GTX and 2.2 ± 0.6 msec after adding DTT. The average parameter values for the 6 channels measured with GTX plus DTT in the *cis* solution were not significantly different from the values measured under control conditions before addition of the redox reagents. Since DTT added to the *cis* chamber did not alter channel activity if channels had not been previously exposed to GTX (Fig. 8 below), the fall in activity with DTT in the presence of GTX (Fig. 4) suggested that *cis* GTX altered channel gating by covalent modification of functionally important cysteine residues.

GTX was dissolved in ethanol at a maximum stock concentration of 8.5 mM. Thus addition of 200 μM GTX was accompanied by addition of 2.4% ethanol. 1% ethanol has been reported to activate pig skeletal RyRs (Haarmann et al., 1999), but does not affect rabbit skeletal, or sheep cardiac, RyRs (Ahern et al., 1999; Eager et al., 1997). In four experiments, rabbit skeletal RyRs were exposed to 3% ethanol in the *cis* solution for 5 min, there was a small reduction in activity in 3 channels, which recovered when ethanol was perfused from the *cis* chamber. The fact that activation with GTX + ethanol was reversed by DTT also argues that activation was not due to ethanol, since an ethanol effect would not have been reversed DTT. Therefore, we conclude that activation was due to an effect of GTX, not ethanol.

Activation of RyR Channels by GTX Applied to the *trans* Solution

GTX in the *trans* chamber activated RyR channels in the same way as GTX added to the *cis* solution. Single channel records showed a substantial increase in the number of channel openings toxin (Fig. 5*A*). In marked contrast to the reversal of *cis* GTX-induced activation by *cis* DTT, addition of 2 mM DTT to the *trans* chamber (i.e., *trans* 200 μM GTX plus 2 mM DTT) after activation by *trans* GTX caused a further increase in the activity of RyRs (Fig. 5*B*). There were increases in RyR activity after adding 200 μM GTX to the *trans* solution in all 15 experiments within 45 sec of toxin addition, with a significant increase in average I' (Fig. 6*A*). Single channel parameters were measured in 9 channels. Seven of the channels showed increases in P_o and T_o, while F_o increased in all 9 channels (Figs. 6*B*, *C* and *D*). The increase in each of the average parameters was significant.

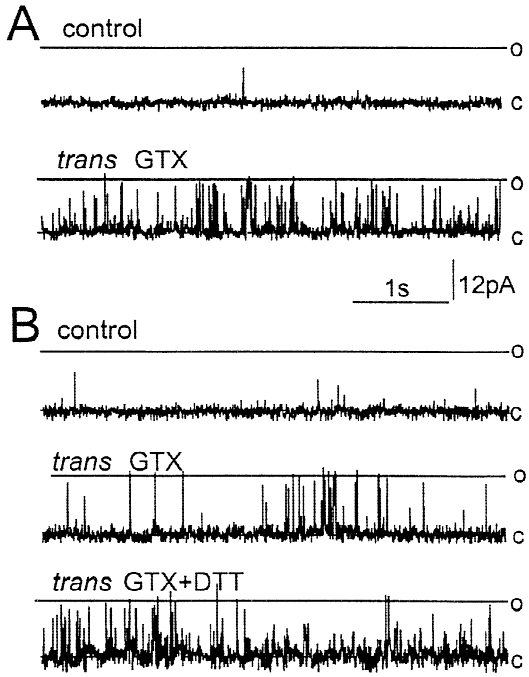

Fig. 5. RyR channel activity (at +40 mV with 100 μM *cis* Ca^{2+}) increases when GTX is added to the luminal solution and channel activity is further enhanced by subsequent addition of DTT to the luminal solution. (*A*) A single RyR channel before (control) and after (*trans* GTX) adding 200 μM GTX to the *trans* solution. (*B*) Activity in a different single RyR channel is enhanced after addition of 200 μM GTX to the *trans* solution and then further enhanced when 2 mM DTT is also added to the trans solution (*trans* GTX + DTT). Channel opening is upward from the zero current level (broken line, labeled C) to the maximum open conductance (continuous line labeled O).

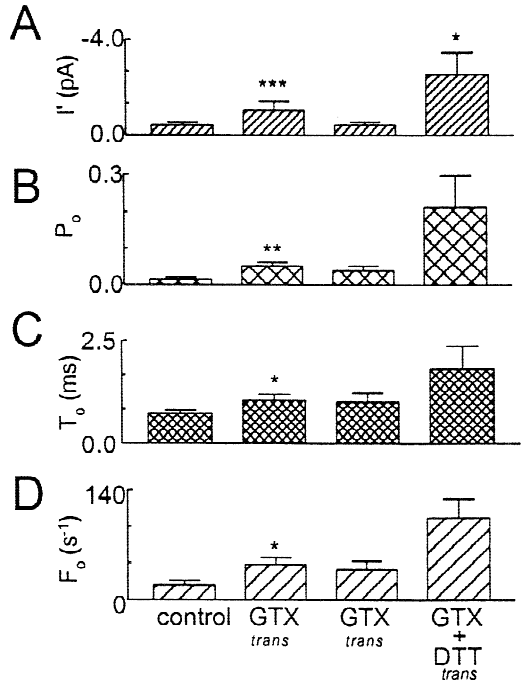

Fig. 6. Addition of GTX to the *trans* chamber increases average mean current and open probability by increasing the frequency and duration of channel opening, while subsequent addition of DTT induces a further increase in mean current. (*A*) Average mean current (I'); (*B*) average open probability (P_o); (*C*) average mean open time (T_o); (*D*) average open frequency (F_o) before (control, first histogram) and after (GTX *trans*, second histogram) addition of 200 μM GTX to the *trans* side of 15 single RyR channels. Average I', P_o, T_o and F_o is then shown for a subset of 7 channels exposed to 200 μM *trans* GTX (GTX *trans*, third histogram) and then exposed to 2 mM *trans* DTT. Asterisks indicate that average parameter values with GTX (second column of histograms) are significantly different from control values in the first column of histograms, or average parameter values with GTX + DTT (fourth column of histograms) are significantly different from those with GTX alone in the third column of histograms (*$P < 0.05$, **$P < 0.01$).

The changes in activity with *trans* GTX were similar to the changes seen with *cis* GTX, with the most consistent effect of the toxin being on the frequency of channel opening and a less consistent effect on channel open time.

Increased activation of *trans* GTX-activated RyRs with the further addition of DTT to the *trans* solution was seen 5 of 7 experiments with a significant increase in the average I' from the 7 experiments within 5–15 sec of adding DTT (Fig. 6). Although all single channel parameters (P_o, T_o and F_o) increased in 5 out of 6 single channel experiments, average parameter values did not increase significantly (Fig. 6).

DTT Activates RyRs When Added to the *trans* Solution, but not the *cis* Solution

The unexpected increase in channel activity after adding 2 mM *trans* DTT suggested that DTT had an independent action of activating RyRs, which depended on its interaction with a site on the luminal side of the channel complex. To test this possibility, 2 mM DTT was added to the *trans* solution in the absence of GTX. A substantial increase in RyR activity was observed, with individual channels showing an increase in both the frequency and duration of channel opening (Fig. 7*A*). A second channel in the bilayer was activated after adding *trans* DTT in the example shown in Fig. 7*A* and is shown to illustrate the extent of channel activation by *trans* DTT. The recording in Fig. 7 was not included in the single channel analysis. An increase in activity was seen in 6 out of 6 experiments, with a significant increase in the average I' (Fig. 7*B*). There were increases in P_o and T_o in 4 out of 5 channels and an increase in F_o in 3 out of 5 channels.

In contrast to *trans* DTT, and in agreement with our previous results (Eager et al., 1997; Haarmann et al., 1999), 2 mM DTT in the *cis* chamber did not alter RyR activity within 45 sec to 2 min after addition to the *cis*

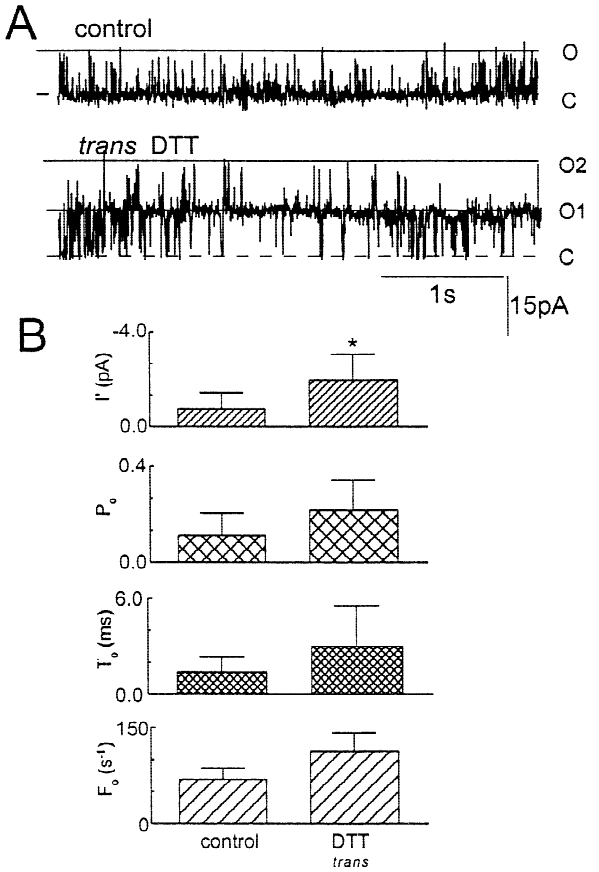

Fig. 7. Single RyR channel activity increases when DTT is added to the luminal solution in the absence of GTX. (*A*) Single channel activity before (control) and after (*trans* DTT) adding 2 mM DTT to the *trans* chamber. Note that a second channel is apparent after adding DTT, so that the experiment in *A* was not used for single channel analysis. Channel opening is upward from the zero current level (broken line, labeled C) to the maximum open conductance of one channel (continuous line labeled O1) or of two channels (continuous line labeled O2). (*B*) Average mean current (I'), open probability (P_o), mean open time (T_o) and open frequency (F_o) before (control, first histogram) and after (DTT *trans*, second histogram) addition of 2 mM DTT *trans* side of 6 single RyR channels. Asterisks indicate that average parameter values with DTT (second column of histograms) are significantly different from control values in the first column of histograms ($*P < 0.05$).

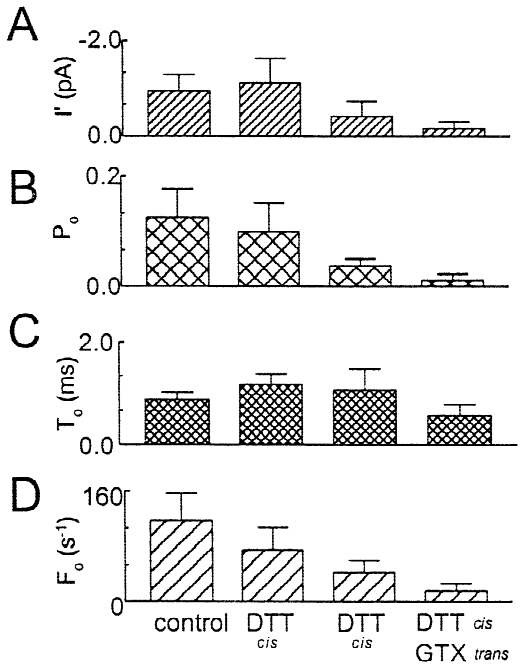

Fig. 8. Single channel activity is unaltered when 2 mM DTT is added to the *cis* chamber and 2 mM DTT in the *cis* chamber prevents activation of RyRs by 200 μM *trans* GTX. (*A*) Average mean current (I'); (*B*) Average open probability (P_o); (*C*) average mean open time (T_o); (*D*) average open frequency (F_o) before (control, first histogram) and after (DTT *cis*, second histogram) addition of 2 mM DTT to the *cis* side of 10 single RyR channels. Average I', P_o, T_o and F_o is then shown for a subset of 5 channels exposed to 2 mM *cis* DTT (DTT *cis*, third histogram) and then exposed to 200 μM *trans* GTX + 2 mM *cis* DTT.

chamber (Fig. 8). I' increased in 6 out of 10 experiments, P_o fell in 5 of 7 single channels, T_o increased in 4 of the channels and F_o fell in 5 channels. The average I', P_o, T_o and F_o remained similar to control values (Fig. 8). The activation of RyRs by *trans* DTT, and the lack of an effect of *cis* DTT, show that DTT had a specific action on the luminal side of rabbit skeletal RyRs.

Site of Action of GTX

DTT, with a pKa of 9 to 10 (Shaked, Szajewski & Whitesides, 1980), is largely uncharged at pH 7 and would, like GTX, rapidly equilibrate into the membrane from the bilayer solution. Both GTX or DTT added to one side of the bilayer, would quickly target either extramembrane protein on that side of the bilayer, or sites in or near the membrane. Because of dilution effects, they would only slowly oxidize/reduce residues remote from the membrane and on the opposite side of the bilayer. Since the effects of GTX were seen at similar times after addition to either the *cis* or *trans* chamber, it is most likely that the toxin oxidized residues in or close to the membrane. If this were the case, oxidation by *trans* GTX would be prevented if the *cis* chamber contained DTT. We added 200 μM GTX to the *trans* chamber 45 sec – 2 min after adding 2 mM DTT to the *cis* chamber. Activation was not seen and I' in fact fell in each of 5 experiments and there was a decrease in P_o, T_o and F_o in each of the 3 channels. There was however no significant change in the average parameters values (Fig. 8).

The failure of *trans* GTX to activate RyRs when the *cis* solution contained DTT was consistent with GTX activating channels by oxidizing −SH groups in or near the membrane. The similar changes in single channel activity with *cis* and *trans* additions of the toxin suggest

that some cysteine residues were oxidized after addition of GTX to either chamber. Conversely, the fact that DTT added to the *cis* and *trans* chambers had very different actions is consistent with the suggestion that the drugs do not equilibrate rapidly in the opposite bulk solution, and that residues on a domain remote from membrane will initially be exposed only to drugs added to the solution bathing that domain.

Since GTX activation sites are located in the membrane region, it is surprising that *trans* DTT did not reduce the activity of RyRs that had been activated by *trans* GTX. It is likely that a decline in activity following reduction of the oxidized groups by DTT was overwhelmed by the DTT-induced activation.

GTX Activates Ca^{2+} Release from Skeletal Muscle SR Vesicles

SR vesicles were loaded with Ca^{2+} and then 500 nM thapsigargin added to suppress SR Ca^{2+}, Mg^{2+}-ATPase activity (Figs. 9*A* and *B*). In control experiments, 1.1% ethanol did not alter the rate of Ca^{2+} release, while 3% ethanol added alone produced a small increase in Ca^{2+} release (Fig. 9*A*). When 30 μM GTX + 1.1% ethanol was added, there was a clear increase in release and 85 μM GTX + 3% ethanol induced a strong increase in the rate of release (Fig. 9*B*). The increase in extravesicular [Ca^{2+}] with thapsigargin and ethanol stopped after addition of ruthenium red, while release induced by GTX was stopped if ruthenium red was added while [Ca^{2+}] was increasing (in all 5 experiments with 57 μM GTX). Figure 9*C* shows that Ca^{2+} release by GTX ($n = 4$) was prevented if ruthenium red was added before the toxin. The results with ruthenium red show that Ca^{2+} release induced by GTX was through RyR channels.

On average, the initial rates of Ca^{2+} release induced by 57 μM and 85 μM GTX were 118 ± 16 ($n = 5$) and 221 ± 43 nmoles/mg/min ($n = 4$) respectively, and were significantly greater than control rates 40 ± 4 nmoles/mg/min ($n = 4$) and 45 ± 9 nmoles/mg/min ($n = 3$) measured with 2 and 3% ethanol respectively (i.e., amounts of ethanol introduced with 57 and 85 μM GTX, in this series of experiments). These results show that GTX activates RyR channels in native SR vesicles as well as in artificial lipid bilayers and that GTX would release Ca^{2+} from SR in intact muscle fibers.

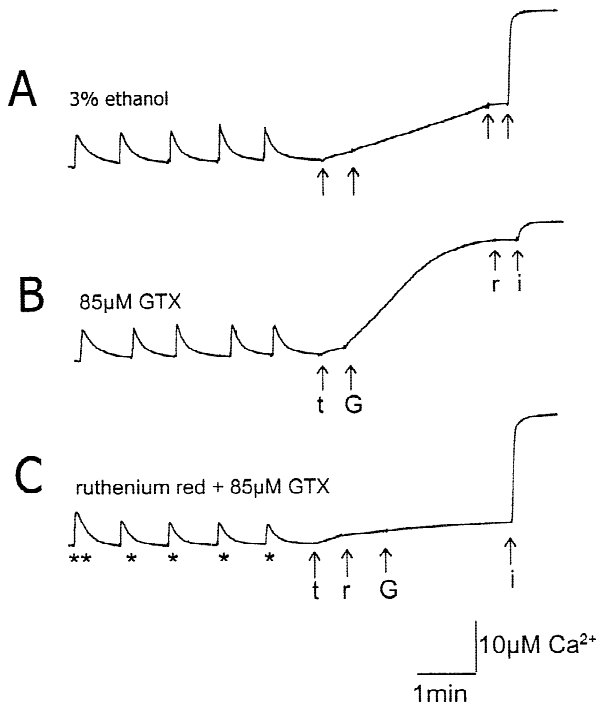

Fig. 9. GTX activates Ca^{2+} release from SR vesicles. *A–D* Show records of OD changes at 710 nm, with changes in extravesicular [Ca^{2+}], measured using antipyrylazoIII (500 μM) as the Ca^{2+} indicator. In each record, extravesicular [Ca^{2+}] increased when TC vesicles were added to the cuvette (**) and then declined as Ca^{2+} was sequestered by the TC vesicles. The next four positive deflections (*) indicate addition of each aliquot of $CaCl_2$ (7.5 μM, final concentration). The first arrow (*t*) indicates addition of 200 nM thapsigargin to block the SR Ca^{2+} ATPase. In *A* and *B*, the second arrow (G) indicates addition of: (*A*) 3% ethanol, (*B*) 85 μM GTX plus 3% ethanol. Ruthenium red (5 μM) was added at third arrow (r), before the ionophore A23187 (last arrow (i), 3 μg/ml). In *C*, 5 μM ruthenium red was added before 85 μM GTX (G). The vertical calibration is in μM Ca^{2+} (addition of 10 μM extravesicular Ca^{2+}, increased OD by 0.2 units).

Discussion

The lipid soluble fungal metabolite, GTX, activates skeletal muscle RyR channels, from either the luminal or cytoplasmic solution, by oxidizing cysteine residues located in or near the membrane. GTX caused an increase in the frequency of opening of all channels and an increase in the open time of some channels. In addition, DTT alone activates RyRs when added to the luminal solution, suggesting that modified cysteine residues on the luminal side of the RyR normally suppresses channel activity.

Activation of RyRs by GTX

The activation of RyRs by GTX is consistent with an increase in RyR channel activity induced by a variety of reactive disulfides (*see* Introduction). Oxidation of cysteine residues associated with the RyR by most reactive disulfides leads to an increase in the duration of channel opening, with little change in the frequency of opening (Boraso & Williams, 1994; Eager & Dulhunty, 1998, 1999; Haarmann et al., 1999). The unusual preferential effect of GTX on the frequency of channel opening suggests that the toxin might oxidize cysteine residues that

are not preferentially oxidized by commonly used reactive disulfides. Curiously, activation of RyRs by S-Nitrosylation or oxidation of thiols in the presence of 10 μM of the NO donor *S*-nitroso-N-acetyl-penicillamine (SNAP) is also caused by a significant increase in the frequency of channel openings (Hart & Dulhunty, 2000). The NO-induced increase in frequency was seen only at −40 mV however, and not at +40 mV where the GTX-induced increase in frequency was observed. Thus it is possible that more than one class of frequency enhancing thiols exist, one which influences activity at −40 mV and the other which influences activity at +40 mV.

Since GTX increased the open time of some channels, the toxin must also target (although less effectively) the cysteine residues which increase open time, i.e., GTX targets two classes of functional thiol groups. The progressive activation of RyRs with increasing [GTX] suggested that oxidation of more than one residue was involved in the increase in channel activation. If each class of functional thiol groups contains a minimum of one residue per subunit, then there would be at least four residues per RyR in each class.

A further difference between GTX and other reactive disulfides was the failure of GTX to inhibit RyR activity during long exposures to the reagent. Both DTDPs and thimerosal, but not DTNB, inhibit RyRs at concentrations of ~1 mM, by oxidizing an inhibiting class of cysteine residue (Eager et al., 1997; Haarmann et al., 1999). This lack of inhibition could indicate that GTX was excluded from the inhibiting thiols because of structural/steric constraints, or that the [GTX] was not high enough to allow the toxin to modify the lower affinity inhibition site. The DTDPs and thimerosal do not inhibit RyRs at concentrations <1 mM (Eager et al., 1997; Marengo, Hidalgo & Bull, 1998). The low solubility of GTX, even in ethanol, meant that concentrations higher than 200 μM could not be tested.

Interactions between GTX and RyRs

GTX could have activated RyRs by forming a stable mixed disulfide, or by the formation of a protein-protein disulfide bridge after further oxidation of an unstable mixed disulfide by a second protein thiol (Glazer, 1970). Indeed both types of reaction may have occurred: one class of –SH residue is S-Nitrosylated by small nitrosothiol compounds, while second and third classes are oxidized to intraprotein disulfides by the same compounds (Xu et al., 1998). Both S-Nitrosylation and oxidation lead to RyR channel activation. The fact that SR proteins showed similar labeling by GTX after 5, 30 and 60 min suggested that GTX was capable of forming stable mixed disulfides with cysteine residues on the protein. Although the stoichiometry of GTX binding to RyRs needs to be determined, there are at least 20 free thiol groups on each subunit of the RyR (Xu et al., 1998) and GTX could have bound to many cysteine residues, not all of which were functionally associated with the ion channel. It is not clear whether the thiols labelled with ^{35}S-GTX were the same as those oxidized under bilayer conditions to activate the channel.

It is also not clear whether the membrane-associated cysteine residues which altered RyR channel activity when modified by GTX were confined to the RyR, or located on regulatory proteins, such as triadin. Since the open time of purified RyRs is increased by oxidation (Boraso & Williams, 1994), the cysteine residues which increase open time when oxidized are likely to be located on the RyR protein.

The Cytoplasmic/Transmembrane Location of the GTX-Oxidation Sites

The location of the functional GTX-oxidation sites in parts of the RyR that are in or near the membrane is consistent with results from different types of oxidizing reagents which show that cysteine residues which modify the gating of RyR channels are located in the cytoplasmic and membrane associated domains of RyRs. The less membrane permeable thimerosal or DTNB activate cardiac or skeletal RyRs from either side of the bilayer and activation is reversed by reducing agents in the opposite chamber, suggesting that the cysteine residues are located in membrane-associated domains (Eager & Dulhunty, 1999; Haarmann et al., 1999).

Effects of DTT on the Luminal Side of the RyR Complex

Activation of rabbit skeletal RyRs by DTT added to the luminal solution suggested that a disulfide bridge or S-Nitrosothiol group on the luminal side of the SR was reduced by 2 mM DTT (DTT is effective in denitrosylation of S-Nitrosothiols as well as in reduction of disulfide bridges, Xu et al., 1998). Results presented by Xu et al. (1998) show higher average P_o recorded after addition of 10 mM DTT to cardiac RyR channels that had been oxidized and activated by CysNO, which suggests that DTT might have had an independent activating action on these channels. It is not clear why DTT activates skeletal RyRs in some cases, but inhibits the channels in other experiments (Eager et al., 1997; Zable, Favero & Abramson, 1997; Marengo et al., 1998). DTT induces a contracture in intact fibers which has been attributed to activation of RyRs (Oba et al., 1996). The effect of DTT is not related to a specific technique used to prepare RyR channels or to the presence or absence of DTT during vesicle isolation. Pig skeletal RyRs (Haarmann et al., 1999) were prepared using a method modified from Meissner (1984) and Ma, Bhat & Zhao (1995) and were

prepared and stored in buffer containing 2 mM DTT, while rabbit skeletal RyRs were prepared using a method modified from Saito et al. (1984) and were prepared and stored in the absence of DTT.

Role of RyR Activation in the Biological Activity of GTX

Biological actions of GTX include immunosuppression and cytotoxicity (Waring et al., 1988) as well as apoptosis and inhibition of T and B cell proliferation after antigen stimulation (Braithwaite et al., 1986; Waring et al., 1988; Meissner, 1994). Clearly, any process that depended on an increase in cytoplasmic Ca^{2+} from intracellular Ca^{2+} stores through RyRs, would be altered by GTX. Although apoptosis induced in thymocytes by 1 μM GTX is independent of changes in intracellular Ca^{2+}, cytoplasmic Ca^{2+} does increase with 50 μM GTX and leads to necrosis and cell death (Beaver & Waring, 1994). RyRs have now been identified in T- and B-cells and a sustained Ca^{2+} increase which depends on Ca^{2+} release through RyRs is essential for immune responses (Guse et al., 1999; Sei et al., 1999). RyRs have been identified in many vertebrate cells (Giannini et al., 1995) as well as in insects (Takeshima et al., 1994), nematodes (Maryon, Saari & Anderson, 1998) and plants (Muir & Sanders, 1996). Thus the function of different cell types in many different organisms could be affected by GTX as a result of its actions on RyR channels. Although GTX caused only a 2- to 3-fold increase in the mean current flowing through RyRs, this increase in activity would be sufficient to deplete Ca^{2+} stores over a prolonged period. Since GTX is covalently associated with RyRs and does not eventually depress channel activity, it would cause long term activation of RyR channels.

In conclusion, the results show that GTX activates skeletal muscle RyR channels in lipid bilayers and in SR vesicles. GTX activates RyRs by preferentially targeting a class of sulfhydryl groups that increases the frequency of RyR channel opening when oxidized and is not preferentially targeted by more commonly used reactive disulfides. GTX provides a new class of reactive disulfide that can activate RyRs and can be used to explore the effects of oxidation reactions on RyR channel activity and on Ca^{2+} release from the SR. The biological actions of GTX include an increase in cytoplasmic $[Ca^{2+}]$ and it is likely that activation of RyRs contributes to this increase in $[Ca^{2+}]$.

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