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Effects of FK506 and rapamycin on excitation–contraction coupling in skeletal muscle fibres of the rat

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1. The effects of the immunosuppressants FK506 and rapamycin were examined in mechanically skinned skeletal muscle fibres of rat in order to determine whether the FK506-binding protein plays a role in the coupling between the voltage sensors and the Ca^{2+} release channels.
2. Both FK506 (1 μM) and rapamycin (1 μM) rapidly and *reversibly* potentiated Ca^{2+} release evoked by either depolarization of the transverse tubular system or caffeine application, suggesting a direct effect of the agents on the Ca^{2+} release channels.
3. In addition, repeated depolarizations in the presence of either FK506 (1 μM) or rapamycin (1 μM) caused irreversible loss of depolarization-induced Ca^{2+} release, without preventing direct activation of the Ca^{2+} release channels by caffeine or low [Mg^{2+}]. If a fibre was exposed to either immunosuppressant for a similar period (10 min) without stimulation, or if the voltage sensors were kept inactivated, there was little if any loss of coupling.
4. The loss of coupling was faster at higher drug concentrations, with 20 μM rapamycin causing 50% inhibition in 7–8 min without stimulation; this was further accelerated by repeated depolarizations in the presence of the drug, but was not noticeably altered by direct activation of the release channels by repeated exposure to caffeine. The irreversible loss of coupling indicates that the FK506-binding protein may play a vital role in enabling the voltage sensors to activate the Ca^{2+} release channels.

It has only recently been recognized that the ryanodine receptor (RyR)–Ca^{2+} release channel complex in skeletal muscle is tightly associated, in a 1:4 ratio, with the 12 kDa FK506-binding protein (FKBP) (Timerman, Ogunbumni, Freund, Wiederrecht, Marks & Fleischer, 1993); presumably, one FKBP is associated with each of the four monomers forming the RyR. It now also appears that the FKBPs in some way co-ordinate the activity of the RyR subunits, eliminating subconductance states and 'stabilizing' the channel complex, so that it shows only fully closed and fully open states (Ahern, Junankar & Dulhunty, 1994; Brillantes *et al.* 1994). FKBP is a proline isomerase, but it has been argued recently that this function is not involved in the ability of FKBP to modulate the Ca^{2+} release channel (Timerman, Wiederrecht, Marcy & Fleischer, 1995). FK506 and another quite different immunosuppressant, rapamycin, bind very tightly to the FKBP, inhibiting its isomerase function and causing its dissociation from the RyR over a time course of minutes to an hour, depending on temperature and drug concentration (Timermann *et al.* 1993; Junankar, Ahern & Dulhunty, 1995). Significantly, when isolated RyRs are no longer associated with FKBP, the Ca^{2+} channel function can still be fully activated with caffeine or a high myoplasmic [Ca^{2+}] (Brillantes *et al.* 1994; Mayrleitner, Timerman, Wiederrecht & Fleischer, 1994). However, it is not known whether the FKBP is necessary for normal excitation–contraction (E–C) coupling, where the voltage sensors in the transverse tubular system (T-system) control the opening of the Ca^{2+} release channels (Melzer, Herrmann-Frank & Lüttgau, 1995). This study examines the effects of FK506 and rapamycin on Ca^{2+} release and contraction in a mechanically skinned muscle fibre preparation that retains voltage sensor control of Ca^{2+} release (Lamb & Stephenson, 1994). Some of these results have been briefly presented previously (Lamb & Stephenson, 1995).

METHODS

Long–Evans hooded rats (*Rattus norvegicus*) were anaesthetized by halothane inhalation (2% v/v) and killed by suffocation, before removal of the extensor digitorum longus (EDL) muscles. As described previously (Lamb & Stephenson, 1994), muscle fibres were mechanically skinned under paraffin oil, mounted on a force transducer (AME875, SensoNor, Horten, Norway), stretched to 120% of rest length and bathed in a high [K^+] solution with 1 mM free Mg^{2+} and 0·1 μM free Ca^{2+} at pH 7·10 (mM: K^+, 126; Na^+, 36; hexamethylene-diamine-tetraacetic acid (HDTA; Fluka, Buchs, Switzerland), 50; total ATP, 8; total magnesium, 8·6; creatine phosphate, 10; NaN_3, 1; EGTA, 0·05; Hepes buffer, 90) at 23 ± 2 °C. All chemicals were obtained from Sigma (USA), unless stated otherwise. The T-system was depolarized by substituting a matched solution in which all K^+ was replaced with Na^+. Only those fibres in which depolarization by Na^+ substitution caused a force response which was at least 50% as large as the maximum Ca^{2+}-

activated force were used for quantitative analysis; this was > 90% of all fibres examined and the remaining fibres showed qualitatively similar effects in all cases. Choline chloride (ChCl) substitution was not used for depolarization (except in the indicated instances after immunosuppressant treatment, when verifying that no depolarizing stimulus could elicit a response), in case the drugs under examination in some way potentiated the ability of ChCl to directly induce Ca^{2+} release, which can occur when the sarcoplasmic reticulum (SR) is heavily loaded, particularly in mammalian fibres (Lamb & Stephenson, 1991 *b*).

Except for the caffeine-induced release experiments (see below), skinned fibres were used with only their initial endogenous level of SR Ca^{2+}, and were exposed to a loading solution (K^+ solution with 2 μM free Ca^{2+} and 50 μM total EGTA) only when it was necessary to show that additional loading did not restore the response to T-system depolarization. In experiments on the effect of the immunosuppressants on caffeine-induced Ca^{2+} release (e.g. Fig. 6*B*), the SR of fibres was initially depleted of Ca^{2+} completely, by exposure for 2 min to the K^+ solution with 30 mM caffeine, 0·05 mM free Mg^{2+} and 0·5 mM EGTA. Then the SR was reloaded to close to the original endogenous level (as judged by the time integral of the caffeine response: Bakker, Lamb & Stephenson, 1996) by exposure for a set period (about 15–30 s) to a K^+ solution with 0·2 μM free Ca^{2+} buffered by 5 mM total EGTA. This load solution was washed out for 1 min and the fibre was equilibrated with or without the immunosuppressant, before being challenged by the standard K^+ solution with 5–7 mM caffeine. The SR was then depleted again and the procedure repeated as required. Maximum Ca^{2+}-activated force was determined using a solution (Max) similar to the K-HDTA solution, but with 50 mM Ca-EGTA (20 μM free Ca^{2+}) replacing all HDTA and 8·12 mM total magnesium giving a free [Mg^{2+}] of 1 mM. The Ca^{2+} dependence of the contractile apparatus was examined with appropriate mixtures of this solution and a corresponding solution with 50 mM free EGTA. FK506 (kindly provided by Fujisawa Pharmaceutical Co., Osaka, Japan) and rapamycin (Calbiochem, La Jolla, CA, USA) were dissolved in ethanol and diluted 1000-fold in the final solution, with an identical amount of ethanol (i.e. 0·1%) added to all corresponding control solutions.

In force traces, unless otherwise indicated, the muscle fibre was bathed in the standard potassium solution. In the text, values given are means ± the standard error of the mean, with *n*, the number of fibres, shown in parentheses. Statistical probability (*P*) was determined with Student's one- or two-tailed paired *t* test, as appropriate.

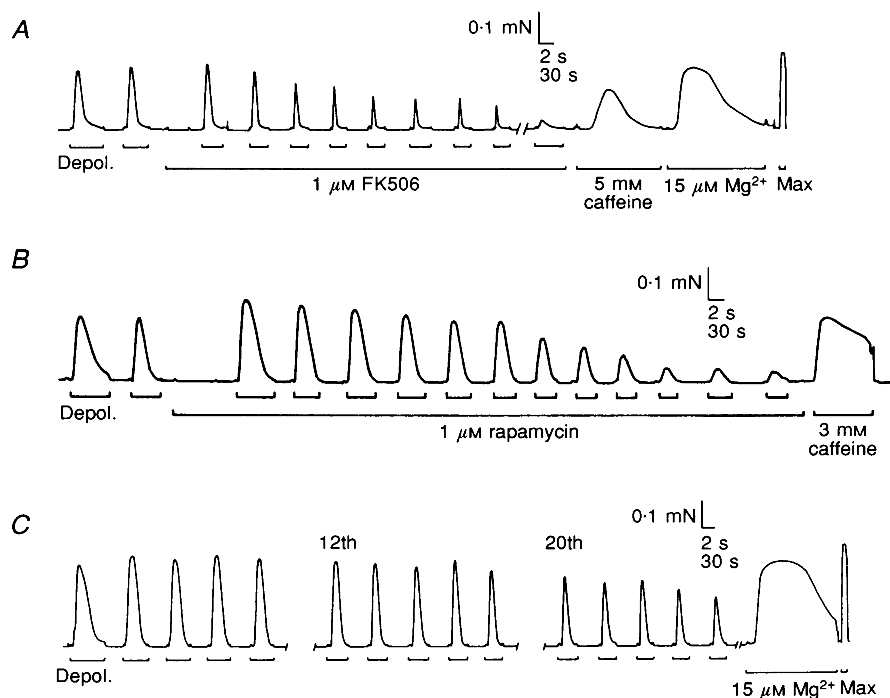

Figure 1. FK506 and rapamycin abolish depolarization-induced force responses in skinned skeletal muscle fibres

A, in the presence of 1 μM FK506, the force response of a rat EDL fibre to successive depolarizations (Depol., upper bars) progressively diminished, even though addition of caffeine (5 mM) or lowering the free [Mg^{2+}] (to 15 μM) could still directly activate the Ca^{2+} release channels. *B*, similar block of depolarization-induced responses in another EDL fibre with 1 μM rapamycin. Note also that the response to depolarization was initially potentiated in rapamycin. *C*, typical depolarization-induced responses in the absence of immunosuppressants in another EDL fibre. Time scale in *A*, *B* and *C*: 2 s during depolarization (by Na^+ substitution) and exposure to caffeine and low [Mg^{2+}], and 30 s elsewhere. Maximum force was determined by direct activation of the contractile apparatus with a solution (Max) with 20 μM free Ca^{2+} (50 mM Ca-EGTA).

RESULTS

Loss of excitation–contraction coupling with FK506 and rapamycin

In the presence of 1 μM FK506, the response to repeated depolarizations progressively diminished until there was little or no force response (e.g. Fig. 1*A*). An almost identical phenomenon was observed with 1 μM rapamycin (Fig. 1*B*). Both immunosuppressant drugs initially caused potentiation of the depolarization-induced response relative to the control response in the same fibre, which had been established by three to five depolarizations before addition of the drug; the mean relative peak force for the first depolarization in 1 μM FK506 was 117 ± 9% ($n = 11$) and in 1 μM rapamycin it was 133 ± 9% ($n = 11$) (both values indicating a significant increase at $P < 0{\cdot}05$). The peak response to further depolarizations then became progressively smaller, dropping to less than 50% of the first response in the presence of the drugs after 9·5 ± 2·5 or 8·3 ± 0·8 depolarizations (45 s apart) in FK506 and rapamycin, respectively (i.e. after a total of 13·8 ± 2·2 or 12·2 ± 0·8 depolarizations, respectively, from the beginning of the experiment). In contrast, in seventeen fibres which were obtained randomly from the same muscles and treated in an identical manner but without exposure to either immunosuppressant, the response did not drop below 50% of the initial level until after 23·5 ± 1·5 depolarizations (e.g. Figure 1*C*). Thus, both drugs caused a significant ($P < 0{\cdot}01$), progressive decrease in the response to depolarization. After treatment with the immunosuppressants, ChCl substitution was no more effective at eliciting depolarization-induced Ca^{2+} release than was Na^+ substitution. Washout of the drugs over many minutes did not restore the response to depolariztion in any fibre examined.

The loss of depolarization-induced responses was not due to depletion of SR Ca^{2+}, nor to complete dysfunction of the Ca^{2+} release channels, because direct stimulation of the channels by application of caffeine (5 mM) or lowering the free [Mg^{2+}] to 15 μM (see Lamb & Stephenson, 1994) induced Ca^{2+} release and a large force response in every fibre (e.g. Figs 1–3). Furthermore, loading fibres with additional Ca^{2+} did not restore the response in any fibre examined (e.g. Fig. 2).

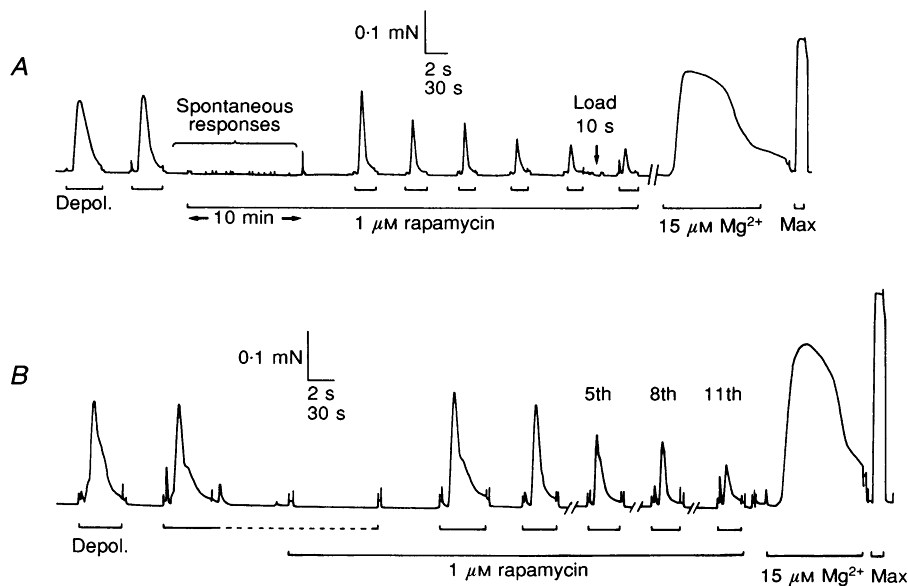

Figure 2. Effects of prolonged exposure to rapamycin and voltage sensor inactivation on depolarization-induced responses

A, after exposure to 1 μM rapamycin for a total of 11 min, the first response to depolarization was slightly larger than the initial control responses. Upon repeated depolarizations in the presence of the drug, the force response became progressively smaller and could not be restored by loading the SR with additional Ca^{2+} (see Methods). Note the small spontaneous force responses (about 5% of maximum force) during the prolonged exposure to rapamycin. *B*, exposure to rapamycin (1 μM) when the voltage sensors were kept inactivated (by continuous exposure to the Na^+ solution; dashed line, slow (30 s) time scale) had no more effect than exposure in the resting state. Time scale in *A* and *B*: 2 s during depolarizations indicated by continuous upper bars and exposure to low [Mg^{2+}], and 30 s between depolarizations and where upper bar is dashed, except for indicated period of 10 min (when the K^+ solution contained an additional 50 μM EGTA to prevent any Ca^{2+} loading).

Effects of exposure without depolarization and voltage sensor inactivation

When a fibre was exposed to the same concentration of immunosuppressant, but not repeatedly depolarized, the loss of E–C coupling was much slower (e.g. Fig. 2*A*). After exposure to 1 μM rapamycin for more than 10 min, the first depolarization-induced response was still potentiated, with the mean response in the four fibres examined being 124 ± 11% of the initial control response; this is similar to the potentiation observed after only 1·5 min in rapamycin (see above), and is strikingly different from the almost complete abolition of responses occurring within 10 min when a fibre was depolarized every 45 s in the drug (e.g. Fig. 1*B*). When the same four fibres were subsequently depolarized at 45 s intervals (e.g. Fig. 2*A*), the response decreased to < 50% after a total of 7·5 ± 2·1 depolarizations in 1 μM rapamycin, which was not significantly different from that found with repeated depolarizations commenced soon after adding the drug.

Interestingly, during the initial 10 min exposure to 1 μM rapamycin, three of the four fibres showed small, brief spontaneous force responses, with peak forces ranging from 4 to 14% of the depolarization-induced response (e.g. Fig. 2*A*). Similar spontaneous responses were observed in other experiments with rapamycin, even when the voltage sensors were inactivated, but they occurred more rarely in fibres treated with FK506, and in fewer than 1% of fibres examined in the absence of any immunosuppressant in this and previous studies. These spontaneous force responses are very similar to those observed in whole muscles after exposure to a high concentration of rapamycin (24 μM) for many minutes (Brillantes *et al.* 1994).

The faster loss of E–C coupling occurring with repeated depolarizations in the presence of the immunosuppressants could be associated with any of a number of processes, such as the *activation* of the voltage sensors and/or the Ca^{2+} release channels or the *inactivation* of the voltage sensors, the latter being the case for the loss of E–C coupling

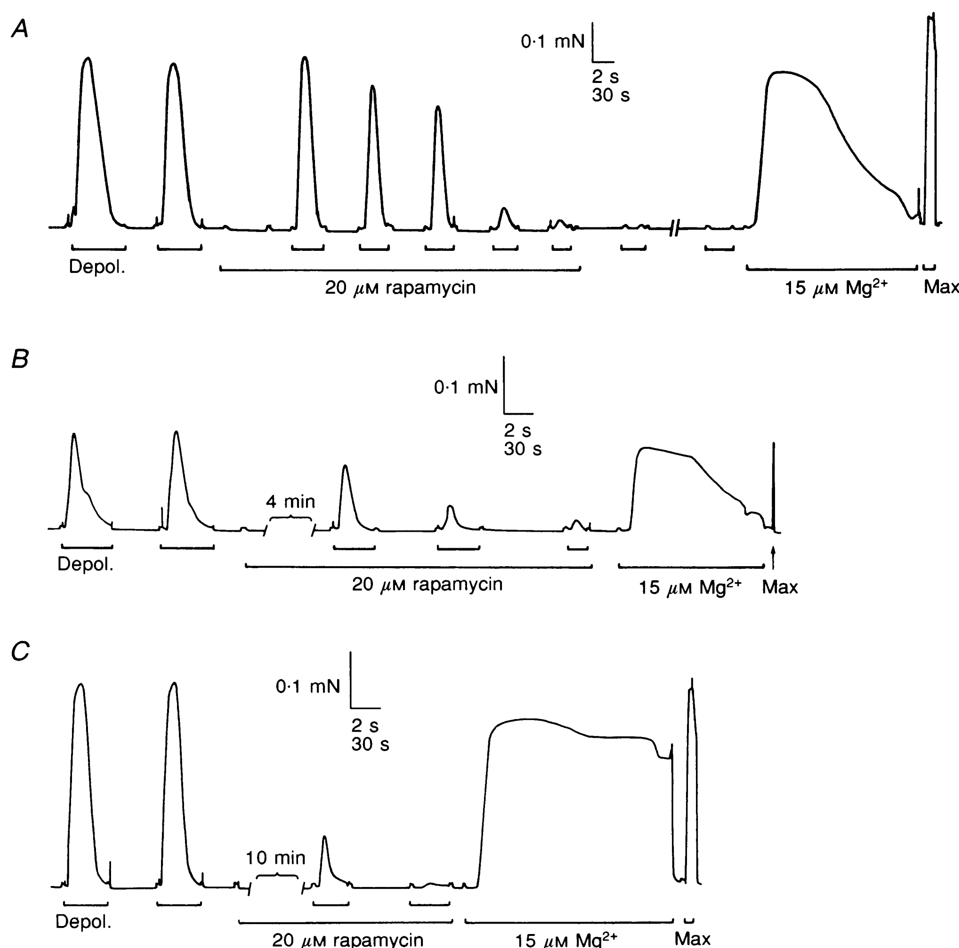

Figure 3. Effect of 20 μM rapamycin on depolarization-induced responses

A, after 1·5 min initial exposure to 20 μM rapamycin, depolarization-induced responses were lost rapidly with repeated depolarizations and could not be restored by washout of the drug for over 3 min. In other fibres, there was a substantial reduction in depolarization-induced responses after 4 min (*B*) or 10 min (*C*) initial exposure to 20 μM rapamycin. Time scale: 2 s during depolarization and exposure to caffeine and low [Mg^{2+}] and 30 s elsewhere.

occurring with D600 (see Melzer *et al.* 1995). As shown in Fig. 2*B*, inactivation of the voltage sensors in the presence of 1 μM rapamycin for 1·5 min, did not cause subsequent loss of E–C coupling, with the response to the first depolarization after repriming being 122 ± 22% of the initial control response ($n = 3$), which was indistinguishable from that for exposure to the drug without inactivation (see above). Depolarization-induced responses then declined to < 50% after a mean of 8·7 ± 1·5 depolarizations in the drug, which was not significantly different from the case for repeated depolarizations without a preceding inactivation period.

Effect of higher concentrations of FK506 and rapamycin

Excitation–contraction coupling was abolished more rapidly at higher concentrations of the drugs. The response to successive depolarizations dropped to less than 50% after only 4·0 ± 0·7 depolarizations (45 s apart) in the presence of 20 μM rapamycin ($n = 8$) (e.g. Fig. 3*A*). The first response to depolarization after 1·5 min in 20 μM rapamycin was not significantly potentiated, the mean being 104 ± 4% of the response in the absence of the drug. This lack of potentiation compared with that with 1 μM rapamycin may have been due to some loss of coupling over the initial 1·5 min exposure at this high concentration of rapamycin, even without depolarizations; such loss was more evident with longer exposures (in other fibres), with responses to a first depolarization in 20 μM rapamycin being 92 ± 13% of the respective control responses after 4 min ($n = 6$; e.g. Fig. 3*B*) and 20 ± 8% after 10 min ($n = 4$; e.g. Fig. 3*C*). Thus, in 20 μM rapamycin, even without repeated depolarizations, there was a progressive loss of E–C coupling over time, with the peak force response to depolarization reduced to only 50% of the initial level after approximately 7–8 min (Fig. 4). If fibres were repeatedly depolarized (e.g. Fig. 3*A*), the loss of coupling with 20 μM rapamycin was even faster (<3·5 min for a 50% decrease; Fig. 4). As was found with 1 μM rapamycin, when the voltage sensors were kept inactivated for the first 1·5 min of the exposure to 20 μM rapamycin, the response to depolarization (after subsequent repriming) was not significantly different from that for a similar exposure when the voltage sensors were in the resting state; the mean response was 112 ± 8% of that before addition of the drug in the same fibres ($n = 5$). With FK506, E–C coupling was also more rapidly lost with repeated depolarizations at 20 μM (response < 50% after 2·4 ± 0·2 depolarizations) than at 1 μM, with the first response after 1·5 min in 20 μM FK506 still being 124 ± 10% of the initial level ($n = 14$); however, the loss of coupling appeared to be accelerated by an additional effect on the inactivated state of the voltage sensor at this high concentration of the drug (not shown).

Ineffectiveness of caffeine activation

In contrast to the effect of activation of the Ca^{2+} release channels by T-system depolarization, direct activation of the channels with caffeine in the presence of the immunosuppressants appeared to have little effect on the time course of the loss of coupling. Over a 4 min period in 20 μM rapamycin, three exposures to 10 mM caffeine, sufficient to induce a 6–10 s force response reaching 25–70% of maximum force, did not cause any significant difference in the size of the first response to depolarization (e.g. Fig. 5) compared with that found after exposure for a similar period without caffeine treatment. The mean data for six fibres treated in this way is shown in Fig. 4, together with the corresponding data for fibres not exposed to caffeine. Similar results were also obtained with and without a single caffeine-induced response during a 1·5 min period in either

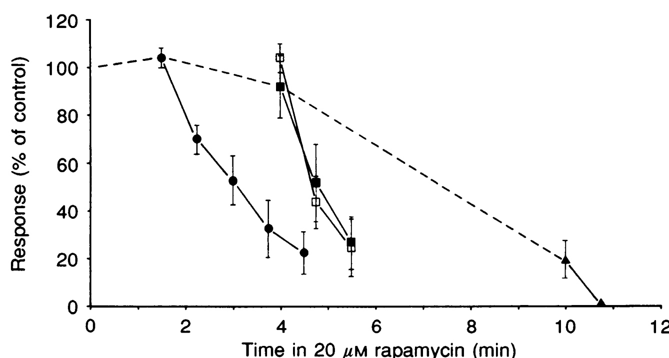

Figure 4. Summarized data of the effect of 20 μM rapamycin on depolarization-induced responses

Mean force responses (± S.E.M.) to first depolarization in the presence of 20 μM rapamycin after an initial exposure for 1·5 min (●, $n = 8$), 4 min (■, $n = 6$) or 10 min (▲, $n = 4$) to the drug; responses expressed as a percentage of the response in the same fibre before addition of rapamycin (e.g. Fig. 3). The dashed line joins the points for the first depolarization-induced response after the indicated time in rapamycin. The mean response to repeated depolarizations following three caffeine-induced responses during an initial 4 min period in 20 μM rapamycin is also shown (□, $n = 6$) (e.g. Fig. 5).

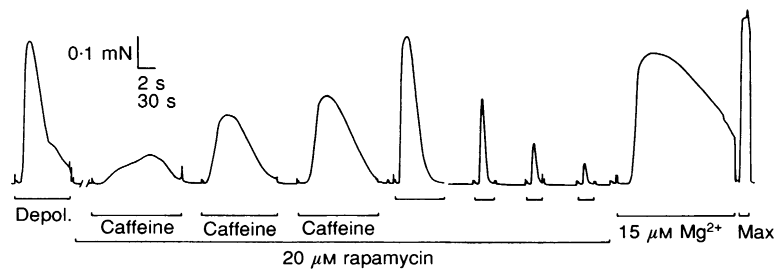

Figure 5. Effect of activating Ca^{2+} release with caffeine in the presence of 20 μM rapamycin on depolarization-induced responses

The first depolarization-induced force response after three caffeine-induced (10 mM) force responses in the presence of 20 μM rapamycin (4 min total exposure time) was very similar to the response before application of rapamycin. Time scale: 2 s during responses to depolarization, caffeine and low [Mg^{2+}] (15 μM) and 30 s between responses.

20 μM FK506 (127 ± 11% of initial response ($n = 4$) *versus* 124 ± 10% ($n = 14$), with and without caffeine) or 20 μM rapamycin (93 ± 4% ($n = 4$) *versus* 104 ± 4% ($n = 8$), with and without caffeine).

Reversibility of potentiation

Unlike the eventual loss of coupling, the prominent potentiation of the depolarization-induced force responses with 1 μM rapamycin could be rapidly reversed by washout of the drug, as shown in Fig. 6*A*. Similar reversibility was found in all four fibres examined in this way. Caffeine-induced force responses were also potentiated in the presence of the immunosuppressants in every fibre examined (e.g. Fig. 6*B*), with the mean response to caffeine (5 or 7 mM) being 8 ± 3% of maximum force before and 26 ± 10% after addition of 1 μM FK506 ($n = 3$), and 2 ± 2% before and 30 ± 9% after addition of 1 μM rapamycin ($n = 4$). In every case, the potentiation of the caffeine-induced response could be reversed by washout of the immunosuppressant during the 4 min period between successive stimulations (e.g. Figure 6*B*). Neither rapamycin nor FK506 (at 2 μM, $n = 3$) had any detectable effect on

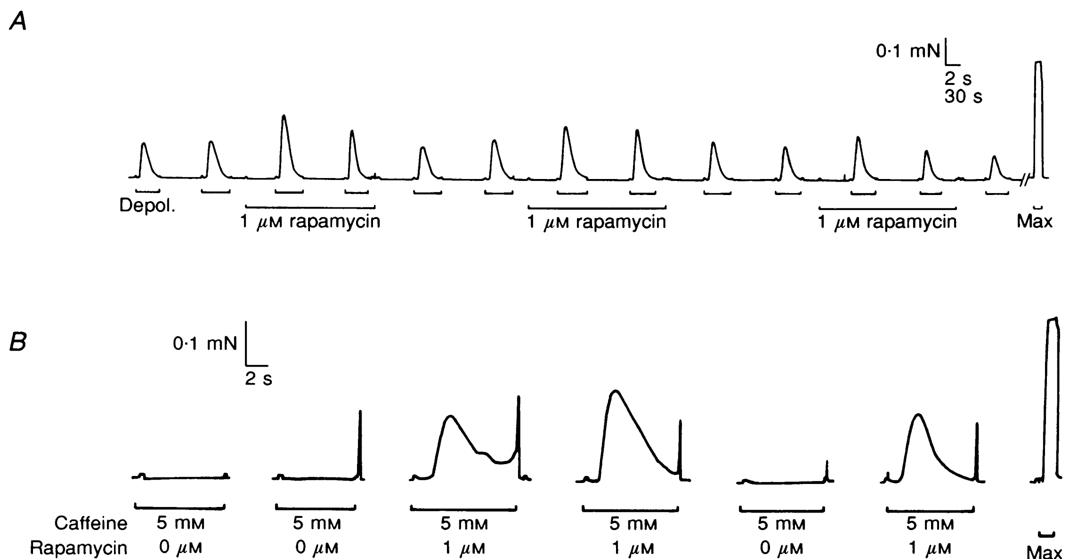

Figure 6. Rapamycin reversibly potentiates responses to both depolarization and caffeine

A, depolarization-induced responses were potentiated after less than 1 min exposure to 1 μM rapamycin, and this effect could be repeatedly reversed and reinstituted, by washout and reapplication of the drug (against a background of progressive decline of the response). Time scale: 2 s during depolarizations and 30 s elsewhere. *B*, the response to 5 mM caffeine was greatly potentiated in the presence of 1 μM rapamycin, and this potentiation was reversed upon washout. The fibre was depleted of Ca^{2+} and reloaded to the same level between successive stimulations (see Methods).

either the maximum Ca^{2+}-activated force or the Ca^{2+} sensitivity of the contractile apparatus (when examined with heavily buffered Ca^{2+} solutions; see Methods), indicating that the observed potentiation of the force responses was indeed attributable to potentiation of Ca^{2+} release.

DISCUSSION

It is shown here that application of 1 μM FK506 or rapamycin causes irreversible loss of normal E–C coupling in skeletal muscle fibres (e.g. Fig. 1). The loss of coupling occurred more rapidly with higher concentrations of the immunosuppressants (e.g. Fig. 3) and with repeated depolarizations in the presence of the drugs (e.g. compare Figs 1 and 2, and see Fig. 4). Importantly, when normal coupling had been lost, the Ca^{2+} release channels could always still be directly activated by caffeine or by lowering myoplasmic [Mg^{2+}] (Figs 1–3). These results suggest that the FK506-binding protein (FKBP) plays an essential role in E–C coupling.

FK506 and rapamycin bind with high affinity ($K_d \approx 5$ nM) to free FKBP, requiring less than 20 min for complete saturation binding with 30 nM at room temperature (Timerman *et al.* 1993). However, when FKBP is associated with the RyR, although the affinity is unchanged, the time course of binding of the immunosuppressants is greatly slowed, requiring more than 1 h to reach saturation with 30 nM FK506. Binding of the immunosuppressants to FKBP results in its dissociation from the RyR, with 0·3 μM FK506 causing approximately 50% dissociation in 1 h at room temperature in SR vesicles (Timerman *et al.* 1993). With 10 μM rapamycin, there is >90% dissociation of FKBP at 37 °C in 15 min, but considerably less dissociation in that time with lower concentrations of rapamycin or at room temperature (Junankar *et al.* 1995). In summary, the binding of the immunosuppressants to FKBP is dependent on both concentration and temperature, and is considerably slower when the FKBP is associated with the RyR. When the immunosuppressants do bind they cause the FKBP to dissociate from the RyR, possibly because they compete with the RyR for the same site on the FKBP.

The results of this study fit very well with the above findings if the binding of the immunosuppressants to FKBP prevents the voltage sensors from activating the Ca^{2+} release channels, either by affecting the function of the FKBP or by inducing the dissociation of FKBP from the RyR. In a resting muscle fibre at room temperature, there was little loss of coupling with 1 μM FK506 or rapamycin over 10 min (e.g. Fig. 2), but approximately 50% loss over 7–8 min with 20 μM rapamycin (see text). Repeated depolarizations in the drugs greatly increased the loss of coupling, possibly implying that the binding site on the FKBP was more easily accessible during the depolarization than in the resting fibre. In fact, the rate of loss of E–C coupling during repeated depolarizations (50% reduction after approximately nine depolarization-induced responses of 1–2 s duration in 1 μM rapamycin or FK506, and after three or four depolarizations in 20 μM) is very similar to the expected binding rate to *free* FKBP for such concentrations of the drugs. Thus, the effect of the immunosuppressants suggests not only that the association of the FKBP and the RyR may be different during depolarization-induced Ca^{2+} release from that during rest, but also that during such activation the drugs have similar access to their binding site to that when the FKBP is free. Interestingly, unlike depolarization, caffeine activation of Ca^{2+} release appeared to be ineffective in causing a loss of coupling in the presence of the immunosuppressants (Figs 4 and 5). This may indicate that the site on the FKBP is preferentially revealed during voltage sensor activation, although this disparity may be simply the result of some difference in the exact opening behaviour (e.g. the average duration of openings) of the Ca^{2+} release channels when activated by the voltage sensor or by caffeine.

It is important to note that after treatment of fibres with the immunosuppressants had caused the complete loss of coupling, the SR still retained most or all of its normal Ca^{2+} load over many minutes (e.g. Figs 1–3). Thus, in the presence of physiological [Mg^{2+}] (1 mM), any leakage of Ca^{2+} out of the SR must have been extremely small and could not have greatly exceeded the very low rate of Ca^{2+} uptake in the solutions used; that is, few if any of the channels could have been open for more than a very small proportion of the time. This is not inconsistent with the treatment having caused dissociation of FKBP from the RyR, because even though the net Ca^{2+} uptake rate of SR vesicles is about 2-fold lower after removal of FKBP (Timerman *et al.* 1993, 1995), this only corresponds to a very small increase in the average open time of the channels, as confirmed directly with isolated channels stripped of FKBP, which remain closed almost all the time in the presence of physiological [Mg^{2+}] and [ATP] (Mayrleitner *et al.* 1994).

Whilst the results of this study would be consistent with the immunosuppressants causing dissociation of the FKBP from the RyR, it is also possible that the interruption to the coupling is caused by the inhibition of the proline isomerase activity of FKBP when it is still attached to the RyR. Such a possibility was recently dismissed by Timerman *et al.* (1995) when they found that FKBP mutants, which lacked isomerase activity, were able to restore the net Ca^{2+} uptake rate in FKBP-stripped SR vesicles (i.e. by 2-fold) in the same way as normal FKBP. However, this small alteration in the opening probability of the Ca^{2+} release channels is approximately 1000-fold less than that occurring during voltage sensor activation of the channels, and clearly does not indicate whether or not the mutant FKBP can play the appropriate role in normal E–C coupling. Consequently, it is quite possible that the voltage sensor activation causes the FKBP to catalyse the isomerization of a proline residue in the RyR, thereby opening the channel.

The apparent role of the FKBP in facilitating voltage sensor activation of the release channels is also suggested by the convergent findings of two other studies. Firstly, it has been proposed, from experiments on the effect of [Mg^{2+}] on E–C coupling, that the voltage sensors activate the Ca^{2+} release channels by lowering their affinity for Mg^{2+}, thereby removing its inhibitory effect (Lamb & Stephenson, 1991*a*). Secondly, it has since been shown that the affinity of the release channels for Mg^{2+} can indeed be lowered by the amount suggested, by the sponge extract, bastadin, which appears to exert its effect via the FKBP (Mack, Molinski, Buck & Pessah, 1994). Thus, together these studies suggest that the voltage sensors may activate the Ca^{2+} release channels by utilizing the ability of the FKBP to lower the affinity of the channel for Mg^{2+}.

Finally, it appears that FK506 and rapamycin also have a second major effect, seemingly unrelated to their effect on the FKBP. Both drugs potentiated the release of Ca^{2+} to stimulation by either depolarization or caffeine (Fig. 6). In contrast to the loss of E–C coupling with the immunosuppressants, this effect was rapid in onset (<30 s at 1 μM rapamycin), did not require previous depolarizations in the presence of the drug (e.g. Fig. 1), and was quickly reversible (Fig. 6). In view of its relatively rapid onset, it appears unlikely that this effect could involve binding to the FKBP when it is associated with the RyR (see above), and considering that both depolarization- and caffeine-induced release were potentiated, it seems more likely that this is a direct effect of the drugs on the RyR. Thus, it may not be correct to ascribe to FKBP dissociation the effects of FK506 and rapamycin in (a) potentiating caffeine-induced channel opening in isolated RyR channels (Mayrleitner *et al.* 1994) and in (b) triggering spontaneous responses and enhancing caffeine-induced responses in intact muscles (Brillantes *et al.* 1994).

In conclusion, irrespective of possible direct effects of the immunosuppressants on the RyR, the results of this study indicate that the FKBP may play a vital role in normal E–C coupling, by enabling the voltage sensors to potently activate the Ca^{2+} release channels.

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