Modulation of type-1 Ins(1,4,5)P_3 receptor channels by the FK506-binding protein, FKBP12

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FK506-binding protein (FKBP12) is highly expressed in neuronal tissue, where it is proposed to localize calcineurin to intracellular calcium-release channels, ryanodine receptors and Ins(1,4,5)P_3 receptors (InsP_3Rs). The effects of FKBP12 on ryanodine receptors have been well characterized but the nature and function of binding of FKBP12 to InsP_3R is more controversial, with evidence for and against a tight interaction between these two proteins. To investigate this, we incorporated purified type-1 InsP_3R from rat cerebellum into planar lipid bilayers to monitor the effects of exogenous recombinant FKBP12 on single-channel activity, using K^+ as the current carrier. Here we report for the first time that FKBP12 causes a substantial change in single-channel properties of the type-1 InsP_3R, specifically to increase the amount of time the channel spends in a fully open state. In the presence of ATP, FKBP12 can also induce co-ordinated gating with neighbouring receptors. The effects of FKBP12 were reversed by FK506. We also present data showing that rapamycin, at sub-optimal concentrations of Ins(2,4,5)P_3, decreases the rate of calcium release from cerebellar microsomes. These results provide evidence for a direct functional interaction between FKBP12 and the type-1 InsP_3R.

Key words: calcium, immunophilin, InsP_3R, ryanodine receptor.

INTRODUCTION

FK506-binding protein (FKBP12) is an abundant cytosolic protein, first identified as the cellular target for the clinical immunosuppressive agent FK506 [1,2]. Immunosuppressant drugs, such as FK506 and rapamycin, bind to FKBP12, resulting in the formation of immunophilin–drug complexes [3]. These complexes disrupt the cytoplasmic portion of T-cell signalling [4] by inhibiting a protein target. The FKBP12–FK506 complex inhibits the protein phosphatase calcineurin [5], whereas the FKBP12–rapamycin complex interacts with FKBP12–rapamycin-associated-protein (FRAP), blocking cell-cycle progression [6,7]. FKBP12 is also abundant in neuronal tissues, where it binds to both major calcium-release channels of the endoplasmic reticulum, ryanodine receptors (RyRs) [8–10] and Ins(1,4,5)P_3 receptors (InsP_3Rs) [10–12]. InsP_3Rs and RyRs play a crucial role in the generation of calcium spikes and calcium waves [13], which act throughout the life of a typical cell from fertilization and development, through cell proliferation, to cell death [14]. FKBP12 is proposed to mediate the interaction of calcineurin with RyR [15] and InsP_3R [12]. There is very strong evidence that FKBP12 modulates the single-channel properties of RyR1 in skeletal muscle by stabilizing the channel in a full conductance state [16], leading to coupled activation of clusters of RyR1 in the transverse cisternae [17,18]. A homologue of FKBP12, FKBP12.6, interacts with the cardiac RyR2 [9,19] but is dissociated by protein kinase A phosphorylation, explaining defective regulation in failing hearts where RyR2 is protein kinase A-hyperphosphorylated [20].

The situation with respect to InsP_3Rs is less clear. Cameron et al. [11,21] showed that FKBP12 associates with and co-purifies with the type-1 InsP_3R from cerebellum and that FK506 affects calcium fluxes in cerebellar microsomes. However, Bultynck et al. [22] failed to show an effect of FK506 on Ins(1,4,5)P_3 (InsP_3)-stimulated calcium release from permeabilized cells and attributed its effect on Ca^{2+} fluxes to an inhibition of the sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pump. More recently, they found that although the type-1 InsP_3R contains a consensus sequence for FKBP12 binding, it failed to bind to immobilized FKBP12, in contrast to RyR, which did [10]. Meanwhile, we showed, using our purification procedure, that the purified receptor did not contain detectable amounts of FKBP12 [23]. This latter finding, however, provided us with the opportunity to study the effects of adding back FKBP12 on the single-channel properties of purified InsP_3R in bilayers. Given the striking changes in RyR channel behaviour caused by FKBP12, it seemed likely that equivalent measurements on InsP_3R would provide a definitive answer to the question of whether or not there is a functional interaction between the immunophilin and the InsP_3R. As pointed out by Bultynck et al. [10], such interactions might be very dependent on experimental conditions. Here we report that recombinant FKBP12, when added to purified InsP_3R incorporated into patch-clamped giant liposomes, causes a substantial change in single-channel properties. Specifically, it increases the amount of time that the channel spends in a fully open or fully closed state, rather than at sub-conductance levels. We also provide evidence that FKBP12 can induce co-ordinated gating between neighbouring receptors. These effects were reversed by FK506. These observations suggested conditions under which FK506 or rapamycin might inhibit InsP_3-stimulated Ca^{2+} release from cerebellar microsomes, and we go on to show that, at sub-optimal concentrations of the non-metabolizable InsP_3 analogue, Ins(2,4,5)P_3, rapamycin decreases the rate constant for Ca^{2+} release with rather little effect on the overall extent of release.

EXPERIMENTAL

Purification

Type-1 InsP_3R was purified from rat cerebellum by the method described by Supattapone et al. [24] with the following modifica-

Abbreviations used: FKBP12, FK506-binding protein; InsP_3, Ins(1,4,5)P_3; InsP_3R, InsP_3 receptor; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase.

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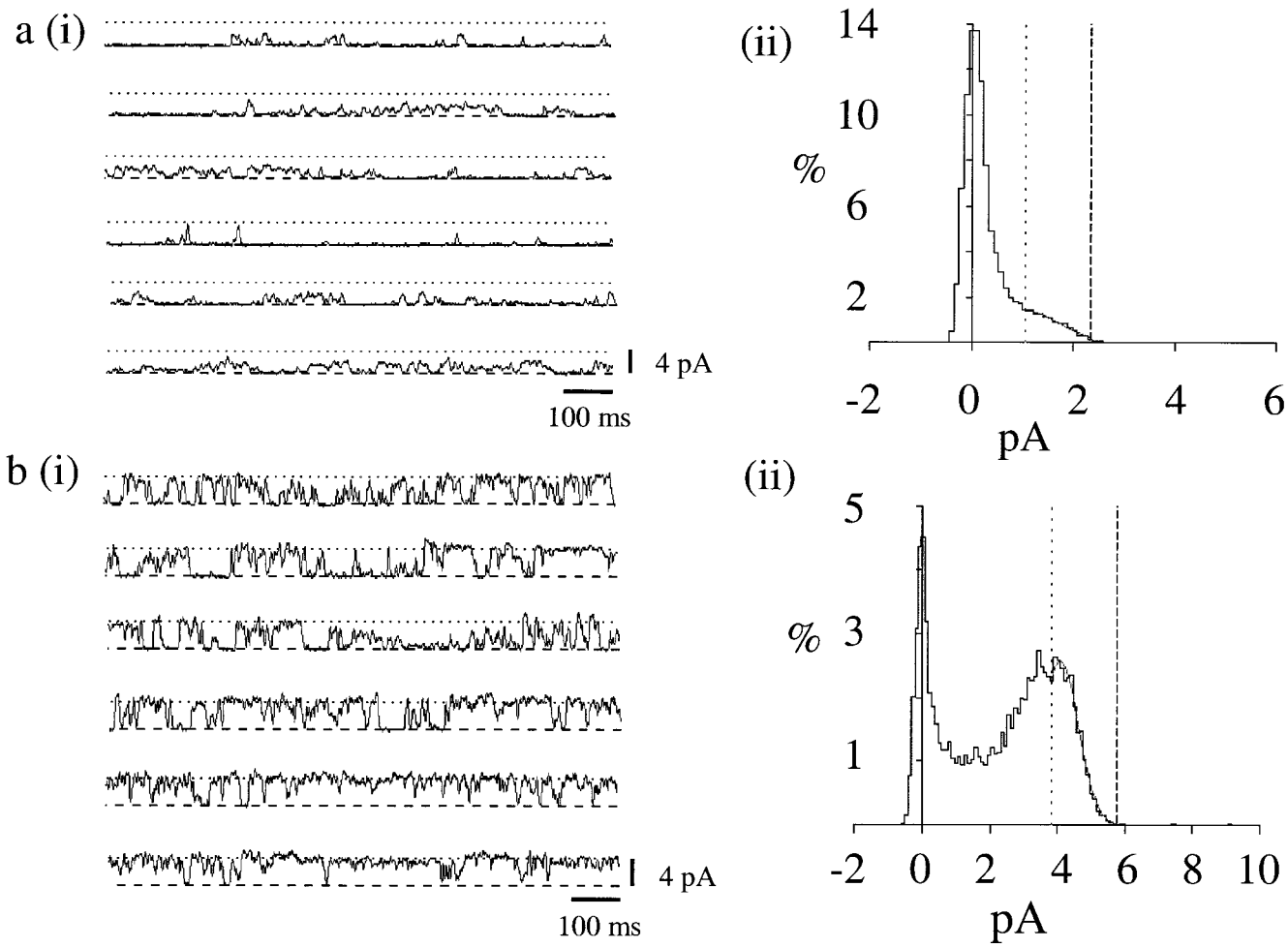

Figure 1 FKBP12 stabilizes single-channel conductance of the type-1 $InsP_3R$ in the presence of high cytosolic free calcium and ATP

A representative experiment ($n = 2$) is shown, performed with a holding potential of +150 mV with 100 μM *cis* and *trans* free calcium, 20 μM $Ins\beta_3$ and 800 μM cytosolic ATP. Single-channel traces are shown in the absence (**a**, panel i) and presence (**b**, panel i) of FKBP12 (183 nM). Upwards deflections from the baseline (long dashes) represent channel openings and the threshold unit current (short dashes) was set at 4.08 pA (27 pS). The mean single-channel current prior to the addition of FKBP12 was 1.08 ± 0.086 pA (7 pS; **a**, panel ii); this increased to 4.08 ± 0.025 pA (27 pS; **b**, panel ii) on addition of FKBP12. The vertical broken and dotted lines in panels ii represent the boundaries between which the Gaussian distributions were fitted.

tions. We used CHAPS instead of Triton X-100 as the detergent for protein solubilization and we excluded the DEAE-cellulose column stage of the procedure. Successful purification was confirmed by SDS/PAGE and competitive [^{3}H]$InsP_3$-binding assays. Protein concentration was determined by a standard bicinchoninic acid assay.

Formation of giant liposomes

Crude liposomes were made by adding lipid (phosphatidylcholine/phosphatidylglycerol, 10:1) to buffer A (100 mM KCl/10 mM Hepes/1 mM $CaCl_2$, pH 7.35) and shaking with glass beads. Crude liposomes were subjected to two freeze–thaw cycles (frozen slowly at −80 °C and thawed at room temperature without agitation). Freeze–thawed liposomes were dehydrated partially in a vacuum dessicator (30 min), and then rehydrated (> 2 h) in patch buffer (250 mM KCl/20 mM Hepes/1 mM *N*′-(2-hydroxyethyl)ethylenediamine-*N*,*N*,*N*′-triacetic acid/1.1 mM $CaCl_2$) to give 100 μM free *cis* calcium (on the bath side of the patch or, functionally, exposed to $InsP_3$, i.e. on the cytosolic face of the receptor). Purified $InsP_3R$ was added during the rehydration procedure to maximize the probability of protein incorporation.

Patch clamping

The patch pipette was filled with patch buffer (see above) to give 100 μM free *trans* calcium (on the pipette side of the patch or, functionally, on the luminal face of the receptor), and 250 mM symmetrical KCl was used as the current carrier. The patch-clamp technique was used to create an inside-out patch and $InsP_3$ was added to the *cis* chamber (bath side) to initiate channel activity. The presence of an $InsP_3R$ in the excised patch was confirmed by observation of channel activity following addition of $InsP_3$. To mimic low cytosolic free calcium, the *cis* calcium was chelated to 300 nM by the addition of EGTA. At the end of experiments, Ruthenium Red (2–50 μM) was added to show that there were no contaminating RyRs, and heparin (100 μg/ml) was added to confirm that the channel activity was due to an $InsP_3R$. Single-channel activity, sampled at 1 ms and filtered at 500 Hz, was recorded directly into Computer Disc Recorder (CDR;

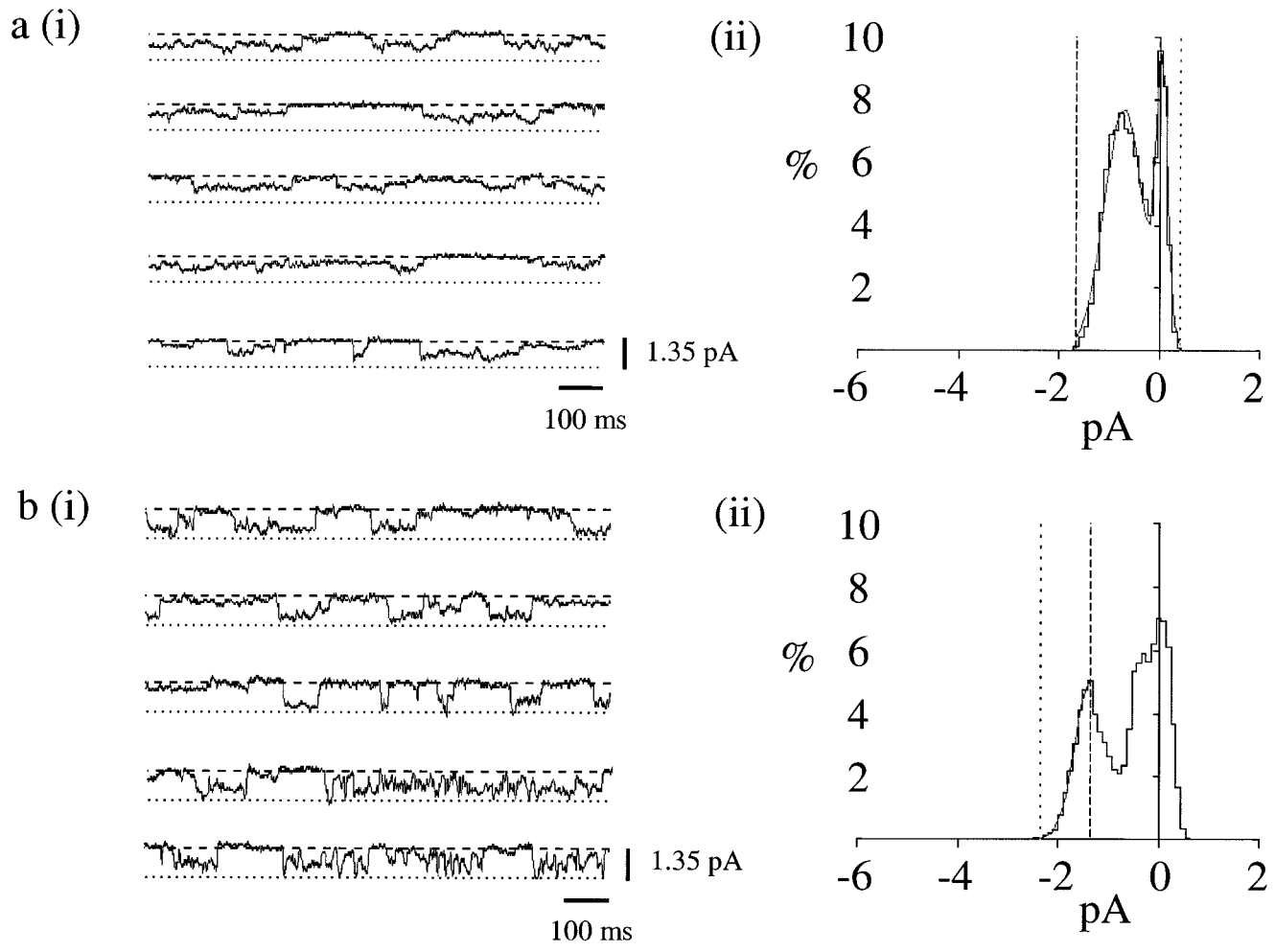

Figure 2 FKBP12 stabilizes single-channel conductance of the type-1 InsP_3R in the presence of low cytosolic free calcium and ATP

A representative experiment ($n = 3$) is shown, performed with a holding potential of -50 mV with 300 nM free *cis* calcium, 100 μM free *trans* calcium, 20 μM InsP_3 and 800 μM cytosolic ATP. Single-channel traces are shown in the absence (**a**, panel i) and presence (**b**, panel i) of FKBP12 (183 nM). Note that in this experiment the channel openings are downwards deflections from the baseline (long dashes) because the applied potential was negative. The threshold current (short dashes) in the channel traces was set to -1.35 pA (27 pS). The mean single-channel current prior to the addition of FKBP12 was -0.71 ± 0.013 pA (14 pS; **a**, panel ii); this increased to -1.35 ± 0.014 pA (27 pS; **b**, panel ii) on addition of FKBP12. Gaussian fits were carried out as defined in the Experimental section.

software from J. Dempster, University of Strathclyde, Glasgow, U.K.).

Analysis of single-channel data

Single-channel data was analysed in Pat v7 (a software package supplied by J. Dempster). All-points amplitude histograms were fitted with Gaussian distribution fits to determine mean single-channel currents. The vertical broken and dotted lines on the histograms in the Figures define the boundaries between which the Gaussian distributions were fitted. Transition detection was carried out with automatic baseline tracking, with an open threshold of 60 % and a closed threshold of 20 %. Mean values for single-channel lifetimes were derived by fitting exponential curves to lifetime histograms (events versus ms). All values are expressed as means $\pm$ S.E.M.

Calcium release from cerebellar microsomes

Rat cerebellar microsomes were prepared as described by Mezna and Michelangeli [25]. Calcium uptake and release were measured in 1 ml of medium containing 100 mM KCl, 20 mM Hepes/KOH, pH 7.0, 100 μg/ml creatine phosphokinase, 10 mM creatine phosphate, 2.5 mM ATP, 2 mM $MgCl_2$, 1 μM Fluo 3 (the impermeant fluorescent Ca^{2+} indicator) and 500 μg of microsomes. The mixture was incubated for 10 min at 30 °C in a rapidly stirred circular cuvette in a PerkinElmer LS50B spectrophotometer (excitation wavelength, 505 nm; emission wavelength, 530 nm) until calcium loading of the microsomes was complete. Calcium release was initiated by addition of Ins(2,4,5)P_3. Again, values are expressed as means $\pm$ S.E.M.

RESULTS

The single-channel properties of the type-1 InsP_3R reconstituted into giant liposomes were characterized using the patch-clamp technique with K^+ as the current carrier. The maximal single-channel conductance was 27 pS and the current–voltage curve was linear (voltage-independent) up to ± 200 mV (results not shown). Channels were activated up to saturating concentrations of InsP_3 (2 μM), but further increases did not alter single-channel

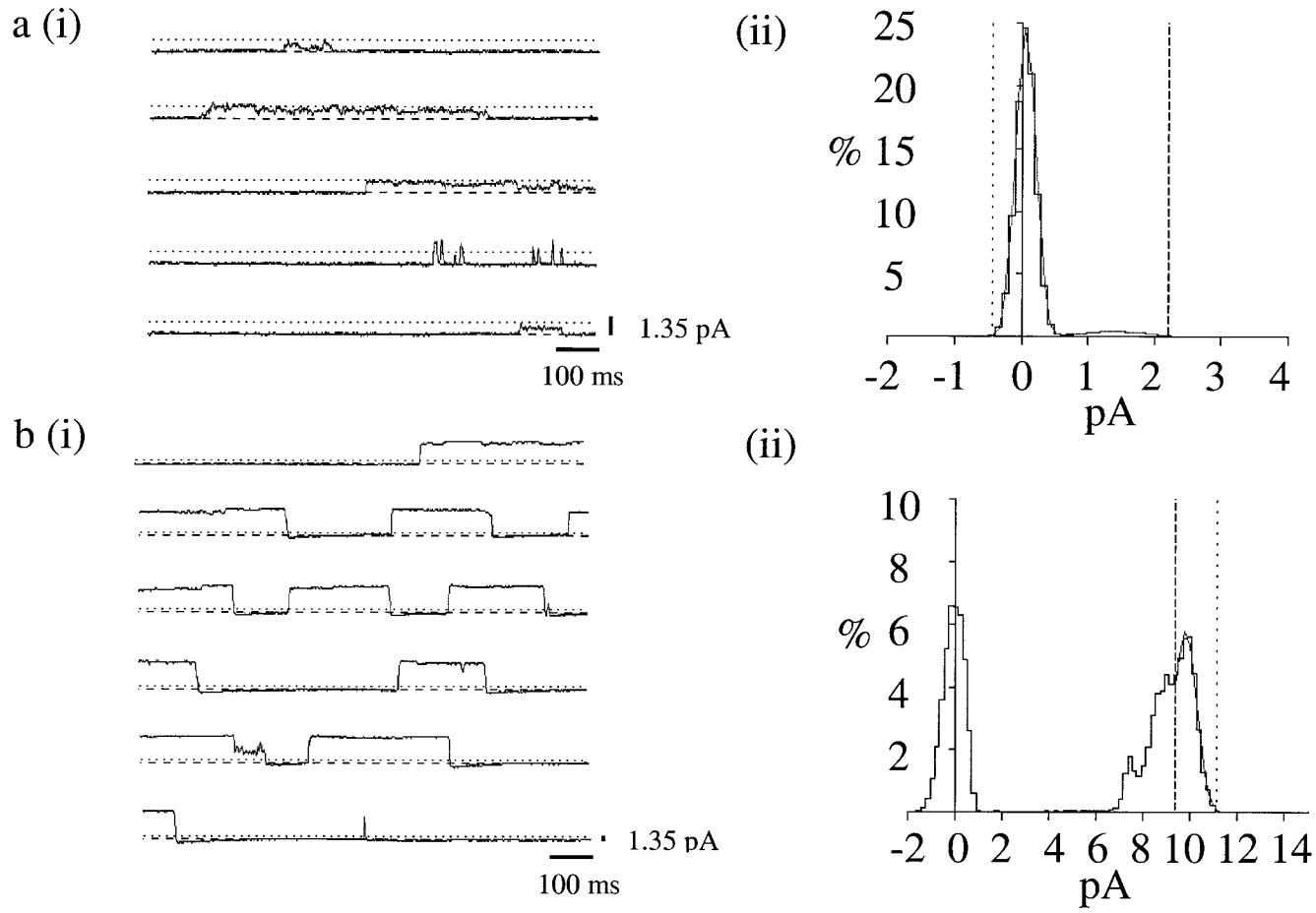

Figure 3 FKBP12 induces co-ordinated gating between neighbouring type-1 $InsP_3Rs$ in the presence of high cytosolic calcium and ATP

A representative experiment ($n = 3$) is shown, performed with 100 μM *cis* and *trans* free calcium with 5 μM $InsP_3$ and 300 μM ATP at a holding potential of +70 mV. Prior to the addition of FKBP12 the maximal single-channel current (determined by fitting a Gaussian curve to the highest peak) was 1.4 ± 0.05 pA (20 pS). Addition of FKBP12 increased the maximal observed current to 9.77 ± 0.021 pA (140 pS), representing the co-ordinated opening of seven 27 pS channels. Channel deflections are observed as upward deflections from the baseline (long dashed line) and the threshold unit current (short dashes) was set at 1.35 pA, which corresponds to the single-channel conductance. Gaussian fits were carried out as defined in the Experimental section.

properties (results not shown). ATP has been shown in our laboratory [23] and by others [26,27] to be a major regulator of single-channel activity of the $InsP_3R$. ATP increases single-channel activity in a dose-dependent manner, activating channel activity in the micromolar range and inhibiting at millimolar concentrations. In contrast to native $InsP_3Rs$, the purified protein was not inactivated by high concentrations of cytosolic free calcium up to 100 μM (consistent with the results of [28]), probably attributable to the lack of calmodulin in our purified preparations [23]. In the experiments described below, we have grouped the results according to whether there appeared to be only one or more than one channel in the patch. In each experiment the data shown are sections from a continuous recording of the same channel(s) before and after the addition of FKBP12.

Initially we studied the effect of FKBP12 at a high cytosolic calcium concentration (100 μM) in the presence of ATP, since this gives maximal stimulation of channel activity. Of five experiments, two appeared to have single channels in the patch, and gave similar results. One of these, at a holding potential of +150 mV with 250 mM KCl as the current carrier, is shown in Figure 1. Typical channel traces and all-points amplitude histograms are shown under the various conditions (Figure 1). Activity was not observed in the absence of $InsP_3$ (results not shown). Addition of 20 μM $InsP_3$ to the *cis* (cytosolic) side then initiated channel activity. Prior to addition of FKBP12, the channel gated continuously, but predominantly to a series of sub-conductance states (Figure 1a, panel i). The mean channel current was low (1.08 ± 0.086 pA, corresponding to a channel conductance of 7 pS; Figure 1a, panel ii). After the addition of 183 nM FKBP12 to the *cis* side, there was a short delay (2 min) followed by the appearance of much more regular channel events. This can be seen in the traces in Figure 1(b) (panel i) and also in the appearance of a more pronounced peak on the all-points amplitude histogram (Figure 1b, panel ii) compared with the situation in the absence of FKBP12 (Figure 1a). The mean channel current increased to 4.08 ± 0.025 pA, which corresponds to a single-channel conductance of 27 pS (Figure 1b, panel ii). The effect of FKBP12 was reversed by addition of FK506, which restored the original appearance of the channels (results not shown). Channel activity was unaffected by the addition of Ruthenium Red, showing that there were no contaminating RyRs, and was completely inhibited by 100 μg/ml heparin, an $InsP_3R$ antagonist (results not shown).

In another series of experiments, the *cis* (cytosolic) free [Ca^{2+}] was decreased to 300 nM. In three out of six experiments there

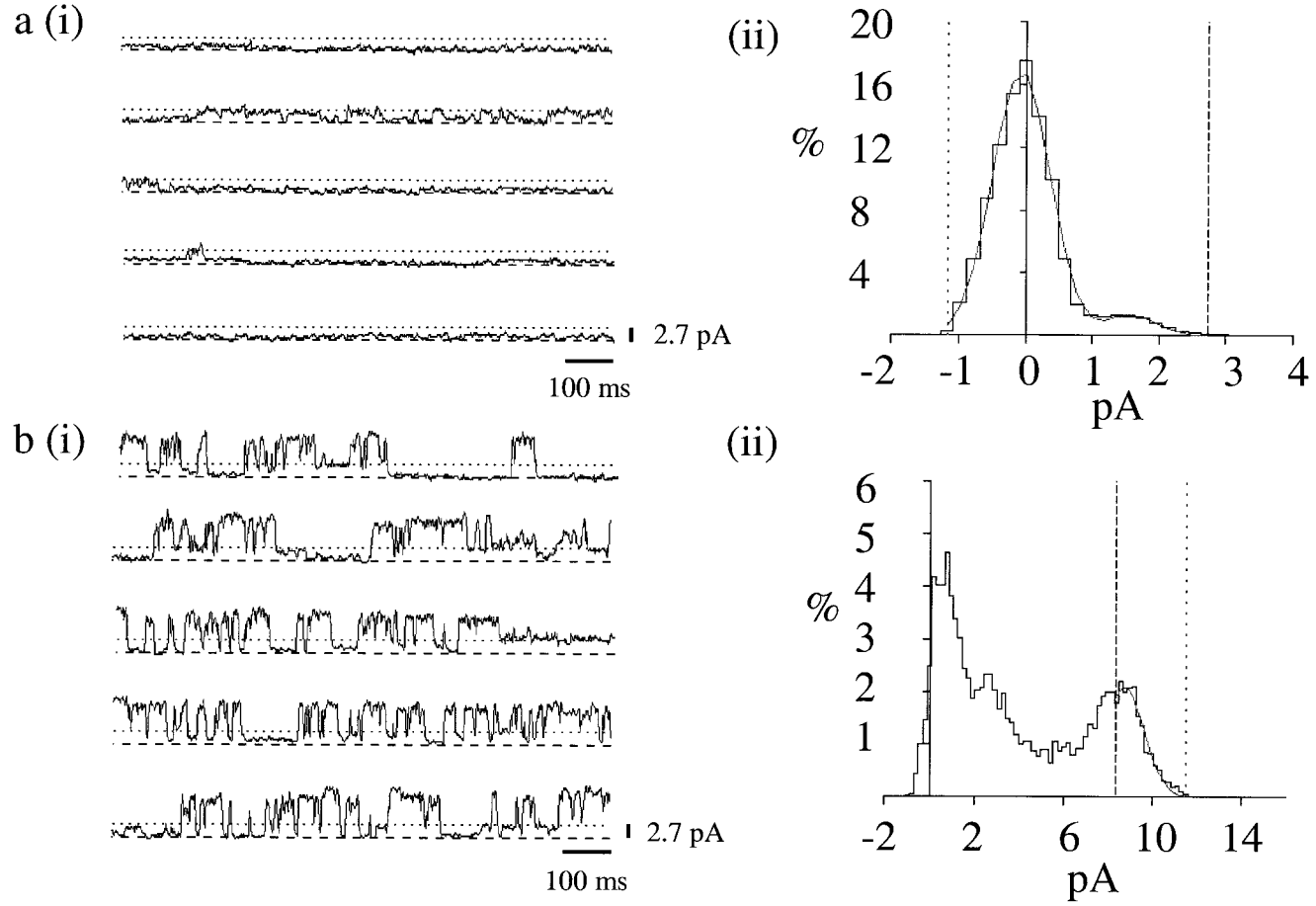

Figure 4 FKBP12 induces co-ordinated gating between neighbouring type-1 InsP_3Rs in the presence of low cytosolic calcium and ATP

A representative experiment ($n = 3$) is shown, performed with 300 nM *cis* free calcium, 100 μM *trans* free calcium, 30 μM InsP_3 and 500 μM ATP at a holding potential of +100 mV. Prior to the addition of FKBP12 the maximal single-channel current (determined by fitting a Gaussian curve to the highest peak) was 1.59 ± 0.107 pA (16 pS). Addition of FKBP12 increased the maximal observed current to 8.75 ± 0.074 pA (87 pS), which corresponds to the co-ordinated opening of three 27 pS channels. Channel deflections are as in Figure 3, and the threshold unit current (short dashes) was set at 2.7 pA (27 pS). Gaussian fits were carried out as defined in the Experimental section.

appeared to be only one channel in the patch. Figure 2 shows data from one of these three experiments (which all showed similar results) at a holding potential of −50 mV with 300 nM free *cis* calcium, 100 μM free *trans* calcium and 800 μM ATP. Again, no activity was observed in the absence of InsP_3 (results not shown) and addition of 20 μM InsP_3 to the *cis* side resulted in the initiation of channel activity. Typical channel traces and all-points amplitude histograms in the absence and presence of FKBP12 are shown in Figure 2. Note that in contrast to the results in Figure 1, channel openings are observed as downward deflections because of the negative applied potential difference. As before, in the absence of FKBP12, the mean channel current was low (0.71 ± 0.013 pA, 14 pS). Addition of FKBP12 caused the appearance of much more regular channel openings and a distinct shift in the 'open' peak on the all-points amplitude histogram (to -1.35 ± 0.014 pA, 27 pS; Figure 2b). Again, Ruthenium Red had no effect on channel activity and addition of heparin (100 μg/ml) completely inhibited channel activity.

In the above experiments at high and low *cis* Ca^{2+} (five out of 11 experiments), there appeared to be only one channel in the patch, since the maximal conductance observed corresponded to the single-channel conductance of 27 pS. However, in other experiments it appeared that there were multiple channels (six out of 11 experiments). In the absence of FKBP12 this was not evident, since no more than one channel was observed to open at any time. Following the addition of FKBP12, openings to conductance states several-fold higher than the single-channel conductance of 27 pS were observed. Since these events went from baseline to maximal conductance in one step we interpret them as the co-ordinated opening of several channels ($n = 6$). This co-ordinated gating was observed at both high and low free calcium (Figures 3 and 4), but the effect was more pronounced at high free calcium. In Figure 4 (b) (panel i), although openings are mostly co-ordinated, many of the closure events appear stepwise. Again, Ruthenium Red was without effect and channel activity was inhibited by heparin.

When the above experiments were repeated in the absence of ATP (results not shown), FKBP12 again stabilized the single-channel conductance (to approx. 27 pS), but co-ordinated gating between channels was not observed ($n = 7$).

To characterize the effects of FKBP12 on single-channel properties further, we carried out a detailed analysis (results not shown) of the channel lifetimes (open, closed and sub-state events). This analysis showed that, under the experimental

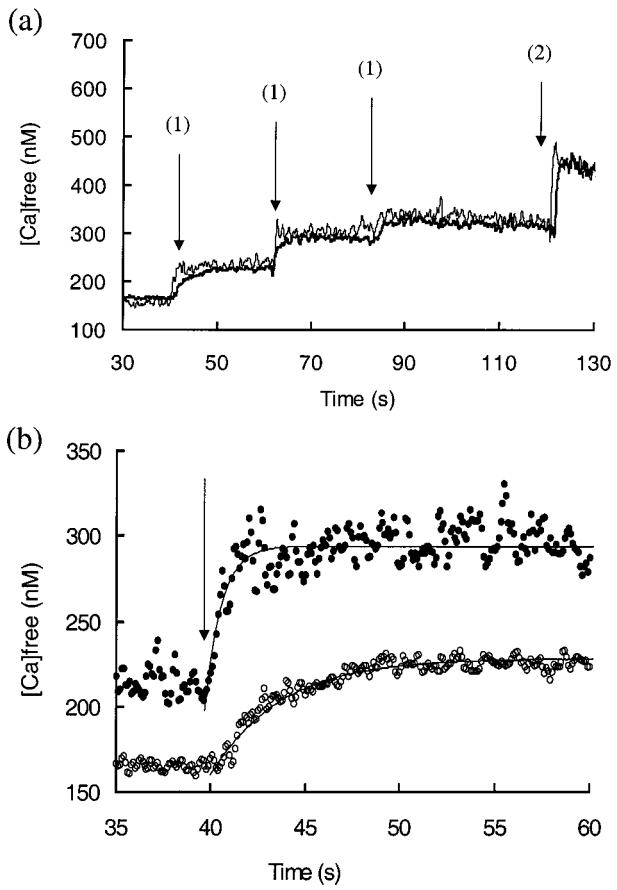

Figure 5 Effect of rapamycin on the kinetics of Ca^{2+} release from cerebellar microsomes

Ca^{2+} uptake and release were measured as described in the Experimental section. (**a**) In the full time course, the arrows marked (1) show the addition of 0.3 μM Ins(2,4,5)P_3, and the arrow marked (2) shows 5 μM InsP_3. The lighter black trace is the control, whereas the heavy black trace indicates an experiment with 10 μM rapamycin, added 5 min before the start of the trace shown. (**b**) An expanded trace showing the first addition of Ins(2,4,5)P_3, with the control trace (●) displaced upwards by 50 nM for clarity. The solid curves are exponentials fitted to the data points. In the control, the rate constant for Ca^{2+} release was 1.2 ± 0.13 s^{-1}, whereas in the presence of rapamycin (○) it decreased to 0.27 ± 0.02 s^{-1}.

conditions described above, there was little systematic change in the $t_{1/2}$ values for these parameters, except that addition of FKBP12 caused the appearance of a new population of open events with a very long $t_{1/2}$ (> 50 ms). Some examples of these events can be seen in the single-channel traces in Figure 1(b) (panel i). Addition of FK506 caused this population to disappear. What is not apparent from the lifetime analysis, but is clear from the all-points histograms, is that at both low (300 nM) and high (100 μM) free *cis* calcium, FKBP12 causes the channel to spend a higher percentage of time in the fully open state. This effect was also observed in both the presence and absence of cytosolic ATP.

The results from the single-channel experiments suggested that FK506, and thus also rapamycin (which also causes dissociation of FKBP12 from its binding sites), might affect the kinetics of calcium release from intracellular stores. Following the observations of Bultynck et al. [22] that FK506 could cause non-specific inhibition of the calcium pump, we decided to look at the effect of rapamycin instead. At the concentrations used we could not see any effects of rapamycin on calcium pumping or calcium leakage from cerebellar microsomes (results not shown). Figure 5 shows the effect of rapamycin on the kinetics of InsP_3-induced calcium release from cerebellar microsomes. After incubation of the microsomes with rapamycin for 5 min, addition of a submaximal dose of the non-metabolizable InsP_3 analogue Ins(2,4,5)P_3 resulted in a much slower release of calcium. However, in agreement with the observations of Bultynck et al. [22], there was very little effect on the overall extent of calcium release.

DISCUSSION

The experiments described above (a total of 18 under various conditions; with or without ATP, in the presence of high or low *cis* calcium) show that the immunophilin protein FKBP12 interacts with purified type-1 $InsP_3R$ to modulate channel activity. The effect of FKBP12 was to cause much more co-ordinated gating of the channel, which is similar to the effect observed for the RyR [16]. FKBP12 specifically increased the percentage of time that the $InsP_3R$ channel spent in a fully open state, an effect that was reversed by FK506. The effect on mean $InsP_3R$ channel lifetimes was less clear. The presence of multiple populations of closed, open and sub-state times made it very difficult to identify significant changes in channel lifetimes. However, detailed lifetime analysis indicated that FKBP12 introduced a new population of long open times with $t_{1/2}$ values of > 50 ms (results not shown), which is consistent with the channel spending a higher percentage of time in the open state.

Does FKBP12 co-purify with $InsP_3Rs$?

Although it is well established that FKBP12 binds tightly to the RyR, and hence routinely co-purifies with it, the situation in the case of the $InsP_3R$ is much more confusing. Cameron et al. [11] have shown that FKBP12 is present in their purified $InsP_3R$ samples, but both we [23] and Bultnyck et al. [10] could not detect FKBP12 in purified $InsP_3R$ preparations. It is possible that our use of CHAPS as the detergent to solubilize the receptor may have disrupted interactions left intact by solubilization with Triton X-100 [24]. The differences in FKBP12 content of purified $InsP_3R$ preparations, and the failure to show direct binding of recombinant $InsP_3Rs$ to immobilized FKBP12 [10], suggests that interaction of FKBP12 with the $InsP_3R$ is either intrinsically much weaker or more dependent on experimental conditions than is the case with the RyR. Although it was not possible to make any estimates of the association constant between the two proteins from our experiments, increasing the FKBP12 concentration from 183 to 366 nM did not increase the effect on channel activity (results not shown). Also, after the addition of FKBP12 to single $InsP_3R$ channels, there was a short lag (1–2 min) followed by a direct transition to the new state with no intermediate steps (results not shown), indicating an all-or-none response.

FK506 effects: a result of calcineurin inhibition or $InsP_3R$–FKBP12 dissociation?

Cameron et al. [12] showed that FKBP12 anchors calcineurin to the $InsP_3R$ and that disruption of this complex by FK506 results in enhanced receptor phosphorylation by protein kinase C *in vitro*, suggesting that modification of $InsP_3R$ calcium-release activity by FK506 is a result of calcineurin inhibition. They proposed a physiological role for FKBP12, in its complex with calcineurin and the $InsP_3R$, as a facilitator of receptor phosphorylation/dephosphorylation, which in turn modulates calcium conduction and participates in the generation of calcium oscillations [12]. Similar findings have been shown recently in adrenal

glomerulosa cells, where both calcium signalling and protein kinase C-mediated phosphorylation of the $InsP_3R$ were modified by FK506 [29]. Kanoh et al. [30] have also studied the effects of FK506 on InsP_3-induced calcium release, and their results suggest that FK506 and rapamycin exert their effects by disrupting the FKBP12–$InsP_3R$ association rather than by interfering with calcineurin-mediated dephosphorylation of $InsP_3Rs$. Our single-channel experiments (Figures 1–4) show for the first time that disruption of the FKBP12–$InsP_3R$ complex by FK506 does modify calcium conduction in the absence of calcineurin, but do not, of course, say anything about the likely additional influences of calcineurin and phosphorylation on channel activity.

Bultynck et al. [22] could not detect any effect of FK506 on InsP_3-induced calcium release, but showed that it inhibited the SERCA Ca^{2+} pump. Our batch of FK506 caused a major addition artifact in calcium-uptake assays, rendering analysis difficult. Instead we used rapamycin, which did not cause an artefact and, at 10 μM, did not affect the calcium pump. However, rapamycin did decrease the rate of calcium release stimulated by sub-maximal concentrations of Ins(2,4,5)P_3, again supporting a functional role of FKBP12 in InsP_3-induced calcium release.

This work was supported by grants from Biotechnology and Biological Sciences Research Council and the Wellcome Trust. We thank Professor Robin Irvine for kindly supplying the inositol phosphates.

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Received 10 September 2001/30 October 2001; accepted 9 November 2001