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Single channel activity of the ryanodine receptor calcium release channel is modulated by FK-506

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Abstract The immunosuppressant drug FK-506 (3–20 μM) increased the open probability of ryanodine receptor calcium release channels, formed by incorporation of terminal cisternae vesicles from rabbit skeletal muscle into lipid bilayers, with *cis* (cytoplasmic) calcium concentrations between 10^{-7} M and 10^{-3} M. FK-506 increased mean current and channel open time and induced long sojourns at subconductance levels that were between 28% and 38% of the maximum conductance and were distinct from the ryanodine-induced subconductance level at about 45% of the maximum conductance. FK-506 relieved the Ca^{2+} inactivation of the ryanodine receptor seen at 10^{-3} M Ca^{2+}. The results are consistent with FK-506 removal of FK-506 binding protein from the ryanodine receptor [15].

Key words: FK-506; Skeletal muscle; Sarcoplasmic reticulum; Excitation–contraction coupling; Ryanodine receptor calcium release channel

1. Introduction

The ryanodine receptor has been identified as a major calcium release channel in internal membranes of many cell types including neurons [1], cardiac [2] and skeletal muscle [3–5]. Ryanodine receptors in skeletal muscle are located at the triad junction and extrajunctional TC membranes [6]. The massive cytoplasmic domains of ryanodine receptors form the 'feet' [7] which span the junctional gap between the transverse (T-) tubule and TC [8].

It has recently been shown that FK-BP12, a 12kDa protein which binds the immunosuppressant FK-506, is tightly associated with ryanodine receptors in muscle [9,10]. FK-BPs form a class of *cis-trans* proline isomerases first identified in human T cells and later in other cells [11,12]. The skeletal muscle ryanodine receptor contains proline residues close to the putative transmembrane segments [13] and a *cis-trans* isomerization of these residues may play a role in channel gating. FK-BP12, localized at the triad junction [9], could be involved in ryanodine receptor activation during excitation–contraction coupling.

A regulatory action of FK-BP12 on ryanodine receptors is suggested by observations that calcium uptake by FK-BP12 deficient TC vesicles falls and caffeine-induced calcium release is enhanced [14]. The open probability of ryanodine receptors is greater in FK-BP deficient vesicles than in native vesicles when cytoplasmic $[Ca^{2+}]$ is 10^{-7} and 10^{-6} M, although little effect of FK-BP dissociation is seen at 10^{-5} M $[Ca^{2+}]$ [15]. FK-BP is removed from the ryanodine receptor by FK-506 [10] and addition of FK-506 to native vesicles activates ryanodine receptor channels when cytoplasmic $[Ca^{2+}]$ was 55 nM [15]. We show here that FK-506 activates ryanodine receptors in TC vesicles over the physiological range of *cis* (cytoplasmic) $[Ca^{2+}]$ between 10^{-7} and 10^{-3} M. FK-506 relieved the calcium-inactivation of ryanodine receptor channels at *cis* $[Ca^{2+}]$ above 10^{-4} M.

2. Materials and methods

TC vesicles were prepared from the back and leg muscle of New Zealand rabbits as described by Saito et al. [16]. All buffers contained the protease inhibitors: leupeptin, 1 μM, pepstatin A, 1 μM, benzamidine 1 mM, PMSF 0.7 mM. Protein was assayed [17] using BSA as a standard. Stock solutions of FK-506, kindly supplied by Fujisawa Pharmaceutical Co., were prepared at either 5 mM in ethanol and stored at 4°C, or 60 mM in DMSO at −20°C. Final concentrations of ethanol and DMSO in the bilayer solution were 0.33% and 0.004%, respectively.

Bilayers were formed from a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2, by volume) [18,19], obtained in chloroform from Avanti Polar Lipids (Alabaster, Alabama). The lipid mixture was dried under N_2 and redissolved in *n*-decane at a final concentration of 50 mg/ml. Lipid bilayers were formed over apertures (diameter 150 μm–200 μm) in the wall of 2 ml delrin cups. The *cis* chamber contained 250 mM CsCl, 10 mM TES and 1 mM $CaCl_2$, pH 7.5 and the *trans* chamber contained 50 mM CsCl, 10 mM TES and 0.6 mM $CaCl_2$, pH 7.5. TC vesicles (final concentration = 10 μg/ml) were added to the *cis* chamber. Cs^+ was the major ion carrying current through the calcium channel. After incorporation, free $[Ca^{2+}]$ was varied by perfusing the *cis* side with solutions containing 2 mM BAPTA and titrated to the desired free $[Ca^{2+}]$ with $CaCl_2$, using a Ca^{2+}-selective electrode (Radiometer).

Voltage was controlled and single channel activity recorded via an Axopatch 200 amplifier (Axon Instruments). The *cis* and *trans* chambers were connected to the amplifier head stage by Ag/AgCl electrodes in agar salt-bridges. Current was monitored on an oscilloscope and stored on video tape using pulse code modulation. Data were filtered at 2 kHz (4-pole Bessel, −3 dB) and digitized via a TL-1 DMA interface (Axon Instruments) at 5 kHz. Data were analysed using an in-house program, Channel 2, developed by M. Smith and P.W. Gage. Parameters measured were mean current (I', the integral of the current divided by time), the open probability (P_o, the sum of the open times/total time, measured over a period of at least 30 s) and the mean open time. Channel opening was defined as any event which reached 10–20% (depending on noise) of the maximum open amplitude. Multiple Gaussian functions were fitted to all-point histograms using PeakFit (Jandel).

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Abbreviations: PMSF, phenylmethylsulphonyl fluoride; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; TES, *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulphonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; TC, terminal cisternae; P_o, open probability; I', mean current; S.E.M., standard error of the mean; *r*, coefficient of correlation.

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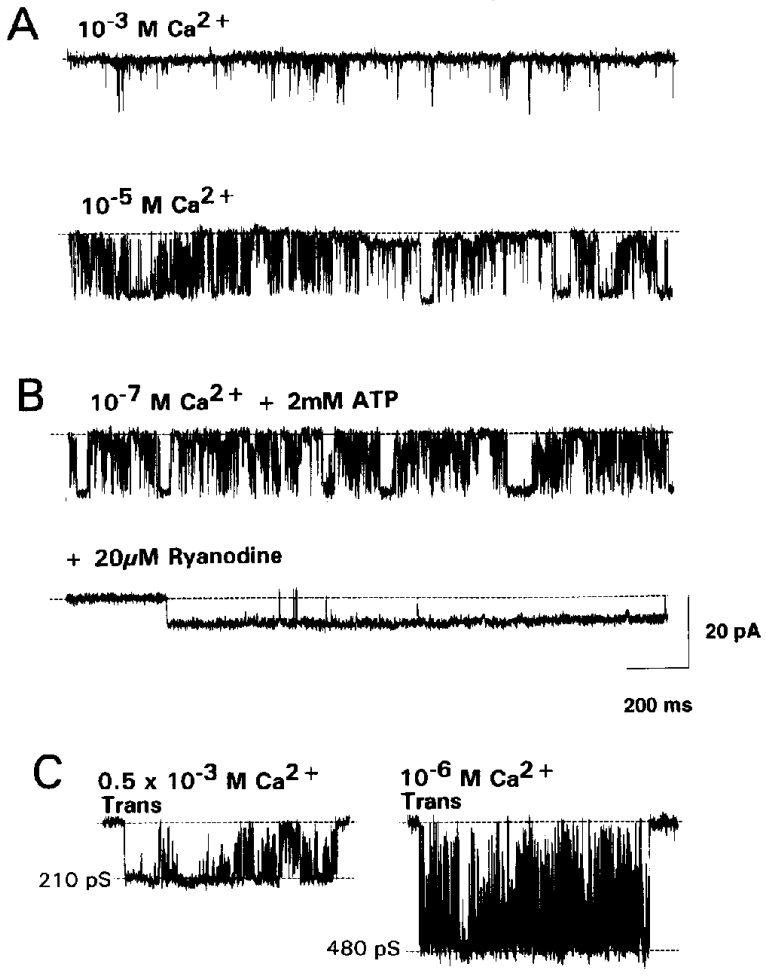

Fig. 1. Characterisation of the ryanodine receptor calcium release channel. (A) Low channel activity with 10^{-3} M *cis* [Ca^{2+}] and activation of the channel by 10^{-5} M *cis* [Ca^{2+}] (with 0.6×10^{-3} M *trans* [Ca^{2+}]). (B) Channel activity with 0.6×10^{-3} M *trans* [Ca^{2+}], 10^{-7} M *cis* Ca^{2+} and 2 mM ATP (top trace) and the effect of 20 μM ryanodine added to the *cis* chamber (lower trace). (C) The effect of reducing *trans* [Ca^{2+}] from 0.5×10^{-3} M to 1×10^{-6} M, with 10^{-5} *cis* [Ca^{2+}].

3. Results

Ryanodine receptor calcium release channel activity was recorded at −40 mV (*trans* potential relative to *cis*), the zero current potential for chloride ions. Under our conditions with 0.6 mM *trans* Ca^{2+} (close to the estimated in vivo luminal [Ca^{2+}]), ryanodine receptor channels showed flickering and bursts of activity with conductances typically between 200 and 250 pS, with classical behaviour [20] in that: (i) the burst activity was more pronounced with micromolar *cis* (cytoplasmic) [Ca^{2+}] than with millimolar *cis* [Ca^{2+}] (Fig. 1A); (ii) the channel was active at 10^{-7}M *cis* [Ca^{2+}] with 2 mM *cis* ATP (Fig. 1B) and (iii) ryanodine (20 μM *cis*) induced a long-lived subconductance state of 80–100 pS. The single channel conductance of 200 to 250 pS was lower than some reported values (see e.g. [21]), but is typical of Cs^+ conductances seen with 250/50 (*cis*/*trans*) Cs^+ and millimolar *trans* [Ca^{2+}], where Ca^{2+} causes a reduction in conductance by competitively displacing Cs^+ from the channel pore [22]. If *trans* [Ca^{2+}] was lowered to 10^{-6} M, single channel conductance increased to values close to 500 pS (Fig. 1C). The more physiological 0.6 mM *trans* [Ca^{2+}] was used in the following experiments.

Addition of 20 μM FK-506 to the *cis* chamber produced a dramatic increase in the activity of channels with *cis* [Ca^{2+}] between 10^{-7} M and 10^{-3} M. The increase in activity was largely due to the appearance of long sojourns at subconductance levels that were around 30% of the maximum single channel conductance, shown as levels F, near −4.7 pA (59 pS) in Fig. 2Ab or −4.5pA (56 pS) in Fig. 2Bb. In addition to the substate activity, openings to the maximum current level were observed with FK-506 but were not significantly increased by exposure to FK-506: the increase in the open probability of the channel (see average results below) being due to the increase in subconductance activity.

The subconductance levels that dominated channel activity in the presence of FK-506 were stabilised, not induced, by the immunosuppressant: flickering activity to levels at about 30% of the maximum current was observed under control conditions (Fig. 2Aa and Ba) and induced asymmetries in the all-points histograms. Gaussian curves fitted to the histograms of control activity in Fig. 2Aa and Ba showed peaks at −4.4 pA and −5.5 pA respectively (see legend to Fig. 2), i.e. at current levels that were close to the dominant subconductance peaks in the histograms for data from the same channels in the presence of

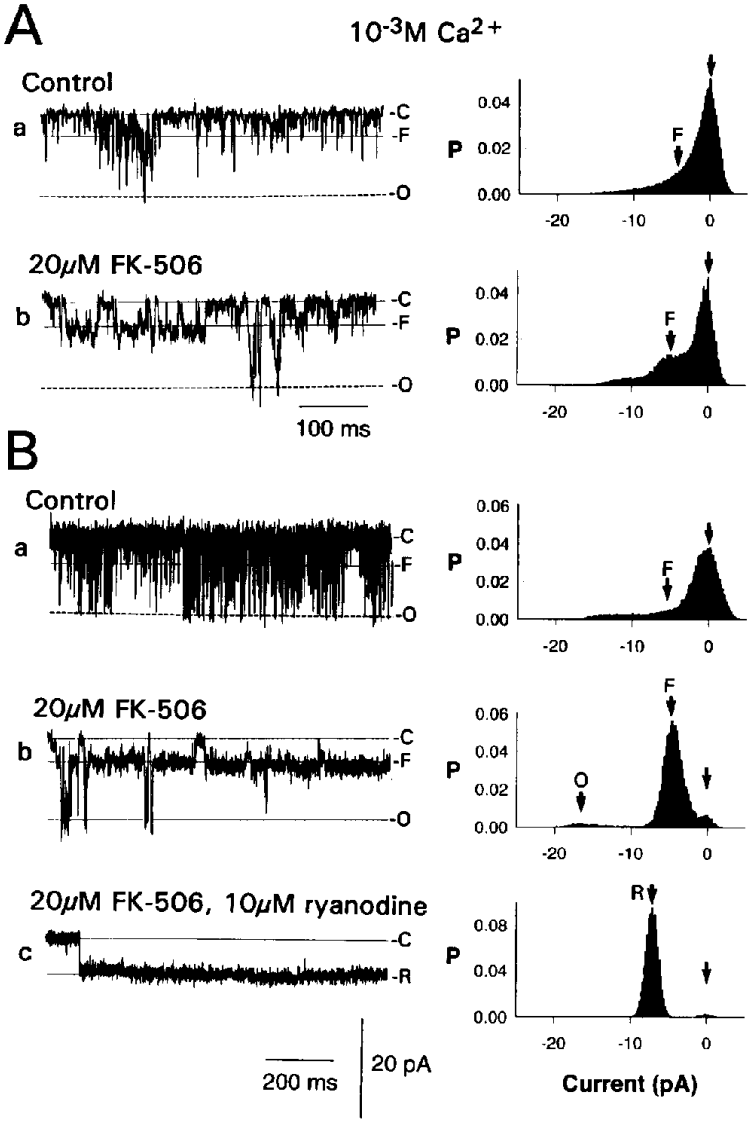

Fig. 2. Examples of the effect of FK-506 on ryanodine receptor calcium release channel activity with 10^{-3} M *cis* [Ca^{2+}] and 0.6×10^{-3} M *trans* [Ca^{2+}]. Two channels are shown: one, (A), showing flickering substate activity and infrequent flickering between the closed (C) and fully open (O) states under control conditions; the second, (B), showing frequent flickering mostly between C and O in the control record. The histograms have been fitted with a multiple Gaussian function: the number of peaks fitted (n), r^2 values for the best fit and current levels (±1S.E.M.) for peaks having the highest probability are given in parentheses for each histogram. Aa: control, (n = 6; r^2 = 0.997; 0 ± 0.01, −2.1 ± 0.1 pA and −4.4 ± 0.1 pA). Ab: enhanced substate activity at F in the same channel several seconds after addition of 20 μM FK-506 (n = 6; r^2 = 0.995; 0 ± 0.01, −2.3 ± 0.1 pA and −4.7 ± 0.1 pA). Ba: control (n = 6; r^2 = 0.985; 0 ± 0.02 pA, −3.1 ± 0.1 pA and −5.5 ± 0.3 pA). Bb: enhanced substate activity at F with 20μM FK-506 (n = 5; r^2 = 0.997; 0 ± 0.07 pA, −3.1 ± 0.1 pA, −4.5 ± 0.02 pA and −16.4 ± 0.1 pA). Bc: 10 μM ryanodine added in the presence of 20 μM FK-506 (n = 2; r^2 = 0.993; −0 ± 0.3 pA and −7.0 ± 0.01 pA). Note that the maximum current at about −16 pA is not resolved in histograms in Aa, Ab or Ba. The all-points histograms include data from a 9 s continuous recording in A, or a 4 s recording in B. Unlabelled arrows in the histograms are at 0 pA, the closed level. Solid lines and arrows labelled C, F and O are placed at levels corresponding to the peaks of Gaussian curves fitted to the histograms. The broken lines Aa, Ab and Ba are drawn by eye through brief transitions to the maximum conductance.

FK-506 (Fig. 2Ab and Bb). Flickering channel openings to the FK-506-stabilised current level (F), recorded under control conditions in the absence of FK-506, were more apparent at lower *cis* [Ca^{2+}] and are particularly well defined in the example of channel activity shown in Fig. 3Ab. In general Gaussian curves fitted to the all-points histograms had very similar peaks under control conditions and after addition of FK-506 (see e.g. Fig. 2 and Fig. 3). The curve fitting procedure detected a second subconductance peak in the histograms, with a probability similar to that of the FK-506-stabilised peak, between −2 pA and −3 pA (see legends to Figs. 2 and 3). This lower current level was seen in control channels and was maintained with FK-506. Marked subconductance activity in the presence of FK-506 has also been reported in FK-BP deficient vesicles [15].

Ryanodine added to the *cis* chamber was able to lock the FK-506-activated channel into a level at about 45% of maximum conductance (a 88pS substate in Fig. 2Bc), as it did in the absence of the immunosuppressant (e.g. Fig. 1B). The ryanodine substate was distinct from the FK-506-stabilised substate at 56 pS: the FK-506-stabilised substate was no longer observed

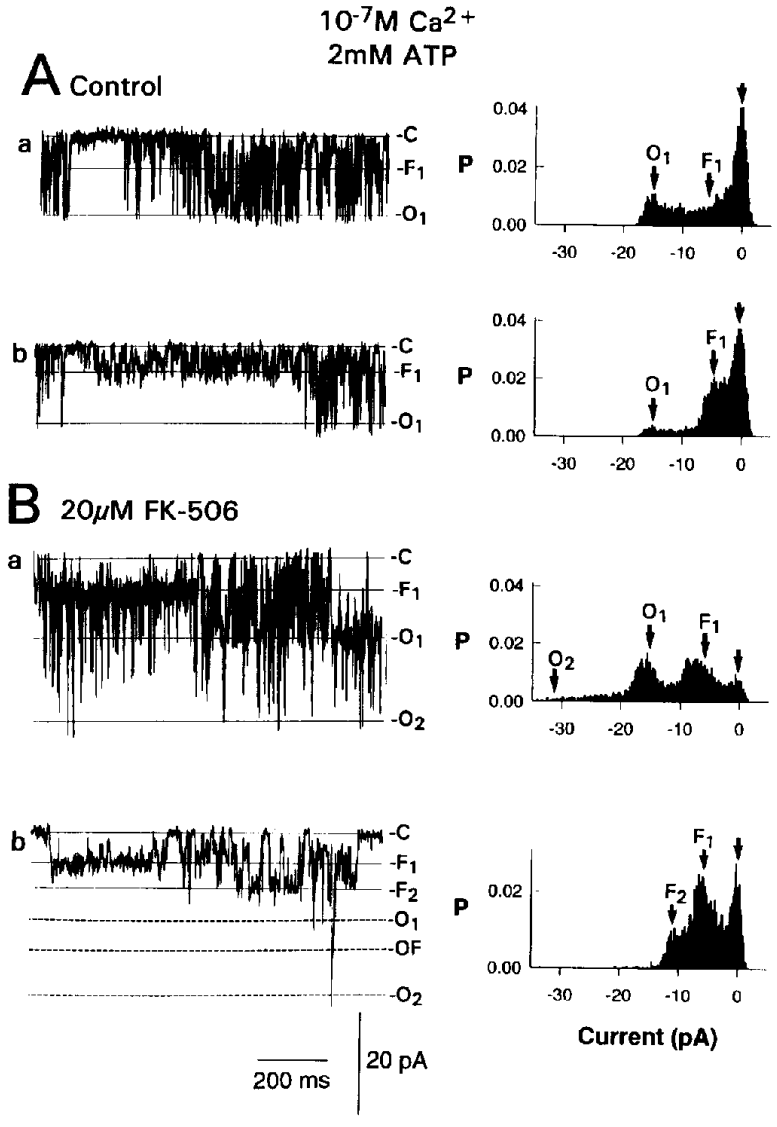

Fig. 3. Activation of multiple ryanodine receptor calcium release channel activity by FK-506 with 2 mM *cis* ATP, 10^{-7} M *cis* [Ca^{2+}] and 0.6×10^{-3} M *trans* [Ca^{2+}]. The segments of recording are from one bilayer and were selected to show clear current levels. The histograms are for the data in each record only and have been fitted with a multiple Gaussian function. The results of the Gaussian fit are given in parentheses, as defined in Fig. 2. A: control activity. Aa: usual channel activity, mostly between closed (C) and fully opening levels (O_1) ($n = 6$; $r^2 = 0.968$; 0 ± 0.02 pA, -3.2 ± 0.1 pA, -6.25 ± 0.2 pA and -15.1 ± 0.1). Ab: an unusual burst of control activity between levels C and F_1 ($n = 6$; $r^2 = 0.986$; 0 ± 0.02 pA, -2.72 ± 0.1 pA, -4.9 ± 0.1 pA and -14.7 ± 0.2 pA). B: channel activity several seconds after addition of 20 μM FK-506 to the *cis* chamber. Ba: full flickering openings in two channels with activity between levels C, O_1 and O_2, and substate activity to level F_1. Due to limitations of our analysis program, the histogram was was split into three segments, 0 to -11 pA ($n = 4$; $r^2 = 0.932$; 0 ± 0.1 pA, -3.3 ± 0.1 pA, -6.1 ± 0.1 and -8.8 ± 0.06 pA), -9 to -22 pA ($n = 5$; $r^2 = 0.906$; -15.3 ± 0.3 and -20.3 ± 0.12 pA) and -18 to -36 pA ($n = 5$; $r^2 = 0.682$; -20.3 ± 0.27 pA and -31.4 ± 0.4 pA). Bb: prolonged openings to levels F_1, F_2 and flickering to O_1 and O_2 ($n = 7$; $r^2 = 0.974$; 0 ± 0.02 pA, -3.3 ± 0.05 pA, -5.9 ± 0.05 pA, and -10.8 ± 0.1 pA). Unlabelled arrows in the histograms are at 0pA, the closed level. Lines and arrows labelled C, F_1, F_2, O_1, O_2 are at levels corresponding to the peaks of the Gaussian curves fitted to the histograms. Broken lines show levels fitted to the data by eye.

in channel activity after addition of ryanodine (Fig. 2Bc). Overall, the conductance of the FK-506-stabilised substate varied between 30% and 40% of the single channel conductance, but was always lower than that of the ryanodine substate when ryanodine was added to an FK-506-activated channel.

In addition to increasing the open probability of individual channels (see average results below), FK-506 induced activity in channels that had previously been silent: openings to two to three times the maximum single channel conductance were often observed, at all *cis* [Ca^{2+}], upon addition of the immunosuppressant. An example of multiple channel activation by FK-506 at 10^{-7} M *cis* [Ca^{2+}] is shown in Fig. 3. Records in this figure were selected to illustrate distinct current levels: it should be noted that the maintained subconductance activity at level F in Ab was not frequently observed under control conditions. One channel was active in the bilayer before addition of FK-506, with 2 mM *cis* ATP (A), and a second channel was activated shortly after addition of FK-506 to the bilayer solution (B). The current levels (see legend to Fig. 3 for details of analysis) suggest that both channels responded in a similar

manner to the immunosuppressant drug. Openings to the maximum single channel conductance (level O_1) and to twice the maximum single channel conductance (level O_2) were observed (Fig. 3Ba), with flickering subconductance activity (e.g. to level F_1) that was similar to the subconductance activity seen in the control records (Fig. 3Aa and Ab). In some segments of the recording (Fig. 3Bb), openings to levels O_1 and O_2 became very brief and longer periods of channel activity at levels F_1 and F_2 were observed. We speculate that F_2 represented a summation of the FK-506-stabilised substates from each of the two channels. Level OF has been drawn at −20 pA, i.e. the sum of $O_1 + F_1$, and some flickering activity at this level was seen.

The data in Fig. 3 also shows that FK-506 induced a similar increase in substate activity with 10^{-7} M *cis* [Ca^{2+}] and with 10^{-3} M *cis* [Ca^{2+}] (Fig. 2). The activation of ryanodine receptor calcium release channels by FK-506 was seen in 20 bilayers with *cis* [Ca^{2+}] between 10^{-7} and 10^{-3} M. The activation resulted in an increase in the mean current (I'), in the open probability (P_o) and open times of single channels (see section 2) where these could be measured. Average data is summarised in the following paragraphs.

The immunosuppressant drug was able to activate channels that had been inactivated by 10^{-3} M *cis* [Ca^{2+}]. This is illustrated in Fig. 2 (above) and was seen in 8 out of 8 bilayers. Four of these bilayers demonstrated only single channel activity before addition of FK-506 and additional channels were activated by the immunosuppressant, with an increase in I' in these four bilayers from an average control value of −0.68 ± 0.35 pA (mean ± S.E.M.) to −3.05 ± 1.23 pA with FK-506. Single channel activity in the remaining four bilayers was maintained after addition of FK-506, and P_o of the single channel increased fivefold from 0.06 ± 0.01 to 0.29 ± 0.12. The average open time increased from 1.56 ± 0.59 ms to 3.6 ± 0.68 ms and I' increased threefold from −1.16 ± 0.32 pA to −3.03 ± 0.25 pA.

Mayrleitner et al. [15] found no difference between P_o in control and FK-BP deficient vesicles at 10^{-5} M *cis* [Ca^{2+}] when 2 mM ATP was present. However, we have found that, in the absence of *cis* ATP, addition of 3–4μ M FK-506 (in DMSO) increased the activity of Ca^{2+} channels in 5 bilayers with 10^{-5} M *cis* [Ca^{2+}]. Multiple channel activity was induced by addition of FK-506 in four of these bilayers. Single channel activity persisted in one of the bilayers after addition of 3 μM FK-506 and P_o in this channel increased from 0.1 to 0.4. The average I' ($n = 5$) increased from −3.6 ± 1.2 to −7.7 ± 2.4 pA. It is probable that, when channels are nearly maximally activated with 2 mM ATP and 10^{-5} M *cis* Ca^{2+}, additional activation by FK-BP removal may not be detected. In our hands, an effect of FK-506 was not obvious in highly active channels. For example in one bilayer containing two active channels, I' was −31 pA before, and −26 pA after addition of 6 μM FK-506 when the [Ca^{2+}] in the *cis* solution was 10^{-4} M Ca^{2+} and the [ATP] = 2 mM.

Several lines of evidence suggested that FK-506 specifically interacted with the ryanodine receptor complex. Addition of ryanodine eliminated the FK-506-stabilised substate and locked the channel into a higher conductance ryanodine substate (Fig. 3Bc above). This observation implied that the submaximal current activated by FK-506 flowed through the ryanodine receptor channel and not a separate channel. In addition, FK-506 (a) did not induce channel activity when added to the bilayer alone, (b) neither altered the activity of chloride channels, nor induced additional channel activity in bilayers demonstrating chloride channels, but not ryanodine receptor calcium channels and (c) addition of 0.33% ethanol alone to the *cis* chamber did not alter ryanodine receptor channel activity ($n = 3$).

The effect of FK-506 could not be reversed by washing the bilayer chambers with drug-free solutions ($n = 2$). In one example with 10^{-4} M *cis* Ca^{2+}, P_o increased from 0.02 to 0.43 upon addition of 6 μM FK-506 and remained high (0.52) following washout of the *cis* chamber. This observation is consistent with the proposition that FK-506 acted by removing FK-BP from the ryanodine receptor, although the FK-506 may have been difficult to remove from the bilayer because of its lipophilic nature.

4. Discussion

The results show clearly that FK-506 alters the gating characteristics of the ryanodine receptor calcium release channel in native TC vesicles. The channel was activated by FK-506 with *cis* [Ca^{2+}] ranging from 10^{-7} to 10^{-3} M. The mean current in bilayers increased because the immunosuppressant activated additional channels and because the single channel occupancy of a subconductance state, at 30–40% of the maximum channel conductance, was increased. Brief transitions to this substate were observed under control conditions, indicating that the subconductance level was stabilised, not induced, by addition of FK-506. Openings to the maximum current level were not eliminated by FK-506. Ryanodine locked the FK-506-treated channel into the usual substate, which had a conductance that was 20–30 pS greater than, and was distinctly different from, the FK-506-stabilised substate. Therefore FK-506 did not modify the ryanodine binding site.

Ryanodine receptor calcium release channels in FK-BP deficient TC display a greater sensitivity to Ca^{2+} for channel activation between 10^{-7} and 10^{-6} M *cis* [Ca^{2+}] and FK-506 has been shown to increase the activity of channels in native vesicles at nanomolar *cis* [Ca^{2+}] [15]. We find that FK-506 increases the P_o of ryanodine receptors in native vesicles over the same range of [Ca^{2+}] and, in addition, alleviates Ca^{2+} inactivation of channel activity at 10^{-3}M *cis* [Ca^{2+}]. Thus FK-BP12 may modify both the Ca^{2+}-activation and Ca^{2+}-inactivation characteristics of the channel.

Calcium-inactivation of calcium release occurs with cytoplasmic calcium concentrations between 10^{-4} and 10^{-3} M [20]. Although bulk myoplasmic [Ca^{2+}] seldom approaches these levels, [Ca^{2+}] near release sites on the TC membrane can rise to levels between 10^{-4} and 10^{-3} M during calcium release [6]. Indeed, calcium-inactivation of calcium release has been observed in cut fibres [23], suggesting that [Ca^{2+}] near the release channel can rise to levels that produce inactivation during normal contraction.

The mechanism by which FK-BP12 modifies channel gating is unknown. The proline isomerase activity suggests a role in protein folding and this is supported by the occurrence of FK-BPs in many cellular compartments [24–26]. Proline residues are found near transmembrane segments of transport proteins [27] and near the M2 segment of the ryanodine receptor [13]. Changing the conformation of peptide chains near the transmembrane region could alter channel gating. Alternatively FK-BP12 binding to the ryanodine receptor per se may modify the conformation of the ion channel and alter its gating.

A remote possibility is that the potentiating action of FK-506 depended on FK-506/FK-BP12 inhibition of endogenous phosphatase activity. The FK-506/FK-BP12 complex binds to and inhibits calcineurin, a Ca^{2+}/calmodulin dependent serine/threonine phosphatase. Phosphorylation of serine-2843 on the skeletal muscle ryanodine receptor activates the calcium channel [28]. Binding of the FK-506/FK-BP12 complex to, and inhibition of, the phosphatase may increase phosphorylation of serine-2843 and thus increase the ryanodine receptor channel activity. However, the recent observation that rapamycin has the same effect as FK-506 ([29], our unpublished results), makes this possibility unlikely, since rapamycin binds to FK-BP12, but does not inhibit phosphatase activity.

In conclusion, FK-506 increases the single channel activity of the ryanodine receptor calcium release channel in native TC vesicles. The activation occurs at 10^{-7} M *cis* (cytoplasmic) [Ca^{2+}] where channel activity is low, as well as at 10^{-5} M *cis* [Ca^{2+}] where channel activity is high and at 10^{-3} M *cis* [Ca^{2+}] where channel activity is inactivated by *cis* Ca^{2+}. It is most likely that the activation produced by FK-506 is due to removal of an inhibitory influence of FK-BP12.

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Note added in proof

A recent publication by Brillantes et al. [29] reports some similar findings.