

Effects of ivermectin and midecamycin on ryanodine receptors and the Ca^{2+} -ATPase in sarcoplasmic reticulum of rabbit and rat skeletal muscle

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1. Ryanodine receptor (RyR) Ca^{2+} channels in the sarcoplasmic reticulum (SR) of skeletal muscle are regulated by the 12 kDa FK506- (or rapamycin-) binding protein (FKBP12). Rapamycin can also activate RyR channels with FKBP12 removed, suggesting that compounds with macrocyclic lactone ring structures can directly activate RyRs. Here we tested this hypothesis using two other macrocyclic lactone compounds, ivermectin and midecamycin.
2. Rabbit skeletal RyRs were examined in lipid bilayers. Ivermectin (*cis*, 0.66–40 μM) activated six of eight native, four of four control-incubated and eleven of eleven FKBP12-‘stripped’ RyR channels. Midecamycin (*cis*, 10–30 μM) activated three of four single native channels, six of eight control-incubated channels and six of seven FKBP12-stripped channels. Activity declined when either drug was washed out.
3. Neither ivermectin nor midecamycin removed FKBP12 from RyRs. Western blots of terminal cisternae (TC), incubated for 15 min at 37 °C with 40 μM ivermectin or midecamycin, showed normal amounts of FKBP12. In contrast, no FKBP12 was detected after incubation with 40 μM rapamycin.
4. Ivermectin reduced Ca^{2+} uptake by the SR Ca^{2+} - Mg^{2+} -ATPase. Ca^{2+} uptake by TC fell to ~40% in the presence of ivermectin (10 μM), both with and without 10 μM Ruthenium Red. Ca^{2+} uptake by longitudinal SR also fell to ~40% with 10 μM ivermectin. Midecamycin (10 μM) reduced Ca^{2+} uptake by TC vesicles to ~76% without Ruthenium Red and to ~90% with Ruthenium Red.
5. The rate of rise of extravesicular $[\text{Ca}^{2+}]$ increased ~2-fold when 10 μM ivermectin was added to TC vesicles that had been partially loaded with Ca^{2+} and then Ca^{2+} uptake blocked by 200 nM thapsigargin. Ivermectin also potentiated caffeine-induced Ca^{2+} release to ~140% of control. These increases in Ca^{2+} release were not seen with midecamycin.
6. Ivermectin, but not midecamycin, reversibly reduced Ca^{2+} loading in four of six skinned rat extensor digitorum longus (EDL) fibres to ~90%, and reversibly increased submaximal caffeine-induced contraction in five of eight fibres by ~110% of control. Neither ivermectin nor midecamycin altered twitch or tetanic tension in intact EDL muscle fibres within 20 min of drug addition.
7. The results confirm the hypothesis that compounds with a macrocyclic lactone ring structure can directly activate RyRs. Unexpectedly, ivermectin also reduced Ca^{2+} uptake into the SR. These effects of ivermectin on SR Ca^{2+} handling may explain some effects of the macrolide drugs on mammals.

The ryanodine receptor (RyR) is a large conductance ion channel which allows Ca^{2+} release from the sarcoplasmic reticulum (SR) of skeletal muscle to initiate contraction in response to sarcolemmal depolarization (Dulhunty, 1992).

The contraction is terminated when Ca^{2+} is pumped back into the SR by the Ca^{2+} - Mg^{2+} -ATPase. The functional skeletal RyR Ca^{2+} -release channel is a homotetrameric complex of RyR monomers (M_r ~560 000) and four FK506-

binding proteins (FKBP12, $M_r \sim 12000$), each of which is tightly bound to a RyR monomer (Timerman *et al.* 1993; Brillantes *et al.* 1994; Wagenknecht *et al.* 1996). The immunosuppressive drugs FK506 and rapamycin bind with nanomolar affinities to FKBP12, causing the binding protein to dissociate from the RyR tetramer as the drug-bound protein complex (Jayaraman *et al.* 1992; Timerman *et al.* 1993). The RyRs, 'stripped' of their FKBP12 co-proteins, display increased channel activity (Mayrleitner *et al.* 1994; Ahern *et al.* 1997*a*). We found that rapamycin also has an FKBP12-independent action on the RyR, since the release channel is activated by rapamycin even when 'stripped' of FKBP12. Thus we postulated that there is a binding site on the RyR for compounds with macrocyclic lactone structures similar in size to rapamycin and FK506 (Ahern *et al.* 1997*b*).

To test this hypothesis, we examined the effects of the anti-helminthic ivermectin (Campbell, 1989) and the antibiotic midecamycin (Hamilton-Miller, 1992; Mazzei *et al.* 1993). The chemical structures of both drugs contain a sixteen-membered macrocyclic lactone ring. Ivermectin (22,23-dihydroavermectin B_{1a}) is a member of the avermectin class of macrocyclic lactones, isolated from *Streptomyces avermitilis*, which are potent antihelmintics and insecticides and are used to treat onchocerciasis (river blindness) in humans (Campbell, 1989). The EC₅₀ for the biological activity of avermectins is strongly correlated with their ability to activate glutamate-sensitive channels and their binding affinity for nematode membrane preparations (Arena *et al.* 1995). The antihelminthic activity of ivermectin is believed to depend on its paralysing the pharyngeal muscle and preventing feeding (Dent *et al.* 1997; Brownlee *et al.* 1997; Geary *et al.* 1998). Avermectin B_{1a} can both activate (nanomolar concentrations) and inhibit (micromolar concentrations) GABA-gated Cl⁻ currents in mammalian neurones (Bloomquist, 1993; Huang & Casida, 1997). There are no previous reports of ivermectin affecting mammalian skeletal muscle, or of midecamycin acting on ion channels. Quinolidomycin A1, which has a 60-membered macrocyclic lactone ring structure, activates Ca²⁺ release from SR vesicles, but only about 50% of this release is inhibited by the RyR channel blockers Ruthenium Red, procaine and Mg²⁺ (Ohkura *et al.* 1996).

The results presented here show that micromolar ivermectin reversibly activated native RyR channels incorporated into planar lipid bilayers. Micromolar midecamycin also reversibly activated some, but not all, channels. Since we found here that these macrocyclic lactones did not dissociate FKBP12 from the RyR, and both drugs reversibly altered FKBP12-'stripped' RyR activity, the results provide strong evidence that macrocyclic lactones exert their effects by acting directly on the RyR channel complex. Ivermectin increased Ca²⁺ release from SR vesicles and from skinned muscle fibres, not only by activating the RyR channels but also by inhibiting Ca²⁺ uptake by the SR Ca²⁺-Mg²⁺-ATPase. Midecamycin reduced Ca²⁺ uptake by the SR, but to a lesser

extent than ivermectin and had no detectable action on Ca²⁺ release from SR vesicles or on skinned fibres. These effects of micromolar concentrations of ivermectin could contribute to symptoms of drug overdose in mammals.

METHODS

Materials

Rapamycin was obtained from Calbiochem and as a gift from Wyeth-Ayerst (Princeton, NJ, USA), ivermectin was a gift from Merck, Sharp & Dohme (Sydney, Australia), and ryanodine was obtained from Calbiochem-Novabiochem (San Diego, CA, USA) and Latoxan (Rosans, France). Other chemicals and biochemicals were from Sigma-Aldrich. Stock solutions of ivermectin and midecamycin were prepared in ethanol or DMSO.

Vesicle preparation

New Zealand White rabbits were killed by captive bolt and the back and leg muscles removed immediately. SR vesicles were isolated as described by Saito *et al.* (1984) with minor modifications (Ahern *et al.* 1994, 1997*a*). TC vesicles were obtained from the 38%–45% sucrose interface, and longitudinal SR (LSR) from the 32%–34% sucrose interface. FKBP12 was removed from TC vesicles by incubation with either 10 or 20 μM rapamycin for 15–20 min at 37 °C. 'Control-incubated' channels were incubated in the same way, but without rapamycin. The amount of FKBP12 remaining bound was assessed by immunostaining Western blots of TC vesicles with anti-peptide antibodies raised against peptides corresponding to the N-terminal sequence of FKBP12. Details of the FKBP12 'stripping' procedure, production of anti-peptide antibodies, electrophoresis and Western blotting are given in Ahern *et al.* (1997*a*).

Lipid bilayers and recording and analysis of single channel activity

Experiments were carried out at 20–25 °C. The techniques are described in detail in Ahern *et al.* (1994) and Laver *et al.* (1995). Bilayers were formed from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (5:3:2 w/w) (Avanti Polar Lipids, Alabaster, AL, USA) across an ~250 μm diameter aperture in the wall of a 1.5 ml Delrin cup (Cadillac Plastics, Australia). TC vesicles (final concentration, 10 μg ml⁻¹) and drugs were added to the *cis* chamber. The normal *cis* solution contained 250 mM CsCl, 1 mM CaCl₂ and 10 mM Tes (pH 7.4 with CsOH), and the *trans* solution contained 50 mM CsCl, 1 mM CaCl₂ and 10 mM Tes (pH 7.4). Changes in free Ca²⁺ concentration in the *cis* solution, and drug washout, were achieved by perfusion with appropriate solutions. Bilayer potential was controlled and currents recorded using an Axopatch 200A amplifier (Axon Instruments). Bilayer potentials are given relative to the *trans* chamber.

Channel activity was recorded at 1 kHz (10-pole low-pass Bessel, -3 dB) and digitized at 2 or 5 kHz. Analysis of single channel records (Channel 2, P. W. Gage & M. Smith, John Curtin School of Medical Research, Australian National University) 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 (\bar{I}). The event discriminator was set above the baseline noise at ~20% of the maximum current, rather than the usual 50%, so that openings to both subconductance and maximum conductance levels were included in the analysis.

Single channel activity of RyRs fell into two modes, either high activity ($P_o > 0.1$), or low activity ($P_o < 0.01$). The mode of the control activity in a channel was maintained for the duration of the experiment and was reflected in the activity recorded in the

Table 1. Solutions for skinned fibre experiments

Solution	Hepes (mM)	Mg _{total} § (mM)	EGTA (mM)	Ca _{total} (mM)	HDTA (mM)	NaN ₃ (mM)	Caffeine (mM)	ATP (mM)	CP (mM)	pCa
MAS	90	10.3 (1)	50	50	—	1	—	8	10	4.2
HRS	90	10.3 (1)	50	—	—	1	—	8	10	12.8
LRS	90	8.6 (1)	0.2†	—	50	1	—	8	10	9.4
LS	90	8.6 (1)	1.0*	—	50	1	—	8	10	7.7
RS	90	2.0 (0.05)	0.5†	—	50	1	30	8	10	9.9
RS _{SM}	90	5.3 (0.25)	0.5‡	—	50	1	15	8	10	9.9

MAS, maximum activating solution; HRS, high relaxing solution; LRS, low relaxing solution; LS, load solution; RS, release solution; RS_{SM}, submaximal release solution. All solutions were adjusted to pH 7.4 using 4 M KOH. *LS was made by adding 100 μ l of HRS and 20 μ l of 49 mM CaCl₂ to 5 ml of LRS (lacking EGTA). †LRS and RS were initially made without EGTA. EGTA was included by adding 20 or 50 μ l of HRS, respectively, to 5 ml of LRS or RS immediately before use. ‡RS_{SM} was made by combining 2.5 ml of LRS (without EGTA) with 2.5 ml of RS (lacking EGTA) and then adding 50 μ l of HRS. §Mg²⁺ was added as MgO; the calculated free [Mg²⁺] is given in parentheses. ||Ca²⁺ was added as Ca(CO₃)₂. HDTA, hexamethylenediamine-tetraacetate; CP, creatine phosphate.

presence of drugs. The different modes led to large standard errors in average data. The different modes were also seen in FKBP12-stripped and control-incubated channels. In some cases, the majority of channels in one preparation showed low activity, while most channels in another preparation showed high activity. This can be seen in data presented in Table 2, where FKBP12-stripped channels in one experiment had an average P_o of 0.007 ± 0.004 , but average P_o values of 0.183 ± 0.117 and 0.297 ± 0.159 in two other experiments (means \pm S.E.M.).

Ca²⁺ uptake into SR vesicles

Ca²⁺ uptake was measured using a procedure based on Chu *et al.* (1989). Extravesicular Ca²⁺ was monitored at 710 nm with the Ca²⁺ indicator antipyrylazo III, using a Cary 3 Spectrophotometer (Varian, Mulgrave, Victoria, Australia). Identical uptake experiments, performed at 790 nm, showed no changes in optical density (OD) which would alter the rate of Ca²⁺ uptake measured from recordings at 710 nm. The temperature of the cuvette solution was thermostatically controlled at 25 °C and the solution was stirred magnetically during experiments. TC or LSR vesicles (100 μ g of protein) were added, to a final volume of 2 ml, to a solution containing (mM): 100 KH₂PO₄ (pH 7), 1 MgCl₂ (86 μ M free Mg²⁺), 1 Na₂ATP and 0.2 antipyrylazo III. Ca²⁺ was added, to a final concentration of 50 μ M, and a loading rate was obtained from the decline in OD at 710 nm (slope *a*, Fig. 5A). Ruthenium Red was then added to a final concentration of 5 μ M and the uptake rate in the absence of a Ca²⁺ efflux through the RyR obtained (slope *b*, Fig. 5A).

Ca²⁺ release from TC vesicles

Ca²⁺ release was measured as described by Timmerman *et al.* (1993). TC vesicles were added to a solution containing (mM): 100 KH₂PO₄ (pH 7), 4 MgCl₂ (370 μ M free Mg²⁺), 1 Na₂ATP and 0.5 antipyrylazo III. Experiments were performed at 710 and 790 nm. Step changes in OD at both wavelengths were seen upon addition of ivermectin or Ruthenium Red to the cuvette and were subtracted from the records shown in Figs 5 and 6. Vesicles were partially loaded with Ca²⁺, after four sequential additions of CaCl₂, each initially increasing the extravesicular [Ca²⁺] by ~ 7.5 μ M (Figs 5 and 6). The ability of ivermectin to potentiate caffeine- (0.5–5 mM) induced release of Ca²⁺ was measured in the presence of thapsigargin (200 nM, added to inhibit Ca²⁺-Mg²⁺-ATPase activity; Sagara & Inesi, 1991) and an additional 5 mM MgCl₂ (added to

depress Ca²⁺ leakage through the RyR which was apparent when the Ca²⁺ pump was blocked). The Ca²⁺ ionophore A23187 (3 μ g ml⁻¹) was added at the completion of each experiment to release the Ca²⁺ remaining in the TC vesicles.

Skinned fibre experiments

Extensor digitorum longus (EDL) fibres were dissected from male Wistar rats, following asphyxiation by CO₂ and cervical dislocation. The fibres were mechanically skinned under paraffin oil (Lamb & Stephenson, 1990). One end of the fibre was attached to an Akers semiconductor force transducer (model AE 875, SensorNor a.s., Horten, Norway) using silk suture and the other end held by clamping forceps. The fibre was lowered sequentially into 2 ml baths containing the following solutions (see Table 1), in order of application: (i) release solution to release all Ca²⁺ remaining in the SR; (ii) maximum activating solution (pCa, ~ 4) to determine the maximum force that could be produced by the contractile proteins; (iii) high relaxing solution (50 mM EGTA) to remove Ca²⁺; (iv) low relaxing solution (50 μ M EGTA) to remove excess EGTA; (v) release solution to confirm that the SR remained depleted of Ca²⁺; (vi) 30 s in high relaxing solution; (vii) 30 s in low relaxing solution.

The SR was loaded with Ca²⁺ by exposing the fibre to load solution (pCa, 7.7) in which Ca²⁺ was moderately buffered. Loading was terminated by exposing the fibre to the low relaxing solution for 30 s. The extent of loading was assessed from the amplitude of the contracture in the release solution (30 mM caffeine, 0.05 mM free Mg²⁺). The load time required for $\sim 50\%$ maximum loading was determined for each fibre. The free [Ca²⁺] in the solutions was calculated using the program 'Bound and Determined' (Marks & Maxfield, 1991).

Caffeine-induced Ca²⁺ release was examined by maximally loading fibres with Ca²⁺, followed by 30 s in the low relaxing solution and then the release solution containing 15 mM caffeine and 0.25 mM free Mg²⁺, which gave $\sim 50\%$ maximal release.

Intact fibre experiments

Methods are described in Dulhunty (1991). Small bundles of EDL fibres from adult male rats were dissected and bathed in a Krebs solution containing (mM): 120 NaCl, 2 MgCl₂, 2 KCl, 2.5 CaCl₂, 11 glucose and 2 Tes (pH 7.4). Twitches were elicited at 0.1 Hz, with fused tetanic contractions elicited at 5 min intervals.

Block of the Ca²⁺ release channel by Ruthenium Red

The Ca²⁺ release channel blocker Ruthenium Red was used to show either (a) that Ca²⁺ release was through the RyR in vesicle experiments, or (b) that single channel recordings were from RyRs incorporated into bilayers. Concentrations of Ruthenium Red ranging from 5 to 80 μM were used for different purposes. Ruthenium Red at 5–10 μM was sufficient to substantially reduce Ca²⁺ release from SR within seconds of application. The same concentrations of Ruthenium Red took 30–60 s to fully block RyRs in bilayer experiments, while 80 μM Ruthenium Red blocked all single channel activity within 10 s. Higher concentrations of Ruthenium Red were used in many bilayer experiments to block the channel before the bilayer broke. Differences in the sensitivity of RyR channels to Ruthenium Red (see e.g. Smith *et al.* 1988) may be explained by differences in the purity (from 10 to 50%) of Ruthenium Red from different sources (P. R. Junankar, unpublished observations). The reason for the difference in sensitivity to Ruthenium Red between RyR channels in vesicles and in bilayers is not clear, but has been observed with other agents (e.g. Lu *et al.* 1994; Mack *et al.* 1994; El-Hayek *et al.* 1995) and suggests that the sensitivity to drugs can be altered by incorporation of channels into bilayers.

Statistics

Average data are given as means \pm 1 s.e.m. The significance of the difference between the logarithms of paired variables was tested using Student's *t* test for paired data. Some data were tested using the Wilcoxon rank-sum test. Differences were considered to be significant when $P \leq 0.05$.

RESULTS

Ivermectin reversibly activates RyRs

Ivermectin increased the activity of native and FKBP12-stripped RyR channels, without affecting single channel conductance. Brief discrete events, apparent in Fig. 1A,

dominated native RyR activity when channels were inhibited by 1 mM *cis* Ca²⁺.

When 1.3 μM ivermectin was added to the *cis* chamber, the frequency of channel openings increased: open events became longer and the mean current (\bar{I}) increased (Fig. 1A). The mean current through native RyRs increased in eleven out of thirteen bilayers with 0.66–4.0 μM ivermectin and increased reversibly with 40 μM ivermectin in four out of four bilayers containing control-incubated channels. Records from bilayers with only one active single channel were analysed further and the data are summarized in Table 2.

With 0.66–4.0 μM ivermectin, open probability (P_o) increased in six out of eight native RyR channels, mean open time (T_o) became longer in all eight channels and the frequency of openings (F_o) increased in seven channels. Native channel activity in one of one experiment fell to control levels when ivermectin was perfused out of the *cis* chamber. P_o , T_o and F_o increased in each of the four control-incubated channels exposed to 40 μM ivermectin. The ratio of the value of each parameter in ivermectin relative to control was calculated for each of the four channels and expressed as percentage, e.g. $(P_{o,ivermectin}/P_{o,control}) \times 100\%$. The average changes were: $2300 \pm 720\%$ for P_o , $242 \pm 41\%$ for T_o and $1065 \pm 342\%$ for F_o . All parameters in each of the four control-incubated channels fell towards control levels after ivermectin was washed out.

Ivermectin also activated FKBP12-stripped channels (Table 2). Stripped channel activity (Fig. 1B) consisted of long openings to a submaximal level (S1) at $\sim 25\%$ maximum current, with brief openings to higher conductance levels

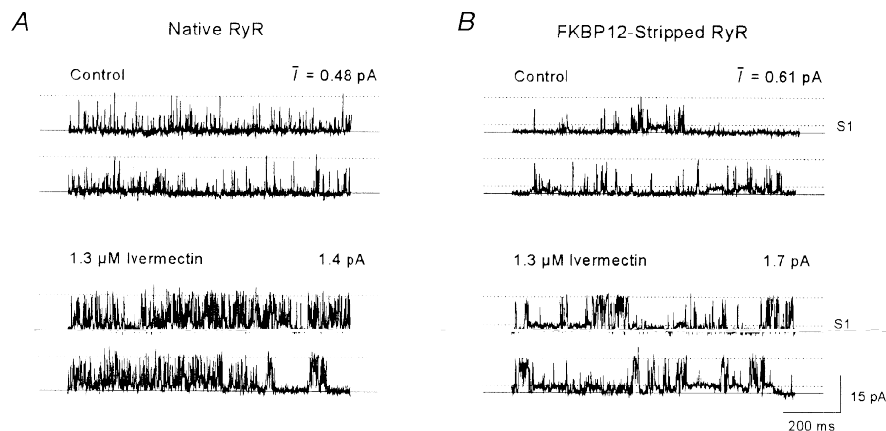


Figure 1. Activation of RyRs by ivermectin

A, continuous channel activity of a single RyR incorporated from native TC vesicles. Upper traces, control recording. Lower traces, recording after addition of 1.3 μM ivermectin. *B*, continuous activity from an FKBP12-stripped RyR, incorporated from FKBP12-stripped TC vesicles. Upper traces, control activity. Lower traces, activity after addition of 1.3 μM ivermectin. Bilayer potential was +40 mV and solutions contained 250/50 mM CsCl and 1/1 mM Ca²⁺ (*cis/trans*). In *A* and *B* the continuous lines show the zero current level, maintained when the channel is closed. The upper dotted line shows the maximal single channel conductance, and the lower dotted line in *B* shows the lowest subconductance level, S1. The mean current, \bar{I} (in pA), was calculated from at least 60 s of activity including that in *A* and *B*.

Table 2. Reversible effects of ivermectin on single channel properties of native, control-incubated and FKBP12-stripped RyRs

RyR	<i>n</i>	<i>P</i> _o	<i>T</i> _o (ms)	<i>F</i> _o (s ⁻¹)
Native (10 ⁻³ M Ca ²⁺)				
Control	8	0.015 ± 0.009	0.71 ± 0.06	21 ± 6
Ivermectin (0.66–4.0 μM)	8	0.079 ± 0.019**	1.21 ± 0.22*	68 ± 10**
Control incubated (10 ⁻⁷ M Ca ²⁺)				
Control	4	0.013 ± 0.004	2.0 ± 0.6	6 ± 3
Ivermectin (40 μM)	4	0.241 ± 0.066**	5.6 ± 2.5**	51 ± 11**
Washout	4	0.096 ± 0.059*	3.0 ± 1.0	26 ± 12*
FKBP12 stripped (10 ⁻⁴ M Ca ²⁺)				
Control	4	0.007 ± 0.003	0.7 ± 0.6	8 ± 4
Ivermectin (4.0 μM)	4	0.023 ± 0.014	0.9 ± 0.2	21 ± 10
FKBP12 stripped (10 ⁻⁴ M Ca ²⁺)				
Control	4	0.007 ± 0.004	0.7 ± 0.2	8 ± 4
Ivermectin (20–24 μM)	4	0.080 ± 0.040†	1.8 ± 0.3*	40 ± 15†
FKBP12 stripped (10 ⁻⁷ M Ca ²⁺)				
Control	7	0.183 ± 0.117	6.3 ± 4.0	15 ± 10
Ivermectin (34–40 μM)	7	0.404 ± 0.133**	12.8 ± 6.4	46 ± 9*
Washout	7	0.274 ± 0.097*	8.2 ± 2.8	40 ± 8

Concentrations of ivermectin and *cis* Ca²⁺ are given for each set of data. Channel activity was measured over ~60 s periods before and after addition of ivermectin. The data are given as means ± s.e.m. for *n* channels. Asterisks indicate the significance of the difference between the control and ivermectin values or between ivermectin and recovered (Washout) values using Student's *t* test on the mean of the logarithm of individual values (* *P* < 0.05; ** *P* < 0.01). † Not significantly different from control when tested with Student's *t* test, but significantly different according to the Wilcoxon rank-sum test (*P* < 0.05).

(described previously, Ahern *et al.* 1997a). The frequency and duration of openings to S1 and to the maximum conductance level increased after addition of 1.3 μM ivermectin to the channel shown in Fig. 1*B*, with an accompanying increase in \bar{I} . *P*_o, *T*_o and *F*_o increased in one of four single FKBP12-stripped channels with 4 μM ivermectin. All of the parameters increased when the same four channels were exposed to 20 or 24 μM *cis* ivermectin, and the parameters also increased in six of seven FKBP12-stripped channels with 34–40 μM ivermectin (Table 2).

When ivermectin (34–40 μM) was perfused out of the *cis* chamber, FKBP12-stripped channel activity returned slowly towards control levels in six out of the seven channels. On average, *P*_o recovered significantly from the exposure to the antihelminthic (Table 2). Ruthenium Red (10 or 80 μM) was added to the *cis* chamber in eleven experiments in which ivermectin had activated channels, and channel activity was abolished in all cases.

Action of the antibiotic midcamycin on RyR channels

Midcamycin (10–20 μM) reversibly activated native RyRs in three of four bilayers. In one experiment (Fig. 2), \bar{I} increased with 10 μM *cis* midcamycin and increased further with a second addition of 10 μM midcamycin.

\bar{I} then fell with midcamycin washout. Two channels became active when the bilayer was again exposed to 10 μM midcamycin. Adding 13 μM *cis* ryanodine then induced long openings in both channels to ~50% maximum conductance (Fig. 2). In the other experiments, 10 μM midcamycin (*n* = 1) increased *P*_o from 0.029 to 0.051 (*T*_o from 0.64 to 0.71 ms, and *F*_o from 44 to 72 s⁻¹), while 16 μM midcamycin (*n* = 1) increased *P*_o from 0.05 to 0.17 (*T*_o from 0.89 to 1.19 ms and *F*_o from 57 to 142 s⁻¹).

Midcamycin (*cis*, 20–30 μM) increased \bar{I} in control-incubated channels (8 of 12 bilayers) and in FKBP12-stripped channels (8 of 14 bilayers). Of eight single control-incubated channels, *P*_o and *F*_o increased in six and *T*_o increased in five channels. For these eight channels, the averages of the percentage changes after exposure to midcamycin were: 323 ± 150% for *P*_o, 117 ± 16% for *T*_o and 345 ± 80% for *F*_o. Of the seven single FKBP12-stripped channels, *P*_o and *F*_o increased in four and *T*_o increased in six channels with midcamycin, with the averages of the percentage change seen upon exposure to midcamycin being: 292 ± 159% for *P*_o, 125 ± 20% for *T*_o and 185 ± 63% for *F*_o.

Perfusing midcamycin out of the *cis* chamber was followed by a reduction in \bar{I} in six of six bilayers with control-

Table 3. Ethanol at 2% does not alter single channel characteristics of control-incubated or FKBP12-stripped RyRs

RyR	<i>n</i>	<i>P</i> _o	<i>T</i> _o (ms)	<i>F</i> _o (s ⁻¹)
Control incubated (10 ⁻⁷ M Ca ²⁺)				
Control	3	0.100 ± 0.060	11.2 ± 10.4	6 ± 2
Ethanol	3	0.088 ± 0.086	4.6 ± 3.9	8 ± 6
FKBP12 stripped (10 ⁻⁷ M Ca ²⁺)				
Control	5	0.297 ± 0.159	10.0 ± 5.0	25 ± 9
Ethanol	5	0.301 ± 0.151	11.0 ± 5.3	23 ± 8

Channel activity was measured over ~60 s periods before and after addition of ethanol. The data are given as means ± S.E.M.

incubated channels, and seven of seven bilayers with FKBP12-stripped RyRs. Channels activated by midecamycin, like those activated by ivermectin, failed to show a preferential increase in activity to submaximal conductance levels. The results suggest that midecamycin altered activity in some native, control-incubated and FKBP12-stripped channels in much the same way as ivermectin. However, the changes with midecamycin were smaller than those with ivermectin and required higher concentrations of the drug.

The effects of ivermectin and midecamycin were not due to the addition of the vehicle. Ethanol (0.3% v/v) alone has no significant effect on the single channel kinetics of skeletal or cardiac RyRs (Ahern *et al.* 1994; Eager *et al.* 1997). In the present experiments, 2% *cis* ethanol failed to alter single

channel characteristics in three control-incubated and five FKBP12-stripped RyRs within 2 min of addition (Table 3).

In addition, channel activity did not change when ethanol was washed out of the *cis* chamber in two of two control-incubated channels or in four of four FKBP12-stripped channels.

Ivermectin and midecamycin do not remove FKBP12 from TC vesicles

Since FKBP12-stripped channels and RyRs containing FKBP12 (native or control incubated) responded in a similar way to applications of ivermectin (or midecamycin), it was likely that removal of FKBP12 was not necessary for channel activation. However, it remained possible that the

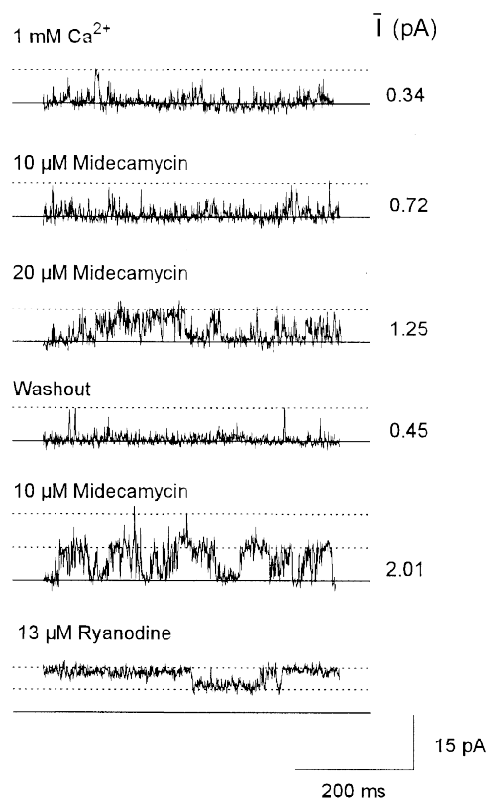


Figure 2. Reversible activation of native RyR channels by midecamycin

Channel activity obtained from one bilayer containing at least two RyR channels. Two sequential additions of 10 μM midecamycin were followed by *cis* chamber perfusion and then 10 μM midecamycin was added once again, followed by 13 μM ryanodine. One channel was active in the bilayer in the top four records; two channels were active in the final two records. Channel activity was recorded at +40 mV with 1 mM *cis* Ca²⁺. The maximum open level for a single channel is indicated by the first dotted line in each record. The summed conductance for the two channels is indicated by the upper dotted line in the last two records. The mean current (\bar{I}) recorded from > 80 s of continuous activity under each condition is shown beside each record (in pA).

drugs could induce FKBP12 dissociation from the RyR. TC vesicles were incubated with ivermectin (40 μM) and midecamycin (40 μM), as well as rapamycin (0.5–40 μM), for 15 min at 37 °C. After the drugs were removed from the vesicles by centrifugation, the resuspended membranes were subjected to SDS-PAGE and Western blotting (Fig. 3) before immunostaining with antibodies to FKBP12.

In contrast to rapamycin, neither midecamycin nor ivermectin caused FKBP12 dissociation from the TC vesicles. Furthermore, unlike bastadin and FK506 (Mack *et al.* 1994), ivermectin did not act synergistically with rapamycin in promoting the removal of FKBP12 from RyRs (P. R. Junankar, unpublished observations).

Ivermectin reduces the rate of Ca^{2+} uptake by TC vesicles

The experiments in this and the following sections examine the possibility that ivermectin and midecamycin alter Ca^{2+} handling by the SR. The rate of removal of Ca^{2+} from the extravesicular solution by TC or LSR was examined in the absence or presence of Ruthenium Red (Fig. 4).

The rate of Ca^{2+} removal by TC vesicles (slope *a*, Fig. 4A) depends on Ca^{2+} uptake by the SR Ca^{2+} - Mg^{2+} -ATPase minus Ca^{2+} release through RyR channels and other Ca^{2+} leakage pathways. Ca^{2+} efflux through RyR channels was blocked when Ruthenium Red (5 μM) was added and the rate of Ca^{2+} removal increased (slope *b*, Fig. 4A). We had predicted that, if Ca^{2+} release alone was enhanced by ivermectin, slope *a* only would be reduced after ivermectin addition to TC vesicles. Surprisingly, both slope *a* and slope *b* were less steep than control with 10 μM ivermectin. Ivermectin caused a concentration-dependent decrease in the rate of Ca^{2+} uptake in both the absence and presence of Ruthenium Red (5 μM), with IC_{50} values (from the best fit of a simple Michaelis–Menton equation to the data) of 3.54 ± 1.37 and 2.01 ± 0.74 μM , respectively (Fig. 4B).

If the only effect of ivermectin was to enhance Ca^{2+} release through RyRs, then slope *a* in LSR vesicles should not be

altered by ivermectin because the vesicles contain little RyR protein (Kourie *et al.* 1996). The paucity of RyR channels was confirmed by a lack of effect of Ruthenium Red on Ca^{2+} uptake rates by LSR (not shown). The rate of Ca^{2+} uptake (slope *a* in Fig. 4C) was reduced with 10 μM ivermectin, with an average reduction to $\sim 40\%$ of control (Fig. 4D). The IC_{50} for the action of ivermectin on Ca^{2+} uptake in LSR was 3.42 ± 0.67 μM , very close to the value obtained for the Ruthenium Red-inhibited TC vesicles. Since ivermectin slowed Ca^{2+} removal from the extravesicular solution in two situations in which RyR activity was minimal, the results suggest that ivermectin reduced the rate of Ca^{2+} uptake by the Ca^{2+} - Mg^{2+} -ATPase.

The control experiments shown in Fig. 4 were performed in the presence of ethanol (the same volume as added with midecamycin; the maximum volume of ethanol added was 0.5% v/v). Ethanol alone reduced Ca^{2+} uptake by TC vesicles in each of three preparations, to $77 \pm 7\%$ of control before adding Ruthenium Red, and to $80 \pm 3\%$ of control after Ruthenium Red.

Experiments were also performed with ivermectin dissolved in DMSO (which was the vehicle used in subsequent skinned fibre experiments, below). Rates with DMSO alone were $115 \pm 12\%$ of control ($n = 5$) before Ruthenium Red and $107 \pm 8\%$ of control after Ruthenium Red was added. Ivermectin (10 μM) in DMSO reduced the rate of Ca^{2+} uptake both before and after adding Ruthenium Red in three out of three preparations, to 66 ± 8 and $54 \pm 5\%$, respectively, of the controls in DMSO. Thus ivermectin substantially reduced Ca^{2+} uptake whether ethanol or DMSO was used as the vehicle.

In contrast to ivermectin, midecamycin (10–40 μM in ethanol) had minimal effects on Ca^{2+} uptake by TC. In one experiment, the uptake rates in 10 and 40 μM midecamycin were 103 and 120%, respectively, of control before Ruthenium Red addition, and 104 and 107% of control, respectively, with Ruthenium Red. In the presence of DMSO, midecamycin (10 μM) reduced Ca^{2+} uptake before and after

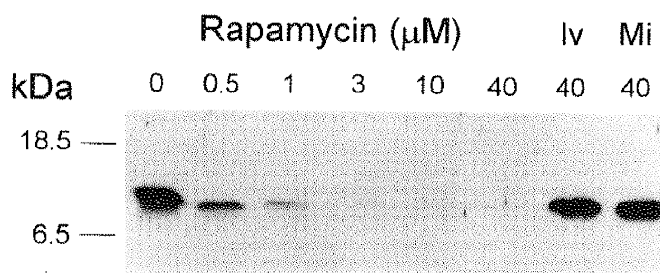


Figure 3. Ivermectin and midecamycin do not remove FKBP12 from TC vesicles, in contrast to the effect of rapamycin

Western blot of TC vesicles which had been pre-incubated with rapamycin (0.5–40 μM), ivermectin (Iv, 40 μM) or midecamycin (Mi, 40 μM). The blot was immunostained with an affinity-purified antiFKBP12-peptide antibody. The positions of molecular mass markers are shown on the left. The immunostaining at ~ 12 kDa in vesicles treated with ivermectin or midecamycin was not decreased compared with the control vesicles. No detectable FKBP12 was observed in SR vesicles preincubated with 10–40 μM rapamycin.

adding Ruthenium Red in three out of three experiments, with averages of 76 ± 11 and $90 \pm 3\%$, respectively, of the control rates with DMSO alone. Although midcamycin reduced Ca^{2+} uptake in this series of experiments (with DMSO as the vehicle), the reduction in Ca^{2+} uptake was substantially less than that seen with ivermectin.

The Ca^{2+} uptake experiments (Fig. 4) provide no information about the effects of ivermectin on Ca^{2+} release through the RyR because the dominant contribution of reduced Ca^{2+} pump activity masked any reduction in Ca^{2+} uptake due to RyR activation. This was not surprising since the Ca^{2+} - Mg^{2+} -ATPase is the most abundant protein in all fractions of SR (Saito *et al.* 1984). The specific effect of ivermectin on Ca^{2+} release was examined in the following experiments.

Ivermectin increases Ca^{2+} release from TC vesicles in the presence of thapsigargin

Native TC vesicles were partially loaded with Ca^{2+} by adding four aliquots of Ca^{2+} to the cuvette, each of which initially increased the extravesicular $[\text{Ca}^{2+}]$ by $7.5 \mu\text{M}$ (Methods, Fig. 5).

Neither ivermectin ($10 \mu\text{M}$) nor ethanol alone (0.5% v/v) altered the resting extravesicular $[\text{Ca}^{2+}]$ when added to the cuvette (Fig. 5A, $n = 5$). The increase in extravesicular $[\text{Ca}^{2+}]$ when A23187 was added indicated that the vesicles were loaded with Ca^{2+} . Either antipyrylazo III was not

sensitive enough to give a measureable response to the Ca^{2+} released by ivermectin and/or any increase in Ca^{2+} release was compensated for by Ca^{2+} - Mg^{2+} -ATPase activity which, although reduced by ivermectin (Fig. 4 above), may have remained sufficient to prevent an increase in extravesicular Ca^{2+} . Thapsigargin (200 nM) was added in subsequent experiments to block the Ca^{2+} - Mg^{2+} -ATPase (Sagara & Inesi, 1991).

Extravesicular $[\text{Ca}^{2+}]$ increased at a rate of $49.8 \pm 1.4 \text{ nmol (mg SR protein)}^{-1} \text{ min}^{-1}$ ($n = 7$) after thapsigargin was added, and ceased after addition of $10 \mu\text{M}$ Ruthenium Red (Fig. 5B), indicating that Ca^{2+} was released through RyR channels. Adding $10 \mu\text{M}$ ivermectin to thapsigargin-treated vesicles enhanced Ca^{2+} release to $81.4 \pm 3.8 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ($n = 4$), also via RyR channels since all release ceased with Ruthenium Red (Fig. 5C). Ca^{2+} release from thapsigargin-treated vesicles remained at $44.4 \pm 3.8 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ($n = 3$) when ethanol (0.5% v/v) was added alone (Fig. 5B).

Midcamycin ($40 \mu\text{M}$) did not affect extravesicular $[\text{Ca}^{2+}]$ in experiments where total MgCl_2 concentration was 9, 4 or 2 mM. Ca^{2+} release in this series of experiments was $77 \pm 8 \text{ nmol mg}^{-1} \text{ min}^{-1}$ ($n = 7$) after adding thapsigargin and this was unchanged with 0.25% (v/v) ethanol ($78 \pm 10 \text{ nmol mg}^{-1} \text{ min}^{-1}$, $n = 4$) or with $40 \mu\text{M}$ midcamycin ($74 \pm 6 \text{ nmol mg}^{-1} \text{ min}^{-1}$, $n = 6$).

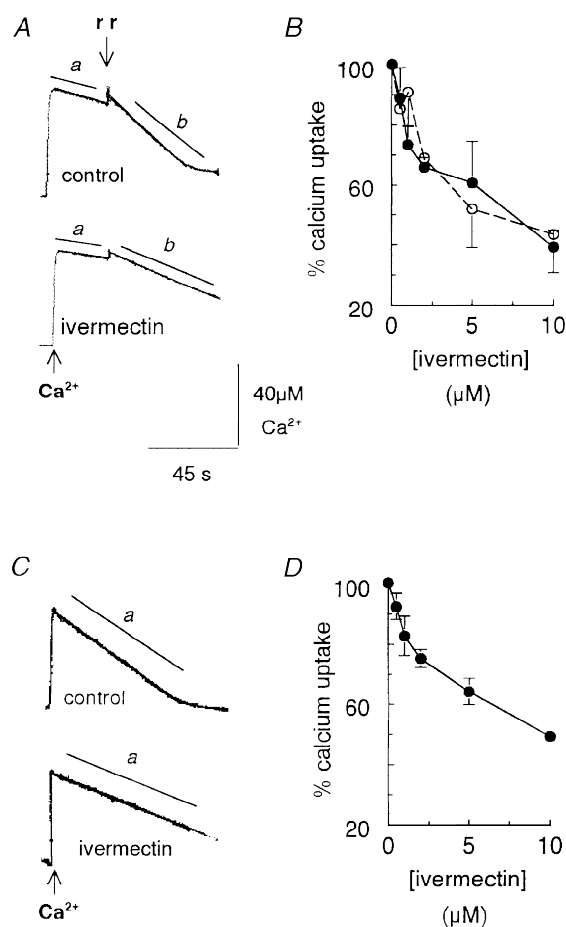


Figure 4. The effect of ivermectin on Ca^{2+} uptake by SR vesicles

A and C, records of changes in OD at 710 nm in response to changes in extravesicular $[\text{Ca}^{2+}]$, using antipyrylazo III ($200 \mu\text{M}$) as a Ca^{2+} indicator. A, Ca^{2+} uptake by TC vesicles after addition of $50 \mu\text{M}$ Ca^{2+} to the cuvette. Slope *a* is the initial rate of Ca^{2+} uptake before adding $5 \mu\text{M}$ Ruthenium Red (rr) (indicated by the discontinuity in the decline in OD). Slope *b* is the initial rate of Ca^{2+} uptake after adding Ruthenium Red. B, the initial rate of Ca^{2+} uptake by TC as a function of ivermectin concentration, expressed as a percentage of the control rate, before (\circ) and after (\bullet) adding Ruthenium Red. Data points are means \pm s.e.m. ($n = 3$); vesicles were from two separate SR preparations. C, Ca^{2+} uptake by LSR vesicles after adding $50 \mu\text{M}$ Ca^{2+} to the cuvette. Slope *a* is the initial rate of Ca^{2+} uptake. D, the initial rate of Ca^{2+} uptake by LSR as a function of ivermectin concentration, expressed as a percentage of the control rate. Data points are means \pm s.e.m. ($n = 4$); vesicles were from three SR preparations. The control records in A and C were obtained after addition of ethanol (0.5% v/v). The ivermectin records were obtained after addition of $10 \mu\text{M}$ ivermectin. The vertical calibration bar shows $[\text{Ca}^{2+}]$, which was routinely determined from the OD response to step increases in $[\text{Ca}^{2+}]$ and was linear over the range of $[\text{Ca}^{2+}]$ values shown in the figure. In this case an increase in $[\text{Ca}^{2+}]$ of $42 \mu\text{M}$ caused an increase of 0.1 OD units.

Ivermectin potentiates caffeine-induced Ca^{2+} release from SR vesicles

Caffeine was used as a means of amplifying Ca^{2+} release in order to examine the effects of ivermectin in the vesicle system. The continued Ca^{2+} release from TC vesicles after addition of thapsigargin and ivermectin meant that insufficient Ca^{2+} remained in the vesicles for examination of caffeine-induced release. Therefore Ca^{2+} release was reduced (Fig. 6A and B) by adding a further 5 mM MgCl_2 to give a total free $[\text{Mg}^{2+}]$ of ~ 0.9 mM, which substantially reduces Ca^{2+} release through the RyR (Meissner, 1994; Laver *et al.* 1997).

The reduced rate of increase in extravesicular $[\text{Ca}^{2+}]$ with Mg^{2+} provides further evidence that Ca^{2+} release from the vesicles was through the RyR. Ivermectin (10 μM), added after 5 mM MgCl_2 , increased Ca^{2+} release (Fig. 6B) thus overcoming the block of RyRs by ~ 0.9 mM free Mg^{2+} . Ethanol alone had no effect on Ca^{2+} release (Fig. 6A). Caffeine-activated Ca^{2+} release was greater with ivermectin (Fig. 6B) than in control (Fig. 6A), and less Ca^{2+} remained in the vesicles to be released by A23187 (final records in Fig. 6A and B).

Caffeine-activated Ca^{2+} release in the presence of 10 μM ivermectin was greater than expected for a simple additive effect of the two drugs. Initial rates of Ca^{2+} release induced by caffeine (0.6–5 mM, measured from records similar to those in Fig. 6A and B) were corrected for 'background' rates in ethanol or ivermectin. Ivermectin potentiated the caffeine-induced release of Ca^{2+} from TC vesicles in which the RyR was inhibited by Mg^{2+} (Fig. 6C), suggesting that ivermectin increased the sensitivity of the RyR to caffeine (see Discussion below).

No enhancement of caffeine- (0.63–6.3 mM) activated Ca^{2+} release from thapsigargin-treated TC vesicles was observed with midcamycin ($n = 7$, Table 4).

On average, caffeine-activated Ca^{2+} release with 0.63 mM caffeine was 109 ± 11 nmol mg^{-1} min^{-1} with ethanol (0.4% v/v) alone and 91 ± 7 nmol mg^{-1} min^{-1} with ethanol plus 40 μM midcamycin. Experiments using FKBP12-stripped vesicles were attempted, but were not successful because the vesicles were 'leaky' and did not accumulate sufficient Ca^{2+} to allow us to examine the ability of the drugs to release Ca^{2+} .

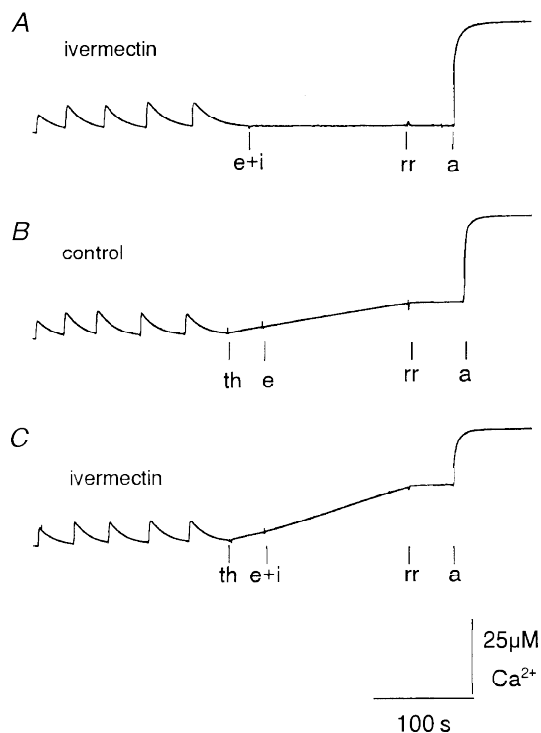
Action of ivermectin on intact and skinned rat EDL fibres

The results described thus far suggest that ivermectin might affect Ca^{2+} stores in intact skeletal muscle. To test this possibility, 40 μM ivermectin was added to the solution bathing small bundles of rat EDL fibres in four experiments. There was no change in twitch or tetanic tension for up to 20 min after adding the drug (preparation run-down prevented an examination of longer exposures). Ivermectin may not have fully partitioned into the cytoplasm and SR within the 20 min of the experiment. Rapamycin and FK506 (at 10 μM) enhance RyR activity (Ahern *et al.* 1994) to a far greater extent than ivermectin (see above) and enhance caffeine-induced contraction in intact rat soleus fibres in Ca^{2+} -free solutions (Brillantes *et al.* 1994). Although the RyR is more sensitive to these agents, they did not increase twitch tension in rat EDL fibres in Ca^{2+} -containing Krebs solutions (J. Mould, unpublished observations).

Ivermectin did alter Ca^{2+} loading and caffeine-activated Ca^{2+} release from many skinned rat EDL muscle fibres. Since ethanol enhanced Ca^{2+} release from skinned fibres (not

Figure 5. Ivermectin releases Ca^{2+} from partially loaded TC vesicles in the absence and presence of thapsigargin

Records of OD changes at 710–790 nm following changes in extravesicular $[\text{Ca}^{2+}]$, using antipyrylazo III (500 μM) as the Ca^{2+} indicator. Extravesicular $[\text{Ca}^{2+}]$ increased by ~ 4 μM at the start of each record when TC vesicles were added to the cuvette and then declined as Ca^{2+} was sequestered by the TC vesicles. The following four positive deflections indicate addition of four aliquots of CaCl_2 (7.5 μM final concentration). A, ivermectin added with ethanol vehicle (e + i, 10 μM). B, thapsigargin (th, 200 nM) added before ethanol control (e, 0.2% v/v). C, thapsigargin (200 nM) added before ivermectin (e + i, 10 μM). In A–C, Ruthenium Red (rr, 10 μM) was added before the ionophore A23187 (a, 3 $\mu\text{g ml}^{-1}$) at the end of each record. The vertical calibration bar shows $[\text{Ca}^{2+}]$ (see legend to Fig. 4). In this experiment an increase in $[\text{Ca}^{2+}]$ of 25 μM caused an increase of 0.3 OD units.



shown), stock ivermectin solutions for these experiments were prepared in DMSO, a solvent vehicle which did not alter load times or tension during release ($n = 8$). Ivermectin ($40 \mu\text{M}$) did not alter the maximum Ca^{2+} -activated force generated by the contractile proteins ($n = 5$; not shown). In Ca^{2+} loading experiments, the amount of Ca^{2+} loaded (i.e. the peak tension in response to the 30 mM caffeine, 0.05 mM Mg^{2+} maximum release solution) increased approximately linearly with time and reached a maximum with a loading time of 2.5 min . The time required for $\sim 50\%$ loading of the SR was $\sim 1.0 \text{ min}$ in the presence of DMSO (0.4% v/v) alone. When $40 \mu\text{M}$ ivermectin was added before, and during, the same 50% loading period as that used in the bracketing DMSO controls (see Methods), peak tension induced by the maximum release solution fell in four of six fibres (see e.g. fibre in Fig. 7A).

On average, peak tension in ivermectin was $92 \pm 3\%$ ($n = 6$) of the DMSO control. Since the size of the tension response

to 30 mM caffeine (0.05 mM Mg^{2+}) reflected the amount of Ca^{2+} loaded (i.e. tension increased with load time until maximum loading was achieved), the fall in tension suggested that ivermectin reduced Ca^{2+} uptake by the SR in the skinned fibres.

Tension during caffeine-activated Ca^{2+} release was enhanced in some fibres that were exposed to $40 \mu\text{M}$ ivermectin before and during release (Methods, Fig. 7B). In this experiment, maximal loading was achieved by 2.5 min exposure to the load solution. After equilibration in low relaxing solution, fibres were exposed to the 15 mM caffeine release solution to produce a contraction with an amplitude of $40\text{--}80\%$ of that recorded with maximum release. This solution was sub-maximal because of the lower caffeine concentration (15 mM) and the higher Mg^{2+} concentration (0.25 mM). Exposure to ivermectin increased tension in five of eight fibres (Fig. 7B), with the average response in ivermectin being $109 \pm 7\%$ ($n = 8$) of the response with DMSO alone.

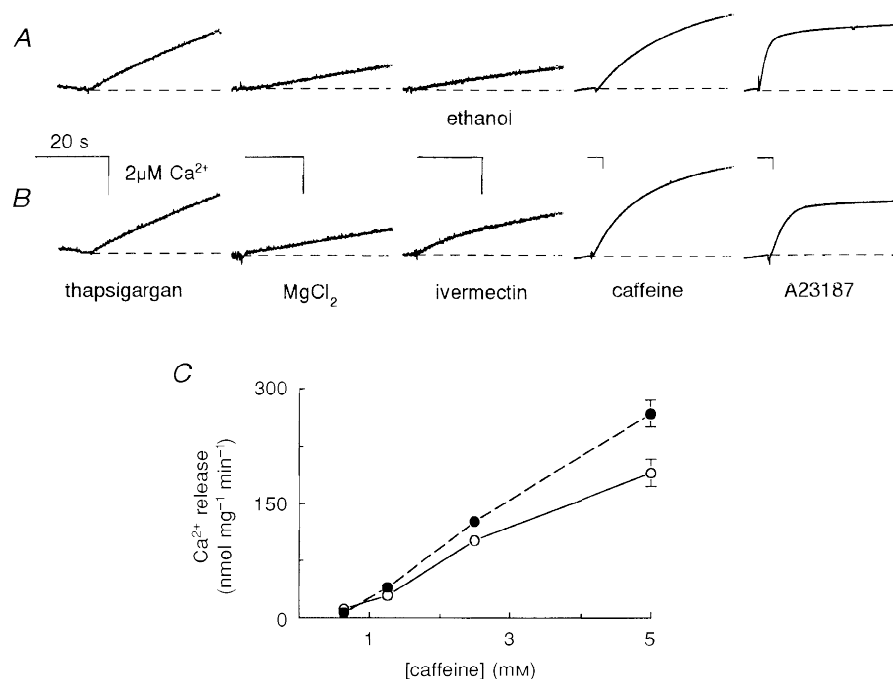


Figure 6. Ivermectin released Ca^{2+} from TC vesicles and enhanced caffeine-induced Ca^{2+} release

A, control records. B, Ca^{2+} release in the presence of ivermectin. A and B show records of OD changes at $710\text{--}790 \text{ nm}$ in response to changes in extravesicular $[\text{Ca}^{2+}]$, using antipyrylazo III ($500 \mu\text{M}$) as a Ca^{2+} indicator. TC vesicles were partially loaded with Ca^{2+} by adding four aliquots of CaCl_2 (each $7.5 \mu\text{M}$ final concentration) as shown in Fig. 5. The scaling of the records has been adjusted (as indicated by the calibration bars for 20 s and $20 \mu\text{M}$ Ca^{2+}) to best display the response to each addition (note the changes in the calibration bars with caffeine and A23187). First record, increase in extravesicular $[\text{Ca}^{2+}]$ with 200 nM thapsigargin. Second record, reduced rate of Ca^{2+} release when MgCl_2 was added to the solution (to give a total Mg^{2+} concentration of 9 mM). Third record, Ca^{2+} release was not altered by addition of 0.25% (v/v) ethanol (A), but was enhanced by $10 \mu\text{M}$ ivermectin (B). Fourth record, enhanced rate of release following 5 mM caffeine addition. Final record, all remaining Ca^{2+} was released from TC vesicles by A23187. C, ivermectin potentiates caffeine-induced Ca^{2+} release from TC vesicles. Initial rates of Ca^{2+} release ($\text{nmol} (\text{mg TC protein})^{-1} \text{ min}^{-1}$) induced by caffeine ($0.6\text{--}5 \text{ mM}$) in control (ethanol, 0.25% v/v, \circ) and with ivermectin ($10 \mu\text{M}$, \bullet). The rate of release shown in C is the initial rate in caffeine minus the preceding rate with ethanol (A) or with ethanol plus ivermectin (B). Data points are means \pm s.e.m. ($n = 7$) using TC vesicles from two SR isolations. The vertical calibration bar shows $[\text{Ca}^{2+}]$ (see legend to Fig. 4). In this experiment an increase in $[\text{Ca}^{2+}]$ of $2 \mu\text{M}$ caused an increase of 0.02 OD units.

Table 4. Individual measurements of initial rate of caffeine-activated Ca^{2+} release with paired experiments using the ethanol vehicle alone (0.4% v/v) and then 40 μM midedcamycin in ethanol

Caffeine concentration (mM)	Ethanol control (nmol mg^{-1} min^{-1})	Midedcamycin (nmol mg^{-1} min^{-1})
0.63	137	92
0.63	98	103
0.63	98	78
1.25	137	180
2.5	650	740
2.5	305*	207*
6.3	560*	431*

All experiments, except those marked with an asterisk, were performed in the presence of 200 nM thapsigargin. The rate given is the initial rate in ethanol or midedcamycin minus the rate of release before addition of ethanol or midedcamycin. The experiments were performed with vesicle preparations giving a stronger response to caffeine than those used for the experiment in Fig. 6.

DISCUSSION

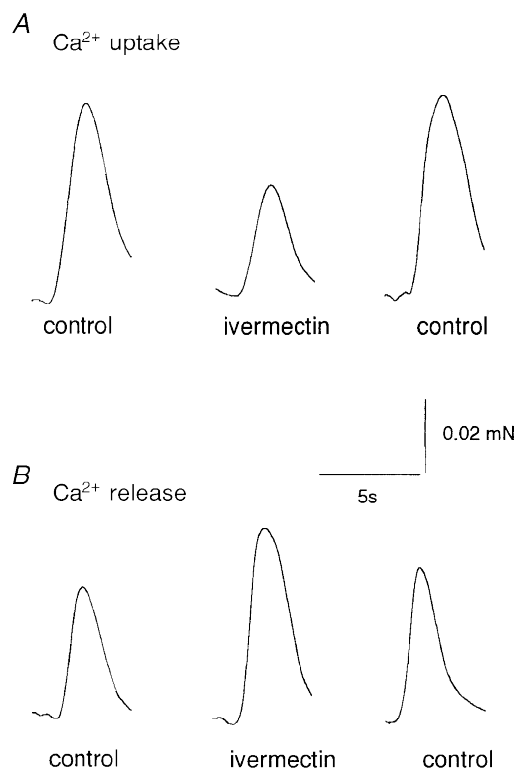
Ivermectin and midedcamycin reversibly increased the single channel activity of native and FKBP12-stripped RyRs in lipid bilayers. Ivermectin and midedcamycin increased the frequency and duration of RyR channel openings and therefore increased the open probability and the mean current. RyR activation was not associated with an increase in the number of channel openings to submaximal conductance levels in native or control-incubated channels.

Indeed an increase in channel openings to the maximum conductance was apparent when ivermectin or midedcamycin activated single FKBP12-stripped channels and when rapamycin activated FKBP12-depleted RyRs (Ahern *et al.* 1997*b*). The increased opening to the maximum conductance was in contrast to the increase in channel openings to submaximal conductance levels seen when FK506 or rapamycin activate native RyR channels (Ahern *et al.* 1994; Brillantes *et al.* 1994). Since neither ivermectin nor midedcamycin removed FKBP12 from TC vesicles, and since both drugs activate FKBP12-stripped RyR channels, the macrocyclic lactones must activate the RyRs through a mechanism that is independent of the FKBP12 molecules tightly bound to the RyR.

It is possible that the macrocyclic lactone quinolidomycin A1, which stimulated $^{45}\text{Ca}^{2+}$ release from loaded SR vesicles via a mechanism that can only partly be blocked by RyR inhibitors (Ohkura *et al.* 1996), acts at the same FKBP12-independent site as rapamycin, ivermectin and midedcamycin. However, there are considerable differences between the structures of these macrocyclic lactones. Rapamycin has a twenty-nine-membered ring structure (Harding *et al.* 1989), ivermectin and midedcamycin both possess sixteen-membered rings, while quinolidomycin A1 has a sixty-membered ring structure. In addition, the number and size of side groups varies widely between the compounds. Although there is no direct evidence that each of the macrocyclic lactones binds to the RyR at the same site, the following observations suggest that they may act at one site: (a) each drug has a lactone group as a part of its macrocyclic structure, (b) each drug produces very similar changes in single channel activity, and (c) the action of the drugs is independent of FKBP12.

Figure 7. Ivermectin (40 μM) reduces Ca^{2+} uptake by SR in skinned EDL muscle fibres and increases caffeine-activated Ca^{2+} release

The records show tension as a function of time. *A*, records obtained from one fibre during maximal Ca^{2+} release (30 mM caffeine, 0.05 mM Mg^{2+}), after submaximal Ca^{2+} loading (see text and Methods). The bracketing control records were obtained with the solvent vehicle DMSO (0.4% v/v) in the low relaxing solution used immediately before loading, and in the load solution. Ivermectin (40 μM : 8 μl of 10 mM ivermectin, i.e. 0.4% v/v) was added to identical solutions in order to obtain the ivermectin record. *B*, records obtained from one fibre during exposure to the submaximal Ca^{2+} release solution (15 mM caffeine, 0.25 mM Mg^{2+}). The bracketing control records were obtained with the solvent vehicle DMSO (0.4% v/v) in the low relaxing solution used immediately before release, and in the release solution. The record labelled ivermectin was obtained with 40 μM ivermectin added to identical solutions.



Other macrocyclic compounds also activate the RyR. Bastadin 5, a heterocyclic compound of similar size to rapamycin but lacking a lactone group, increases single channel open time but does not alter the open probability (Mack *et al.* 1994). Rapamycin and ivermectin, on the other hand, increase P_o by increasing both the frequency and duration of openings. Channels activated by midcamycin also showed increases in open probability, event duration and frequency as well as mean current. It has also been proposed (Mack *et al.* 1994) that bastadin 5 acts synergistically with FK506 to help remove FKBP12 from RyR, a property not shared by ivermectin (P. R. Junankar, unpublished results). Therefore it is likely that bastadin 5 activates RyRs by a mechanism that is different from that of rapamycin, ivermectin and midcamycin.

Ivermectin increases Ca^{2+} release from SR vesicles

The effects on Ca^{2+} handling by SR vesicles and Ca^{2+} release from skinned fibres were consistent with the ability of ivermectin to increase RyR activity. Ivermectin-induced Ca^{2+} release from TC vesicles was seen when the Ca^{2+} - Mg^{2+} -ATPase was blocked by thapsigargin. In addition caffeine-activated Ca^{2+} release from TC vesicles and from skinned fibres was enhanced by this antihelminthic drug. It is possible that the enhanced caffeine-activated Ca^{2+} release from TC vesicles was due to the higher extravesicular Ca^{2+} concentration after addition of ivermectin ($\sim 6 \mu\text{M}$ compared with $\sim 5 \mu\text{M}$, Fig. 6A and B) before caffeine was added. However, the effect of Ca^{2+} on RyR activation by caffeine (in the presence of 1 mM Mg^{2+} and 1 mM AMP-PCP, a non-hydrolysable form of ATP), assessed from [^3H]ryanodine binding, is maximal at less than 1 μM Ca^{2+} and independent of [Ca^{2+}] between 1 and 100 μM Ca^{2+} (Pessah *et al.* 1987).

An unexpected finding was that ivermectin reduced Ca^{2+} uptake into the SR by the Ca^{2+} - Mg^{2+} -ATPase. Bastadin 5 (10 μM) also decreases Ca^{2+} uptake by junctional SR vesicles (Pessah *et al.* 1997) but contrary to our results with ivermectin, the uptake was double the control when the RyR was inhibited by Ruthenium Red or ryanodine. This indicates that bastadin activated the Ca^{2+} pump whereas our results in the presence of Ruthenium Red, and with LSR, show that ivermectin inhibited the Ca^{2+} - Mg^{2+} -ATPase.

That midcamycin was less effective than ivermectin in reducing Ca^{2+} uptake by the SR, and did not release Ca^{2+} from SR vesicles or skinned fibres, was not surprising since the macrocyclic antibiotic was less effective in increasing single RyR channel activity. The minimum concentration of midcamycin required to influence the open times of single channels was 10 μM , in contrast to the minimum concentration of ivermectin which was 1.3 μM . The reason for the less effective action of midcamycin on single channels is not clear. It is possible that the binding of midcamycin is much weaker than ivermectin and that concentrations in excess of 40 μM would be required to observe gross channel activation and activation of Ca^{2+} release in vesicles and skinned fibres.

Effects of ethanol

Ethanol (0.5–2% v/v) enhanced Ca^{2+} release from skinned fibres, slowed Ca^{2+} uptake into SR vesicles, and did not alter Ca^{2+} release from TC vesicles or single RyR channel activity. Oba *et al.* (1997) also found that ethanol (up to 1% v/v) did not alter single frog RyR channel characteristics when added alone, but enhanced the activity of caffeine-activated channels and also enhanced caffeine-activated Ca^{2+} release from SR vesicles. These conflicting results are typical of variations often noted when different experimental preparations are used. For example, low pH enhances Ca^{2+} transients in intact fibres (Westerblad & Allen, 1993), but strongly inhibits RyR channel activity (Ma *et al.* 1988). Another example is dihydropyridine receptor (DHPR) II–III loop peptides which activate [^3H]ryanodine binding to SR, and Ca^{2+} release from SR vesicles at micromolar concentrations, yet activate single RyR channels at nanomolar concentrations (Lu *et al.* 1994; El-Hayek *et al.* 1995). A final example is bastadin, which enhances Ca^{2+} release from the SR, but has no effect on single channel open probability (Mack *et al.* 1994). These results are difficult to reconcile, but suggest, not unexpectedly, that the regulation of RyR channels is very dependent on other factors present in the system. For example, signalling systems which may be disrupted with vesicle isolation, or geometrical features that might be lost with vesicle incorporation into bilayers.

Implications of the action of ivermectin on Ca^{2+} handling by skeletal SR

The biological action of ivermectin on invertebrates has been attributed to its action on the neuromuscular system. Inhibitory glutamate and GABA_A-gated Cl^- channels are activated at picomolar to nanomolar concentrations of ivermectin (Rohrer & Arena, 1995; Duce *et al.* 1995). These concentrations would not be sufficient to deplete Ca^{2+} stores unless the sensitivity of invertebrate RyRs is higher than mammalian RyRs. Toxic effects of ivermectin overdoses have been reported in mice, rats, dogs, cats and sheep, and these effects include mydriasis, muscle tremors, ataxia and weakness (Campbell, 1989; Lovell, 1990). In one case study, hyperthermia was reported in a dog (Hopkins *et al.* 1990). Malignant hyperthermia in pigs and humans is a result of a mutation in the RyR which leads to a higher than normal Ca^{2+} efflux from the SR (Ohta *et al.* 1989). Therefore this and some of the other side effects of ivermectin might result from its action on the skeletal RyR and SR Ca^{2+} pump which would deplete Ca^{2+} stores and increase myoplasmic Ca^{2+} .

Alterations in Ca^{2+} uptake and release may explain both the positive and the negative side effects of other macrolide drugs. The macrolide immunosuppressant FK506 is associated with well-known nephro- and neurotoxicities, some of which do not depend on the ability of the FK506–FKBP12 complex inhibiting calcineurin. More recently, FK506 and non-immunosuppressive macrolides have been shown to promote neurite outgrowth in cell cultures and regeneration in nerve

crush models, by calcineurin-independent mechanisms (Gold *et al.* 1997; Steiner *et al.* 1997*a,b*). In addition, macrolide antibiotics are protective against glutamate excitotoxicity; the protection is structure dependent with larger ringed macrolides being more effective (Manev *et al.* 1993). The molecular basis for these neuronal effects is unknown, but it is possible that they are partly mediated by altered Ca^{2+} transport across the endoplasmic reticulum, similar to those reported here in the SR.

In conclusion, our findings confirm the hypothesis that drugs containing a macrocyclic lactone ring structure can activate the RyR channel by binding to a site on the RyR tetramer that is independent of FKBP12. Ivermectin also potentiated caffeine-induced release of Ca^{2+} from isolated TC vesicles and from SR in skinned muscle fibres, providing further evidence for an activating effect on the RyR Ca^{2+} release channel. An additional action of ivermectin was its inhibition of the SR Ca^{2+} pump in SR vesicles, a result that was consistent with reduced uptake of Ca^{2+} in skinned fibres. These observations may explain some of the side effects of ivermectin poisoning and macrolide drugs in mammals.

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