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Augmentation of SR Ca^{2+} release by rapamycin and FK506 causes K^+-channel activation and membrane hyperpolarization in bladder smooth muscle

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1 The immunosuppressants rapamycin and FK506 are known to relax smooth muscle despite facilitating Ca^{2+} release through ryanodine-receptors of the sarcoplasmic reticulum (SR). The apparent contradiction was studied in isolated guinea-pig urinary bladder myocytes.

2 Modulation of spontaneous SR Ca^{2+} release was monitored by means of spontaneous transient outward currents (or STOCs) in isolated smooth muscle cells voltage-clamped to -20 mV. Rapamycin (10 μM, $n=18$) significantly increased amplitude ($50\pm12\%$, mean $\pm$ s.d.), life time ($77\pm19\%$), and time integral of STOCs ($113\pm22\%$), and it reduced the interval between STOCs ($20\pm7\%$). FK506 (20 μM, $n=24$) increased amplitude ($15\pm7\%$), life time ($50\pm7\%$), time integral ($104\pm26\%$). Cyclosporin A (20 μM, $n=18$) had no significant effects on STOCs.

3 The basal cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) measured by Indo1-fluorescence was insensitive to rapamycin or FK506. Pretreatment with rapamycin (20 μM, 2 min) did not impair the SR Ca^{2+} load as can be concluded from caffeine-induced Ca^{2+}-transients.

4 As it was expected from the enhanced STOC activity, the non-clamped membrane was hyperpolarized by rapamycin (15 ± 2 mV) or by FK506 (15 ± 3 mV).

5 The data are consistent with the idea that rapamycin and FK506 augment spontaneous SR Ca^{2+} release by removal of FK-binding proteins from the RyR-complex. Smooth muscle relaxation is interpreted as negative Ca^{2+} feedback: augmented Ca^{2+} activation of STOCs induces membrane hyperpolarization that reduces Ca^{2+} influx through voltage gated channels.
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Keywords: STOCs; smooth muscle cells; relaxation; Ca^{2+} release; Ca^{2+} feedback; FKBP

Abbreviations: $[Ca^{2+}]_c$, concentration of Ca^{2+} ions in the cytosol; DMSO, dimethyl sulphoxide; FKBP, FK binding protein (target for FK506); FK506, macrocyclic triene antibody from Streptomyces hydroscopicus; HEPES, N-[2-Hydroxyethylpiperazine-N′-[2-ethanesulphonic acid]; $I_{K(Ca)}$, Ca^{2+} activated potassium current; PSS, physiological salt solution; Rapamycin, $C_{44}H_{69}NO_{12}$, r-INN Tacrolimus; RyR, Ryanodin-receptor Ca^{2+}-release channel; SR, sarcoplasmic reticulum; STOC, spontaneous transient outward current; Tween 80, Polyoxyethylenesorbitan monooleate

Introduction

Rapamycin and FK506 are drugs used as immunosuppressants that may cause intestinal and cardiovascular side-effects (e.g. Drake *et al*., 1996; Bielefeldt *et al*., 1997; Lo Rŭsso *et al*., 1997; for structure of FK506 see Figure 1). Some of these side-effects may be independent of the immunosuppression and result from an interaction with the FK binding proteins. FK-binding proteins (FKBPs) are a family of peptidylprolyl *cis-trans*-isomerases that modulate the function of a variety of intracellular targets. In the cytosol, the total concentration of FKBPs may range between 5 and 25 μM (Zarnt *et al*., 1995). Rapamycin or FK506 have been reported to remove FKBP12 from the ryanodine receptor (RyR) Ca^{2+} release channel (Wagenknecht *et al*., 1996). Single channel analysis of RyR1 (skeletal isoform) and RyR2 (cardiac isoform), reconstituted into lipid bilayer membranes, indicated that supramaximal concentration of rapamycin (10 μM) or FK506 (20 μM) prolonged the RyR channel open time and suggested that binding of FKBP12 to RyR1 accelerates RyR channel inactivation (Ahern *et al*., 1997; Chen *et al*., 1994; Mayrleitner *et al*., 1994; Kaftan *et al*., 1996; Xiao *et al*., 1997). The *in-vitro* results are in line with *in-vivo* confocal Ca^{2+} imaging studies of spontaneous SR Ca^{2+} release events (sparks), demonstrating that FK506 prolonged the life time and increased the frequency of sparks as can be expected from the longer RyR2 open state (rat ventricular myocytes: Xiao *et al*., 1997).

For RyR from smooth muscle, the importance of FKBPs for RyR gating is essentially unknown. Although one may extrapolate the results from skeletal and cardiac muscle and assume that also the Ca^{2+} release events of smooth muscle cells may be prolonged, such an extrapolation is questionable because smooth muscle cells express both isoforms RyR2 and RyR3 (for bladder smooth muscle see Wendt-Gallitelli *et al*., 1998). Both *in-vitro* and *in-vivo* studies on the effects of rapamycin and FK506 on individual SR Ca^{2+} release events are missing.

Recently, Bielefeldt *et al*. (1997) reported for smooth muscle strips from rat jejunum that 10 μM FK506 suppressed the spontaneous contractions as well as the tension induced by 100 μM carbachol. In addition, for cultured human jejunal smooth muscle cells FK506 was reported to reduce the amount of caffeine-releasable Ca^{2+}. The authors postulated that FK506 would prolong the RyR open time with the consequences of augmentation of Ca^{2+} release, deprivation of SR Ca^{2+} load and impairment of contractile activation.

In smooth muscle cells, SR Ca^{2+} load and SR Ca^{2+} release are under the control of the membrane potential (e.g.

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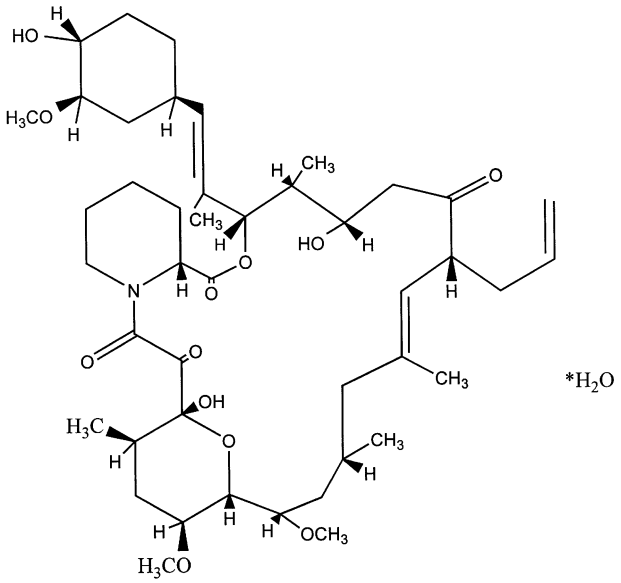

Figure 1 Structure of FK506.

Ganitkevich & Isenberg, 1991; 1993). Hence, we have re-investigated Bielefeldt's hypothesis under the conditions of the voltage-clamp. Individual Ca^{2+} release events and their change by FK506 or rapamycin were monitored by spontaneous transient outward currents (STOCs) that flow through clusters of high conductance Ca^{2+} activated K^+ channels (synonymous BK_{Ca}-channels). STOCs have been described to be a good and convenient indicator of the SR Ca^{2+} release (Benham *et al*., 1985; 1986; Trieschmann & Isenberg 1989; Ganitkevich & Isenberg, 1990) and of the local Ca^{2+} concentration in the subsarcolemmal space (Stehno-Bittel & Sturek, 1992; Ganitkevich & Isenberg, 1996; Bolton & Imaizumi, 1996; Nelson *et al*., 1995; Porter *et al*., 1998). The old speculation that STOCs arise from spontaneous release of Ca^{2+} from SR localized close to the sarcolemma (Benham *et al*., 1985; 1986; Trieschmann & Isenberg, 1989) has been supported by the recent studies on Ca^{2+} sparks of smooth muscle cells that were attributed to brief focal releases of SR Ca^{2+} (Nelson *et al*., 1995; Gordienko *et al*., 1998; Porter *et al*., 1998).

The results of the present study indicate that rapamycin and FK506 (but not cyclosporin A) augment the activity of STOCs by an increase in the STOC amplitude, frequency and duration. They do not confirm that rapamycin and FK506 deprive the SR of releasable Ca^{2+}, i.e. the drugs did not reduce but facilitated the caffeine-induced Ca^{2+} release. Hence, we suggest that relaxation of smooth muscle by rapamycin and FK506 may be caused by a rather indirect mechanism, that is, augmentation of STOCs hyperpolarizes the membrane thereby closing L-type Ca^{2+} channels and reducing the cellular Ca^{2+} load.

Methods

Cell preparation and solutions

Guinea-pigs of ca. 250 g were killed by cervical dislocation, and then the urinary bladder was removed. Single bladder smooth muscle cells were enzymatically isolated according to Klöckner & Isenberg (1985). In brief, chunks of smooth muscular tissue were stirred for 10 min in a Ca^{2+} free medium composed of (in mM): NaCl 140, KCl 5, $MgCl_2$ 1.2, glucose 10, taurine 20, HEPES 5, adjusted with NaOH to pH 7.4. After wash out of Ca^{2+}, the chunks were incubated in the same Ca^{2+} free medium complemented with 5 mg% collagenase (Boehringer, Mannheim), 2 mg% pronase (Serva, Heidelberg) and 100 μM $CaCl_2$. Cells released after 20 min stirring were discarded, those for the experiments were released after an additional 20 min stirring in fresh enzyme medium.

The acutely isolated cells were continuously superfused with a physiological salt solution (PSS) containing (mM) NaCl 150, $CaCl_2$ 3.6, $MgCl_2$ 1.2, KCl 5.4, glucose 20, HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution was composed of (in mM) KCl 140, $MgCl_2$ 0.5, EGTA 0.01, HEPES 5, adjusted with NaOH to pH 7.2. When ratiometric $[Ca^{2+}]_c$ measurements were performed, 0.1 mM Na_5Indo-1 was added to the pipette solution (see Ganitkevich & Isenberg, 1991). The Ca^{2+} signals were measured by a pair of photomultipliers and an analogue divider that delivered the ratio $R = F_{410}/F_{470}$ where at F_{410} and F_{470} are the Ca^{2+} sensitive and the Ca^{2+} insensitive fluorescence intensities of Indo-1 (for details see Ganitkevich & Isenberg, 1991). The Ca^{2+} signals will be presented as normalized signals $\Delta R/R_o$ where R_o is the baseline and $\Delta R = R - R_o$ is the caffeine-induced deviation from R_o. All experiments were performed at room temperature (22°C).

Voltage clamp

Whole-cell membrane currents were recorded with patch pipettes of 2–3 MΩ resistance connected to a RK-400 patch-clamp amplifier (Biologic, Echirolles, France). The currents were filtered at 1 kHz and recorded with a thermo-writer (Gould, TA 550, Cleveland, Ohio, U.S.A.). The currents were not corrected for leakage. A microcomputer in combination with a CED-1401 interface (Science Park, Cambridge, U.K.) was used for generating the voltage-clamp commands and for storing the digitized data (digitizing frequency 2 kHz). For measurements of the membrane potential, the RK-400 amplifier was switched into the current-clamp mode. To make sure that cell dialysis was not a severe problem, measurements were repeated by using nystatin-perforated instead of ruptured patches ($n = 4$).

Drugs

Rapamycin was obtained from Sigma (St. Louis, U.S.A.). It was prepared as a 10 mM stock solution in DMSO and frozen until use. Before the experiment, the stock solution was diluted by adding PSS to give a final concentration of 10 μM. FK506 was a gift from Fujisawa, Japan. A 10 mM stock solution of FK506 was prepared by using a mixture of 82% ethanol plus 18% Tween 80 as solvent, it was diluted with PSS to a final concentration of 20 μM. The 10 mM cyclosporin A stock solution used the same mixture of 82% ethanol plus 18% Tween 80, it was diluted with PSS to a final concentration of 20 μM.

Possible effects of the solvents

The final concentrations of the solvents were 0.2% DMSO, or 0.018% Tween 80 plus 0.08% ethanol. Since the solvents may modulate the membrane currents of interest, in a series of control experiments guinea-pig urinary bladder myocytes were clamped to −20 mV and the STOC activity was measured for 2 min of superfusing control PSS. Thereafter, the superfusate was changed to PSS containing either 0.4% DMSO ($n = 5$). When the STOC activity in absence and presence of the solvents was compared, no significant differences could be

detected (paired t-test, $P<10\%$). That is, the presence of DMSO insignificantly increased the life time of the STOCs by $4.1\pm5.3\%$, the interval between the STOCs by $1.3\pm5.2\%$, the amplitude by $6.4\pm7.3\%$ and the time integral over the STOCs by $3.7\pm4.4\%$. The presence of 0.03% Tween 80 plus 0.2% ethanol ($n=5$) insignificantly increased the life time of the STOCs $5.0\pm1.9\%$, the interval by $1.1\pm2.9\%$, the amplitude by $8.1\pm7.2\%$ and the area by $4.1\pm4.8\%$.

Statistics

The results are presented as means with the standard deviation (s.d.). For statistical evaluation of significance, we compared data that were recorded from the same cell before and after addition of the drug with a paired t-test. Significance of the drug effects was tested at the 5% level, when other significance levels were tested this will be indicated in the text.

Results

Spontaneous transient outward currents (STOCs) at −20 mV

STOCs that flow through clusters of Ca^{2+} activated 200 pS K^+ channels (BK_{Ca}-channels) have been described for a variety of smooth muscle cells, including those from the urinary bladder of the guinea-pig (for references see Bolton & Imaizumi, 1996). The STOC activity is significantly enhanced after depolarization of the membrane from the resting potential (ca. −40 mV, Klöckner & Isenberg, 1985) to −20 mV, hence the present study analysed the STOC activity at −20 mV after a 2 min period of stabilization. Examples are illustrated in Figure 2.

The amplitudes of STOCs broadly distributed around an average of 65 pA. The fit with a Gaussian distribution yielded a standard deviation (s.d.) of 27 pA (59 cells). The 'STOC life time' was defined as the period during which the STOC amplitude exceeded the 10 pA threshold value. The individual life times were grouped in bins of 8 ms and plotted in a life time histogram (e.g. Figure 3b). The fit with a Gaussian distribution provided a life time of 32 ms (36 ± 3 ms, $n=59$). Similarly, the interval between the STOCs was evaluated as the period during which the current did not exceed the 10 pA threshold, fits with the Gaussian distribution yielded for the interval between the STOCs a value of 920 ± 60 ms. The averaged STOC activity was calculated by integrating the STOCs over a 100 s period of time and dividing the result by 100 s. The value of averaged STOC activity was 2.5 ± 0.2 pA ($n=59$). Superfusion of a solution that contained the solvents for rapamycin (DMSO) or for FK506 (Tween 80 plus ethanol)

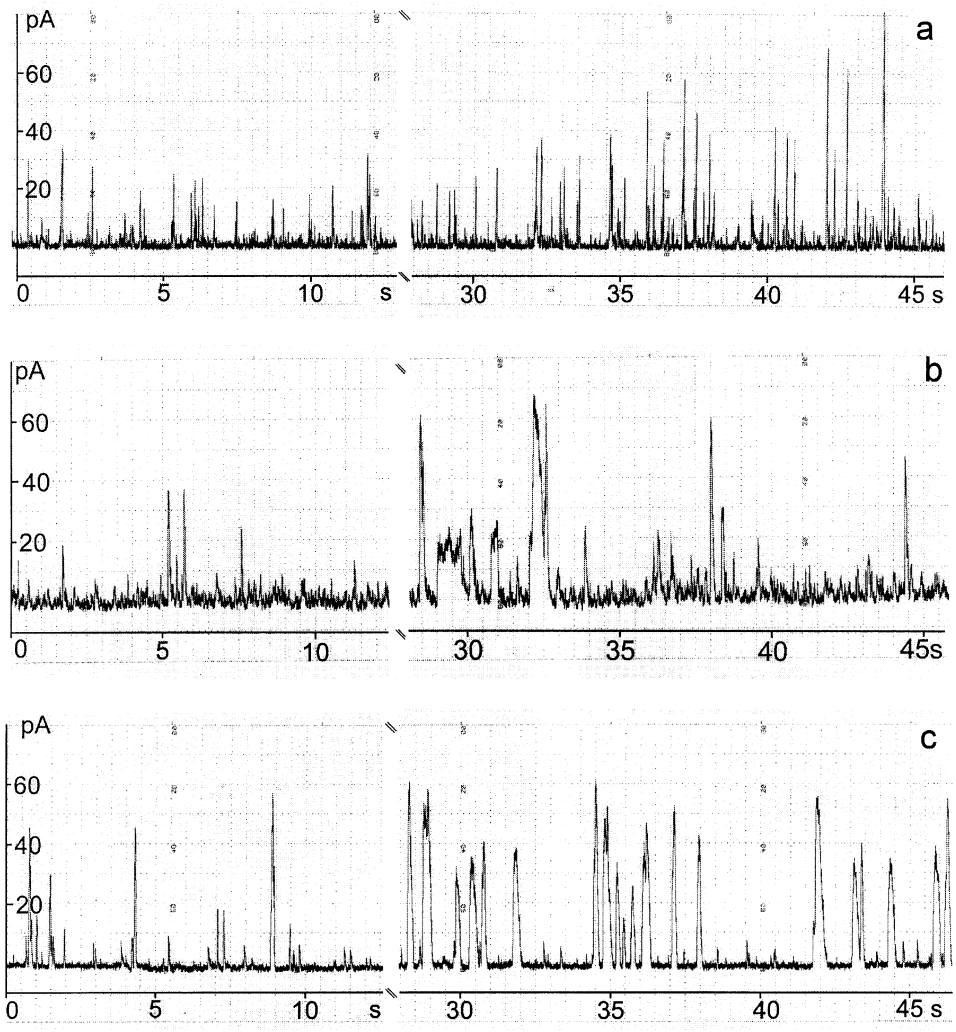

Figure 2 Modulation of spontaneous transient outward currents (STOCs) by 20 μM rapamycin (a) or 20 μM FK506 (b). Holding potential −20 mV. The gap after the control represents the 10 s period of the solution change.

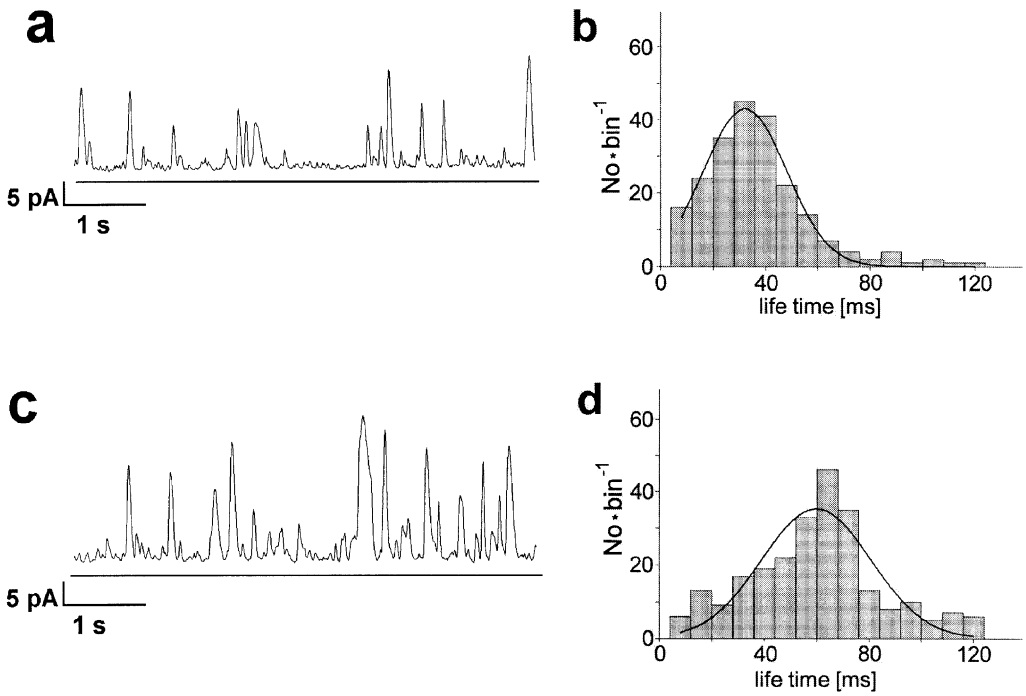

Figure 3 Modulation of STOCs by 10 μM rapamycin. Left: Original traces showing STOCs before (a) and 2–5 min after addition of 10 μM rapamycin (c). Right: Lifetime histograms representing the duration of the STOCs at control (b) and in presence of rapamycin (d). The life time was defined as period during which the STOC exceeds a threshold value of 10 pA. Observed life times were grouped in bins of 8 ms. Distributions were fitted with Gaussian curves, the peak of these curves occurred at 38 ms in absence and at 62 ms in presence of rapamycin.

without the drugs did not change the life-time, the interval between the STOCs or the STOC activity (see Methods).

Augmentation of STOCs by rapamycin

The effects of rapamycin on STOC activity were analysed at 10 μM, a concentration that has been shown to be supramaximal (Bielefeldt *et al*., 1997; Xiao *et al*., 1997). The experiments started with a 2 min control period. Application of rapamycin increased the STOC activity without changing the current between them (Figure 2a). Similar rapamycin effects were recorded with nystatin perforated patches (Figure 2b). The augmentation of STOCs stabilized within 30 s and was stable for at least 5 min. The rapamycin-effects were quantified by comparing in the same cell the STOC activity before and 2–5 min after addition of the drug.

Rapamycin increased frequency, amplitude and duration of the STOCs. Figure 3 illustrates the effects by records from a typical experiment by a computer play-back. Paired comparison ($n=20$) indicates that rapamycin significantly increased the STOC amplitude by $50\pm12\%$. The histogram of a typical experiment shows a life-time of 32 ms before and of 56 ms after addition of rapamycin (Figure 3b), this increase in life time was by $77\pm19\%$ on average. Figure 3c shows that rapamycin increases the STOC frequency by reducing the interval between the STOCs ($20\pm7\%$). The results were reproduced by those from experiments with nystatin perforated patches ($n=4$) where rapamycin increased the STOC amplitude by $48\pm22\%$, the life time by $81\pm32\%$ and reduced the interval between the STOCs by $31\pm19\%$.

Augmentation of STOCs by FK506

FK506 was applied at 20 μM a concentration that had been shown to be supramaximal in terms of relaxation of vascular smooth muscle strips (Bielefeldt *et al*., 1997). Original traces from a representative experiment (Figure 2c) indicate that FK506 increased duration and amplitude of the STOCs but had no influence on the current between the individual STOC events.

Figure 4 compares the STOC activity before and 2–5 min after addition of FK506, longer STOCs can be attributed to $15\pm7\%$ ($n=24$) longer life times (from 40 to 56 ms in Figure 4b). FK506 increased the STOC-amplitude by $15\pm7\%$. FK506 did not reduce the interval between the STOCs (insignificant increase by $20\pm16\%$). FK506 increased the averaged STOC activity from 1.7 to 3.4 pA or by $104\pm26\%$ (calculated from the STOC time integral).

STOCs are insensitive to cyclosporin A

Cyclosporin A is an immunosuppressive drug like rapamycin or FK506 that interacts with calcineurin phosphatases. It also interacts with peptidylprolyl cis-trans-isomerases, however, not like rapamycin or FK506 with the subfamily of the FK-binding proteins but with the cyclo-philine family. Hence, the analysis of a possible cyclosporin A effects on STOCs was performed to test whether the effects of rapamycin and FK506 were specific for their interaction with the FK binding proteins. A concentration of 10 μM cyclosporin A was used because this concentration was reported to be supramaximal in terms of vascular relaxation (Lo Rŭsso *et al*., 1997). The traces of Figure 5 suggest that cyclosporin A rather reduced than increased the STOC activity, paired comparison indicated an insignificant reduction by $8\pm12\%$ ($n=18$, $P=0.83$). The interval between the STOCs was insignificantly increased ($42\pm25\%$, $P=0.36$). In the experiment of Figure 5, the lifetime of the STOCs was 32 ms before and 32 ms after addition of cyclosporin A (insignificant increase by $8\pm12\%$, $P=0.36$). The average activity of STOCs did not significantly change either (increase by $13\pm32\%$, $P=0.26$).

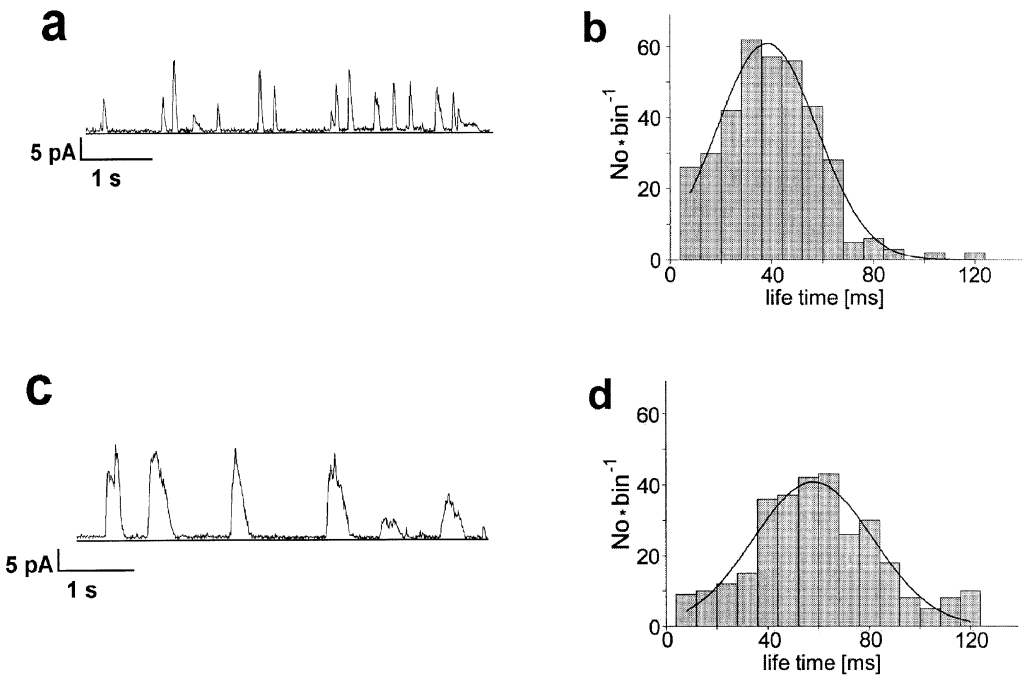

Figure 4 Modulation of STOCs by 20 μM FK506. Left: Original traces showing STOCs before (a) and 2–5 min after addition of FK506 (c). Right: Life time histograms, the fitting Gaussian curves peaked at a life time of 35 ms before (b) and of 54 ms after addition of FK506 (d).

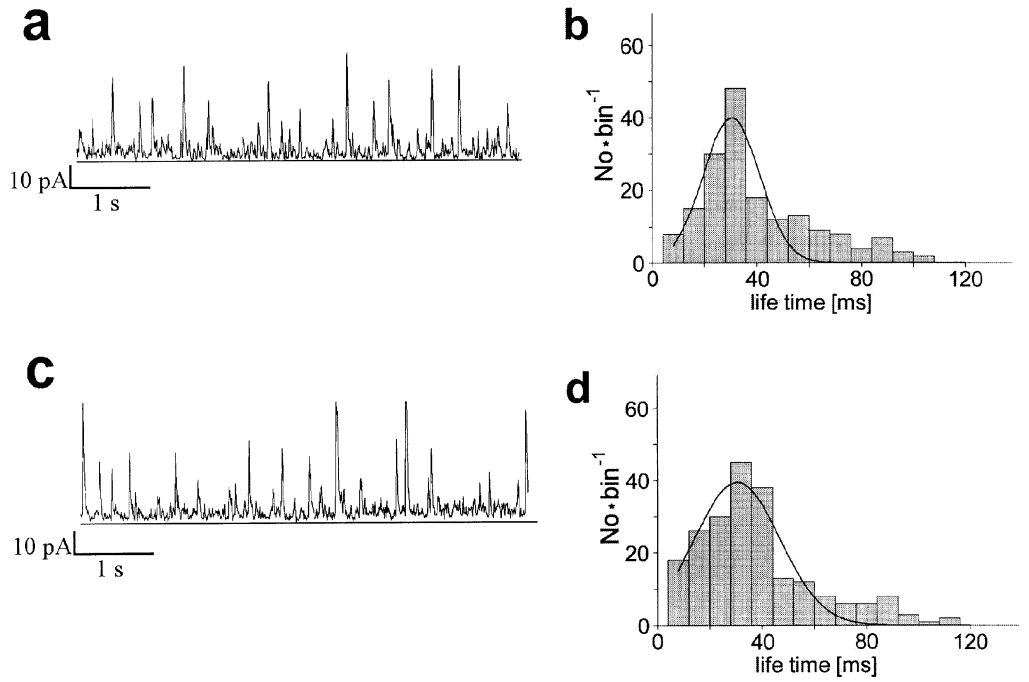

Figure 5 STOCs are insensitive to 20 μM cyclosporin A. Left: Original traces showing STOCs before (a) and 2–5 min after addition of cyclosporin (c). Right: Life time histograms, the fitting Gaussian curves peaked at a life time of 39 ms in absence (b) and of 33 ms in presence of cyclosporin A (d).

Rapamycin-pretreatment does not abolish caffeine induced SR Ca^{2+} release and $I_{K(Ca)}$

It has been suggested that rapamycin and FK506 relax vascular smooth muscle cells by depriving the SR of releasable Ca^{2+}-ions (Bielefeldt *et al*., 1997). The conclusion was based on the result that FK506 blocked caffeine-induced force and Ca^{2+} transients, hence, we repeated this type of experiments in acutely isolated bladder smooth muscle cells under the conditions of the voltage-clamp.

Figure 6 illustrates the effects of rapamycin on the caffeine induced outward currents (trace labelled I_K) and Ca^{2+} signals. In the absence of 20 μM rapamycin, the first application of 20 mM caffeine induced an outward current that peaked within 0.6 s more than 80 pA and then decayed (Figure 6a). Caffeine increased the Ca^{2+} fluorescence $\Delta R/R_o$ along the following time course: from the baseline R_o, $\Delta R/R_o$ rose at a maximal rate of $0.52 \pm 0.14\ s^{-1}$, peaked within 1.22 ± 0.22 s to 2.54 ± 0.55 and then fell at a maximal rate of $-0.18 \pm 0.05\ s^{-1}$. After a 5 min period of wash, caffeine was

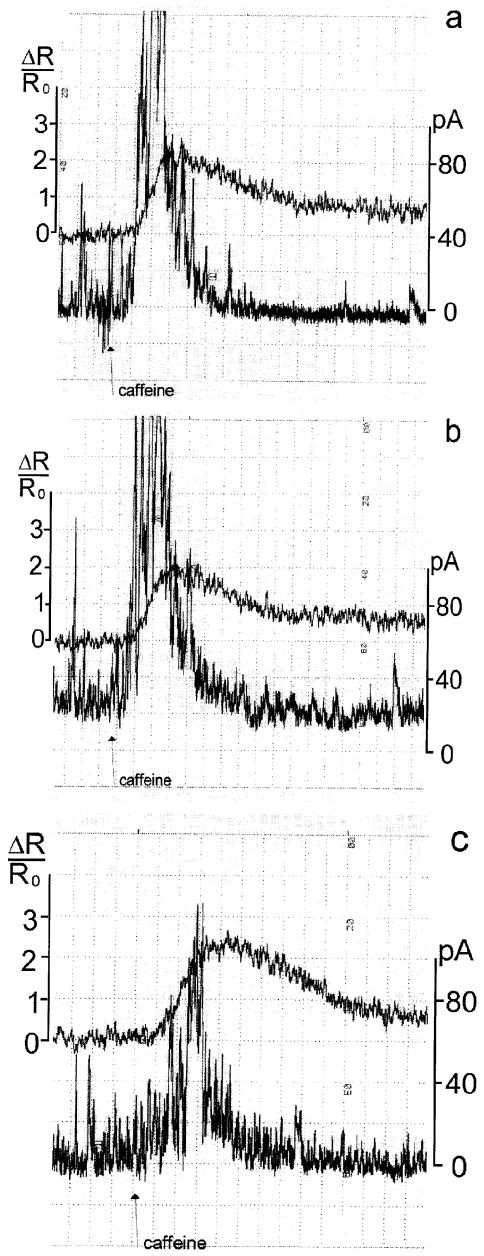

Figure 6 Rapamycin does not block the caffeine-induced Ca^{2+} signals. Membrane currents (STOCs and outward currents) are calibrated on the left, normalized Ca^{2+} signals $\Delta R/R_o$ at the right. (a) Control. First application of 20 mM caffeine. STOCs added up to a Ca^{2+} activated K^+ current $I_{K(Ca)}$ that peaked values slightly above 80 pA. $\Delta R/R_o$ increased at a maximal rate of 0.55 s^{-1} within 1.1 s to a peak of 2.0 and then decayed. (b) Second caffeine-application after a wash-out period of 5 min. Effects on $I_{K(Ca)}$ and on $\Delta R/R_o$ resemble those during the first caffeine-application. (c) Caffeine-application 2 min after continuous superfusion of 20 μM rapamycin. $I_{K(Ca)}$ peaked to 60 pA. $\Delta R/R_o$ increased at a rate of 0.6 s^{-1} within 2.0 s to a peak of 2.2 and then decayed.

applied a second time ($n=5$), these second responses to caffeine did not differ from those evaluated from the first caffeine application (Figure 6b).

Rapamycin was applied during the first wash out of caffeine. Five minutes after wash out of caffeine and 2 min after addition of rapamycin, the caffeine-induced Ca^{2+} signal was measured again (Figure 6c): $\Delta R/R_o$ rose at a maximal rate of 1.16 ± 0.15 s^{-1} ($n=6$), peaked within 1.0 ± 0.14 s to 2.55 ± 0.36 and decayed at a rate of -0.26 ± 0.08 s^{-1}. The comparison of the caffeine-induced Ca^{2+} signals in absence and presence of rapamycin indicates that rapamycin increased the rate of rise and shortened the time to peak whilst it had no effect on the amplitude of the peak or the rate of the decay ($P<0.05$). In four cells superfused with rapamycin, caffeine could be applied a second time. Then, the Ca^{2+} signal increased at lower rate (0.72 ± 0.1 s^{-1}) to a similar peak (2.75 ± 0.41) that occurred at later times (2.5 ± 0.26 s) and decayed more slowly (-0.13 ± 0.03 s^{-1}).

Rapamycin and FK506 hyperpolarize the membrane

In vivo, the cell membrane is not voltage-clamped, and the activation of K^+ channels (underlying the increased STOC activity) is expected to shift the membrane potential towards the K^+-equilibrium potential. Since membrane hyperpolarization has been reported to cause smooth muscular relaxation (see Discussion), we analysed the effect rapamycin and FK506 on the membrane potential under current clamp conditions (external current clamped to zero).

Figure 7a illustrates that 10 μM rapamycin hyperpolarized the membrane from ca. -37 mV to ca. -53 mV. The membrane hyperpolarization of 15 ± 2 mV ($n=5$) was stable for at least 5 min. Figure 7b shows a similar hyperpolarization induced by 20 μM FK506 (15 ± 3 mV, $n=4$).

Discussion

The results of this study indicate that rapamycin and FK506 modulate the events of spontaneous SR Ca^{2+} release as they can be monitored by STOCs. The missing influence of cyclosporin A on STOC activity suggests that the rapamycin- and FK506-induced modulation of STOCs is not related to the immunosuppressant effect of these drugs and that it is specific for their interaction with the FK binding proteins.

Our analysis attributed the rapamycin- and FK506-mediated increase in STOC activity to an increase in both STOC amplitude and STOC life time. We speculate that both effects result from a prolonged RyR open state (reconstituted RyR1 and RyR2: Ahern *et al*., 1997; Mayrleitner *et al*., 1994; Xiao *et al*., 1997). The Ca^{2+} release flux is thought to generate a cloud of elevated $[Ca^{2+}]$ in the 'fuzzy' space between the peripheral SR and the sarcolemma (e.g. Yoshikawa *et al*., 1996; for review see Bolton & Imaizumi, 1996), prolongation of the Ca^{2+} release flux should increase $[Ca^{2+}]$ in this cloud and increase its spatial dimensions. With this hypothesis we interpret the observed elevation of STOC amplitude as a recruitment of BK_{Ca} channels to the cluster of BK_{Ca} channels generating the single STOC. The high local $[Ca^{2+}]_c$ may propagate and activate Ca^{2+} induced Ca^{2+} release from neighboured SR tubules, a mechanism that could explain the observed increase in STOCs frequency. Alternatively, the increase in STOC frequency could be caused by a direct activation of RyR due to removal of FKBP12 by rapamycin (Ahern *et al*., 1997).

The present results do not clarify the functional importance of the RyR3 isoform for spontaneous SR Ca^{2+} release in urinary bladder smooth muscle cells. If Ca^{2+} release through RyR3 would significantly contribute to the activation of STOCs then one should postulate that interaction of the FKBP12 with the RyR3 would accelerate the inactivation of the RyR3 channel isoform similarly as reported for the skeletal and cardiac isoform of the ryanodine-receptor (Chen *et al*., 1994; Mayrleitner *et al*., 1994; Kaftan *et al*., 1996; Xiao *et al*., 1997). Alternatively, the present results could be explained

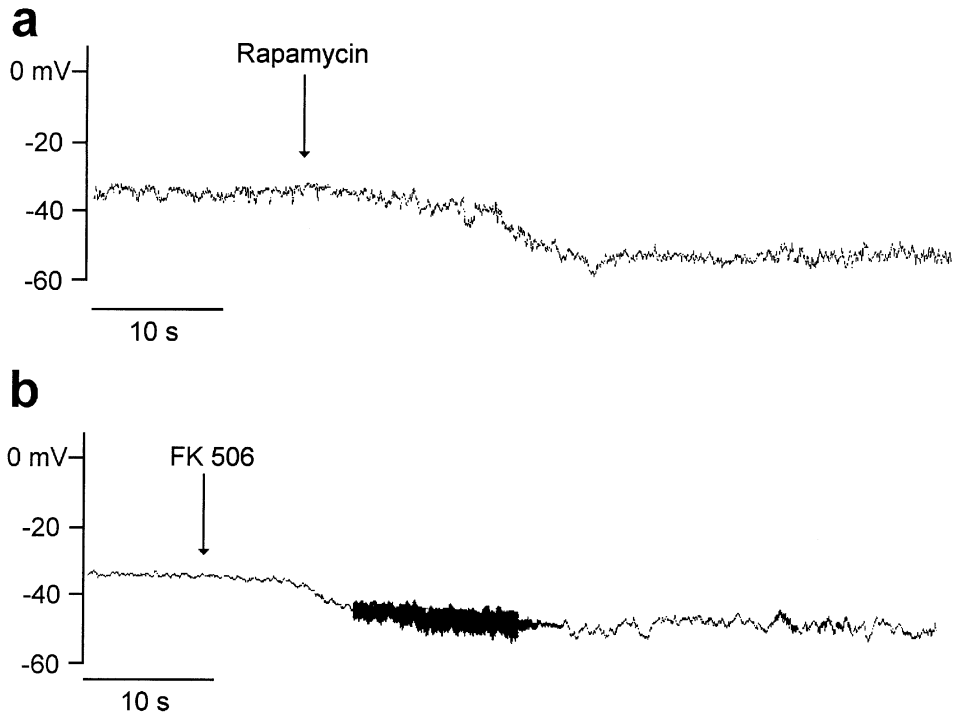

Figure 7 FK506 and rapamycin hyperpolarize bladder smooth muscle cells. Whole-cell recording, amplifier in current-clamp mode. (a) 10 μM; (b) 20 μM. Note: the high frequency noise is an artifact of the solution change.

with the assumption that Ca^{2+} release flux through RyR3 does not significantly contribute to the generation of STOCs.

Our main finding, rapamycin and FK506 augmenting spontaneous Ca^{2+} release, is in line with the suggestion of Bielefeldt *et al.* (1997) that these drugs would relax smooth muscle by promoting Ca^{2+} release flux. However, the present results do not support the interpretation that rapamycin and FK506 would relax the smooth muscle cell by depleting the SR of releasable Ca^{2+}. Firstly, the depletion of releasable Ca^{2+} should have reduced or abolished the activity of STOCs with the consequence of membrane depolarization, just contrary to the effects demonstrated here. Secondly and different to the report of Bielefeldt *et al.* (1997), we did not find that rapamycin abolished the caffeine-induced SR Ca^{2+} release, i.e. in absence or presence of rapamycin the amplitudes of the caffeine-induced signals did not differ. We interpret that the effects of augmented spontaneous SR Ca^{2+} release (STOCs) on the SR Ca^{2+} filling (peak of the caffeine-induced Ca^{2+} signal) were compensated by Ca^{2+} reuptake *via* the SR Ca^{2+} ATPase (SERCA). The rate of rise of the caffeine-induced Ca^{2+} transient was increased by rapamycin, as if rapamycin had increased the rate of the Ca^{2+} release flux *via* a higher RyR open probability (see above).

The present results and those from Bielefeldt *et al.* (1997) are not necessarily exclusive because they were obtained in smooth muscle cells from different specimen, in the present study from guinea-pig urinary bladder, in the study of Bielefeldt *et al.* (1997) from the jejunum. Different smooth muscular specimens are known to deprive their releasable Ca^{2+} after application of acetylcholine more rapidly or more slowly depending on the balance between the Ca^{2+} release flux and RyR Ca^{2+} reuptake *via* SERCA (Missiaen *et al.*, 1992). Hence, it might be possible to generalize the consequences of rapamycin-augmented SR Ca^{2+} release from one smooth muscle to the other. Thus, we do not exclude the Ca^{2+} deprivation hypothesis as an alternative mechanism that mediates relaxation of smooth muscle cells.

The present data suggest that rapamycin or FK506 could relax smooth muscle cells by an additional mechanism which is called 'negative Ca^{2+} feedback'. Our current clamp measurements demonstrated that rapamycin and FK506 induced a 15 mV membrane hyperpolarization. The mechanism, augmented SR Ca^{2+} release leading to Ca^{2+} activation of K^+ channels and membrane hyperpolarization, has been described in a variety of smooth muscular specimen (Klöckner & Isenberg, 1989; Brayden & Nelson, 1992; for references see Nelson & Quayle, 1995); the membrane hyperpolarization is called 'negative Ca^{2+} feedback' because it turns down Ca^{2+} influx through voltage-operated L-type Ca^{2+} channels (see also Porter *et al.*, 1998). The contribution of this Ca^{2+} influx to the concentration of Ca^{2+} ions that finally activate contraction varies between smooth muscle cells from different tissues. The present results suggest that this negative Ca^{2+} feedback on Ca^{2+} influx can have a larger influence on Ca^{2+} homeostasis and SR Ca^{2+} filling than the stimulation of spontaneous SR Ca^{2+} release, at least in non-stimulated smooth muscle cells from the guinea-pig urinary bladder.

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