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RESEARCH PAPER

Regulation by FK506 and rapamycin of Ca^{2+} release from the sarcoplasmic reticulum in vascular smooth muscle: the role of FK506 binding proteins and mTOR

D MacMillan and JG McCarron

Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK

Background and purpose: The sarcoplasmic reticulum (SR), regulates the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) in vascular smooth muscle. Release from the SR is controlled by two intracellular receptor/channel complexes, the ryanodine receptor (RyR) and the inositol 1,4,5-trisphosphate receptor (IP_3R). These receptors may be regulated by the accessory FK506-binding protein (FKBP) either directly, by binding to the channel, or indirectly via FKBP modulation of two targets, the phosphatase, calcineurin or the kinase, mammalian target of rapamycin (mTOR).
Experimental approach: Single portal vein myocytes were voltage-clamped in whole cell configuration and $[Ca^{2+}]_{cyto}$ measured using fluo-3. IP_3Rs were activated by photolysis of caged IP_3 and RyRs activated by hydrostatic application of caffeine.
Key results: FK506 which displaces FKBP from each receptor (to inhibit calcineurin) increased the $[Ca^{2+}]_{cyto}$ rise evoked by activation of either RyR or IP_3R. Rapamycin which displaces FKBP (to inhibit mTOR) also increased the amplitude of the caffeine-evoked, but reduced the IP_3-evoked $[Ca^{2+}]_{cyto}$ rise. None of the phosphatase inhibitors, cypermethrin, okadaic acid or calcineurin inhibitory peptide, altered either caffeine- or IP_3-evoked $[Ca^{2+}]_{cyto}$ release; calcineurin did not contribute to FK506-mediated potentiation of RyR- or IP_3R-mediated Ca^{2+} release. The mTOR inhibitor LY294002, like rapamycin, decreased IP_3-evoked Ca^{2+} release.
Conclusions and implications: Ca^{2+} release in portal vein myocytes, via RyR, was modulated directly by FKBP binding to the channel; neither calcineurin nor mTOR contributed to this regulation. However, IP_3R-mediated Ca^{2+} release, while also modulated directly by FKBP may be additionally regulated by mTOR. Rapamycin inhibition of IP_3-mediated Ca^{2+} release may be explained by mTOR inhibition.
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Keywords: FK506; rapamycin; FK506-binding proteins; ryanodine receptor; inositol 1,4,5-trisphosphate receptor; mTOR; calcineurin; vascular smooth muscle; calcium

Abbreviations: $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; CiP, calcineurin inhibitory peptide; F, fluorescence counts; F_0, baseline fluorescence counts; FKBPs, FK506-binding proteins; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; mTOR, mammalian target of rapamycin; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum

Introduction

Regulation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) is vital to the control of vascular tone. The intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), critically regulates $[Ca^{2+}]_{cyto}$, by controlling Ca^{2+} release (Bootman *et al.*, 2001; McCarron *et al.*, 2006). Release from the SR is mediated by two intracellular receptor complexes, the inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR; nomenclature follows Alexander *et al.*, 2008). IP_3Rs are activated primarily by IP_3 generated via G-protein- or tyrosine kinase-linked receptor activation (Bootman *et al.*, 2001). In smooth muscle RyRs may also be activated pharmacologically (e.g. by caffeine) or when the Ca^{2+} content of the SR exceeds normal physiological levels (Burdyga and Wray, 2005; McCarron *et al.*, 2006).

RyR and IP_3R are regulated by accessory proteins. Of particular importance are the FK506-binding proteins (FKBPs)

Correspondence: JG McCarron, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR, UK. E-mail: john.mccarron@strath.ac.uk
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(Cameron *et al.*, 1995b; Dargan *et al.*, 2002; Tang *et al.*, 2002; Zheng *et al.*, 2004) which were identified initially as the cytoplasmic receptor for the clinical immunosuppressant drugs FK506 and rapamycin (Harding *et al.*, 1989). The association between FKBP and IP_3R or RyR is disrupted by FK506 and rapamycin, each of which binds to the accessory protein to form a drug-immunophilin protein complex and displaces FKBP from the channels (Cameron *et al.*, 1995b; Bultynck *et al.*, 2001a; Dargan *et al.*, 2002).

The two major isoforms of the FKBP recognized as regulators of Ca^{2+} release channels are FKBP12 (the 12 kDa form of the protein) and FKBP12.6 (the 12.6 kDa form) (Tang *et al.*, 2002; Wang *et al.*, 2004; MacMillan *et al.*, 2005b). FKBP12 and FKBP12.6 associate with IP_3R in various cell types (Cameron *et al.*, 1995b; Cameron *et al.*, 1995a; MacMillan *et al.*, 2005b) suggesting that FKBP-dependent modulation of channel function may have an important role in Ca^{2+} signaling. Indeed, in some investigations removal of FKBP12 from the channel decreased IP_3R-mediated Ca^{2+} release (MacMillan *et al.*, 2005b) and addition of exogenous FKBP12 increased IP_3R channel activity in bilayer studies (Dargan *et al.*, 2002). Furthermore, rapamycin, which disrupts FKBP-IP_3R, decreased phenylephrine-induced contractions in rat vas deferens (Scaramello *et al.*, 2009). These results suggest that FKBP12 potentiates IP_3R activity. In other studies FKBP12 may inhibit channel activity and removal of FKBP12 from the channel increased IP_3R-mediated Ca^{2+} release (Cameron *et al.*, 1995b; Cameron *et al.*, 1997).

FKBP12 also associates with RyR2 and RyR3 (Bultynck *et al.*, 2001a; MacMillan *et al.*, 2008) and with the skeletal muscle isoform, RyR1 (e.g. Carmody *et al.*, 2001). An association between FKBP12.6 and RyR occurs in tracheal, pulmonary and coronary artery smooth muscle (e.g. Tang *et al.*, 2002; Wang *et al.*, 2004). RyR may be modulated by FKBPs to inhibit activity of the channel. Removal of FKBPs by either FK506 or rapamycin increased RyR channel open probability in lipid bilayers from coronary arterial smooth muscle (Tang *et al.*, 2002) and cardiac muscle (Kaftan *et al.*, 1996) or $[Ca^{2+}]_{cyto}$ in intestinal, colonic, bladder and pulmonary artery myocytes (Weidelt and Isenberg, 2000; Zheng *et al.*, 2004; MacMillan *et al.*, 2008). Conversely rebinding either FKBP12 or FKBP12.6, following their removal, decreases channel opening (Brillantes *et al.*, 1994; Barg *et al.*, 1997; Bultynck *et al.*, 2001b).

However, evidence is not universally supportive of a role of FKBPs in regulating either RyR or IP_3R activity. In some studies no interaction between either RyR (Carmody *et al.*, 2001; Wang *et al.*, 2004; Zheng *et al.*, 2004) or IP_3R (Bultynck *et al.*, 2001a; Carmody *et al.*, 2001; Zheng *et al.*, 2004) and FKBP12 occurred. Other studies failed to demonstrate an association between FKBP12.6 and either RyR (types 1&3) or IP_3R (types 1–3) in pulmonary artery (Zheng *et al.*, 2004). Functional studies also failed to detect any effect of FK506 or FKBP12 on IP_3-mediated Ca^{2+} release in various cell types, including smooth muscle (Bultynck *et al.*, 2000; Bultynck *et al.*, 2001a). Neither did removal nor addition of FKBP12 or FKBP12.6 alter RyR channel function (Timerman *et al.*, 1996; Barg *et al.*, 1997; Xiao *et al.*, 2007). Nor did FK506 alter Ca^{2+} release via RyR in porcine coronary artery (Yasutsune *et al.*, 1999) or cardiac myocytes (duBell *et al.*, 1997) or induce contraction in renal, mesenteric, coronary or carotid arteries (Epstein *et al.*, 1998).

In addition to binding to the channel, FKBPs may modulate signaling pathways by regulating kinase and phosphatase activity. Thus FKBPs may modulate Ca^{2+} release either directly by binding to the channel or indirectly via the kinase and phosphatase pathways modulated by FKBP. The different signaling pathways regulated by FKBPs, may account, at least in part, for the contradictory findings on FKBP modulation of IP_3R and RyR. For example, following the removal of FKBP from either IP_3R or RyR by FK506, the FK506-FKBP complex formed binds to and inhibits the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin (Liu *et al.*, 1991) Indeed, calcineurin may regulate RyR and IP_3R by forming part of the FKBP-channel complex (Cameron *et al.*, 1995b; Cameron *et al.*, 1995a; Cameron *et al.*, 1997; Bandyopadhyay *et al.*, 2000a; Shin *et al.*, 2002). In support, calcineurin inhibitors increased caffeine- and ryanodine-induced Ca^{2+} release and the frequency and amplitude of Ca^{2+} oscillations in cardiac and skeletal muscle (Bandyopadhyay *et al.*, 2000a; Shin *et al.*, 2002). In COS-7 cells and cerebellar microsomes inhibition of calcineurin increased ATP-induced Ca^{2+} release and IP_3R activity respectively (Cameron *et al.*, 1995a; Bandyopadhyay *et al.*, 2000b).

Calcineurin may also regulate Ca^{2+} release via RyR (Bultynck *et al.*, 2003; MacMillan *et al.*, 2008) or IP_3R (Bultynck *et al.*, 2003) independently of FKBPs or not at all (Kanoh *et al.*, 1999). Indeed, the comparatively few studies in vascular smooth muscle failed to confirm a role for calcineurin in regulating $[Ca^{2+}]_{cyto}$. Drugs which can inhibit calcineurin, cypermethrin and okadaic acid, for example, did not alter Ca^{2+} release via either RyR (MacMillan *et al.*, 2008) or IP_3R (MacMillan and McCarron, unpublished data) in aortic myocytes. Neither did okadaic acid alter $[Ca^{2+}]_{cyto}$ in porcine coronary artery (Ashizawa *et al.*, 1989; Hirano *et al.*, 1989). Cyclosporin A, a calcineurin inhibitor which does not interact with FKBPs, did not alter $[Ca^{2+}]_{cyto}$ in pulmonary (Zheng *et al.*, 2004) and coronary artery myocytes (Frapier *et al.*, 2001) or SR $[Ca^{2+}]$ in aorta myocytes (Avdonin *et al.*, 1999).

Rapamycin, a structural analogue of FK506, also binds to FKBP12 resulting in the formation of a rapamycin-FKBP12 complex. This complex does not inhibit calcineurin but binds to FKBP12-rapamycin-associated protein (Heitman *et al.*, 1991; Peterson *et al.*, 2000) also named mammalian target of rapamycin (mTOR) and inhibits its function (Brown *et al.*, 1995). mTOR is a 289 kDa serine/threonine protein kinase and classified as a member of the phosphatidylinositol kinase-related kinase (PIKK) family. mTOR regulates a myriad of intracellular processes which include cell cycle progression and growth. Inhibition of mTOR by FKBP12-rapamycin may mediate the pharmacological actions of rapamycin (Brown *et al.*, 1995; Sun *et al.*, 2005) including the regulation of IP_3-mediated Ca^{2+} release in colonic smooth muscle (MacMillan *et al.*, 2005b).

In view of the controversy which surrounds the effects of the drugs FK506 and rapamycin and the mechanism of action of FKBPs on intracellular Ca^{2+} release in vascular smooth muscle the present study was carried out in single myocytes from portal vein. Cells were voltage-clamped to avoid $[Ca^{2+}]_{cyto}$ changes which may have occurred via Ca^{2+} influx as a result of

FK506- or rapamycin-evoked changes in membrane potential. The use of photolysed caged IP_3, to activate IP_3R, minimized the number of second messenger systems activated. This study has shown that pharmacological removal of FKBP from RyR with either FK506 or rapamycin, augmented caffeine-evoked Ca^{2+} release in portal vein myocytes. On the other hand, FK506 (FKBP-calcineurin inhibitor) augmented whereas rapamycin (mTOR inhibitor) reduced the IP_3-evoked $[Ca^{2+}]_{cyto}$ release. Neither cypermethrin nor okadaic acid (drugs which can inhibit calcineurin) altered caffeine- or IP_3-evoked $[Ca^{2+}]_{cyto}$ release. Thus, calcineurin is not required for the potentiation of RyR or IP_3R Ca^{2+} release to occur. The mTOR inhibitor LY294002, like rapamycin, decreased IP_3R Ca^{2+} release; mTOR inhibition may mediate the rapamycin-induced decrease in IP_3R Ca^{2+} release. Our results, in portal vein myocytes, suggest that RyR- and IP_3R-mediated Ca^{2+} release are modulated directly by FKBP. IP_3R-mediated Ca^{2+} release may be additionally modulated by mTOR.

Methods

Preparation of portal vein myocytes

All animal care and experimental procedures complied with the Animal (Scientific Procedures) Act UK 1986. Male guinea pigs (500–700 g) were humanely killed by cervical dislocation and immediate exsanguination, and the portal vein was removed quickly and transferred to an oxygenated (95% O_2–5% CO_2) physiological saline solution of the following composition (mM): NaCl 118.4, $NaHCO_3$ 25, KCl 4.7, NaH_2PO_4 1.13, $MgCl_2$ 1.3, $CaCl_2$ 2.7 and glucose 11 (pH 7.4). From this tissue single vascular smooth muscle cells were isolated using a two-step enzymatic process. The vessel was initially treated (12 min at 34.5°C) with papain (1.7 mg·mL^{-1}), DTT (0.7 mg·mL^{-1}) and BSA (1.64 mg·mL^{-1}) in a low Ca^{2+} solution which contained (mM): sodium glutamate, 80; NaCl, 55; KCl, 6; $MgCl_2$, 1; $CaCl_2$, 0·1; Hepes, 10; glucose, 10; 0·2, EDTA (to remove heavy metals) (pH 7·3). During a second incubation, the tissue was further digested (14 min at 34.5°C) in the low Ca^{2+} solution containing BSA (1.64 mg·mL^{-1}), by collagenase (type F; 1.3–2 mg·mL^{-1}) and hyaluronidase (0.8–1 mg·mL^{-1}). The tissue was then rinsed several times with enzyme-free low Ca^{2+} solution containing BSA and then with the enzyme- and BSA-free low Ca^{2+} solution. Single smooth muscle cells were dispersed by trituration with a Pasteur pipette, stored at 4°C and used the same day. All experiments were conducted at room temperature (20–22°C).

Electrophysiological experiments

Cells were voltage clamped using conventional tight seal whole-cell recording (MacMillan *et al.*, 2005a; 2008). The composition of the extracellular solution was (mM): sodium glutamate 80, NaCl 40, tetraethylammonium chloride (TEA) 20, $MgCl_2$ 1.1, $CaCl_2$ 3, HEPES 10 and glucose 30 (pH 7.4 adjusted with NaOH 1M). The pipette solution contained (mM): Cs_2SO_4 85, CsCl 20, $MgCl_2$ 1, HEPES 30, pyruvic acid 2.5, malic acid 2.5, KH_2PO_4 1, MgATP 3, creatine phosphate 5, guanosine triphosphate 0.5, fluo-3 penta-ammonium salt 0.1 and caged Ins (1,4,5) P_3-trisodium salt 0.025 (pH 7.2 adjusted with CsOH 1M). Whole cell currents were amplified by an Axopatch 1D amplifier (Axon instruments, Union City, CA, USA), low pass filtered at 500 Hz (8-pole bessel filter; Frequency Devices, Haverhill, MA, USA), and digitally sampled at 1.5 kHz using a Digidata interface, pCLAMP software (version 6.0.1, Axon Instruments) and stored on a personal computer for analysis.

Assay of $[Ca^{2+}]_{cyto}$

$[Ca^{2+}]_{cyto}$ was measured as fluorescence using the membrane-impermeable dye fluo-3 (penta-ammonium salt) introduced into the cell via the patch pipette (MacMillan *et al.*, 2005a,b). Fluorescence was quantified using a microfluorimeter which consisted of an inverted microscope (Nikon diaphot) and a photomultiplier tube with a bi-alkali photo cathode. Fluo-3 was excited at 488 nm (bandpass 9 nm) from a PTI Delta Scan (Photon Technology International Inc., London, UK) through the epi-illumination port of the microscope (using one arm of a bifurcated quartz fibre optic bundle). Excitation light was passed through a field stop diaphragm to reduce background fluorescence and reflected off a 505 nm long-pass dichroic mirror. Emitted light was guided through a 535 nm barrier filter (bandpass 35 nm) to a photomultiplier in photon counting mode. Interference filters and dichroic mirrors were obtained from Glen Spectra (London, UK). To photolyse caged IP_3 (25 μM) the output of a xenon flash lamp (Rapp Optoelektronik, Hamburg, Germany) was passed through a UG-5 filter to select UV light and merged into the excitation light path of the microfluorimeter using the second arm of the quartz bifurcated fibre optic bundle and applied to the caged compound. The nominal flash lamp energy was 57 mJ, measured at the output of the fibre optic bundle and the flash duration was approximately 1 ms. Fluorescence signals were expressed as ratios (F/F_0) of fluorescence counts (F) relative to baseline (control) values (taken as 1) before stimulation (F_0).

Statistical analysis

Results are expressed as means ± SEM. Student's t-tests were applied to test and control conditions, a value of $P < 0.05$ was considered significant.

Materials

Caged Ins (1,4,5) P_3-trisodium salt was purchased from Invitrogen (Paisley, UK). Fluo-3 penta-ammonium salt was purchased from TEF labs (Austin, Texas, USA). Rapamycin, cypermethrin and okadaic acid were each purchased from Calbiochem-Novabiochem (Beeston, Nottingham, UK) and papain was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). All other reagents were purchased from Sigma (Poole, Dorset, UK). IP_3 was released from its caged compound by flash photolysis. Caffeine (10 mM) was applied by hydrostatic pressure ejection using a pneumatic pump (PicoPump PV 820, World Precision Instruments, Stevenage, Herts, UK). The concentration of caged, non-photolysed IP_3 refers to that in the pipette. Caffeine was dissolved in extracellular bathing solution, FK506 was

dissolved in 100% ethanol (final bath concentration of the solvent, 0.05%, was by itself ineffective). Rapamycin, cypermethrin and okadaic acid were each dissolved in dimethyl sulphoxide (final bath concentration of the solvent, 0.01%, was by itself ineffective). Each drug (with the exception of caffeine) was perfused into the solution bathing the cells (~5 mL per min). The calcineurin inhibitory peptide (CiP) based on the autoinhibitory fragment (ITSFEEAKGLDRINER-MPPRRDAMP) was obtained from Sigma (Poole, UK) and introduced to the cell via the patch pipette filling solution.

Results

To determine the role of FKBPs in regulating Ca^{2+} release via RyRs and IP_3Rs, the effects of FK506 and rapamycin, each of which disrupt the FKBP-channel association, were examined on caffeine- and IP_3-induced Ca^{2+} release in voltage-clamped, single portal vein myocytes. Caffeine (10 mM), which activates RyR (Figure 1A), and photolysed caged IP_3 (25 μM, Figure 2A), which activates IP_3R, each reproducibly increased $[Ca^{2+}]_{cyto}$. FK506 (10 μM; Figure 1A) and rapamycin (10 μM;

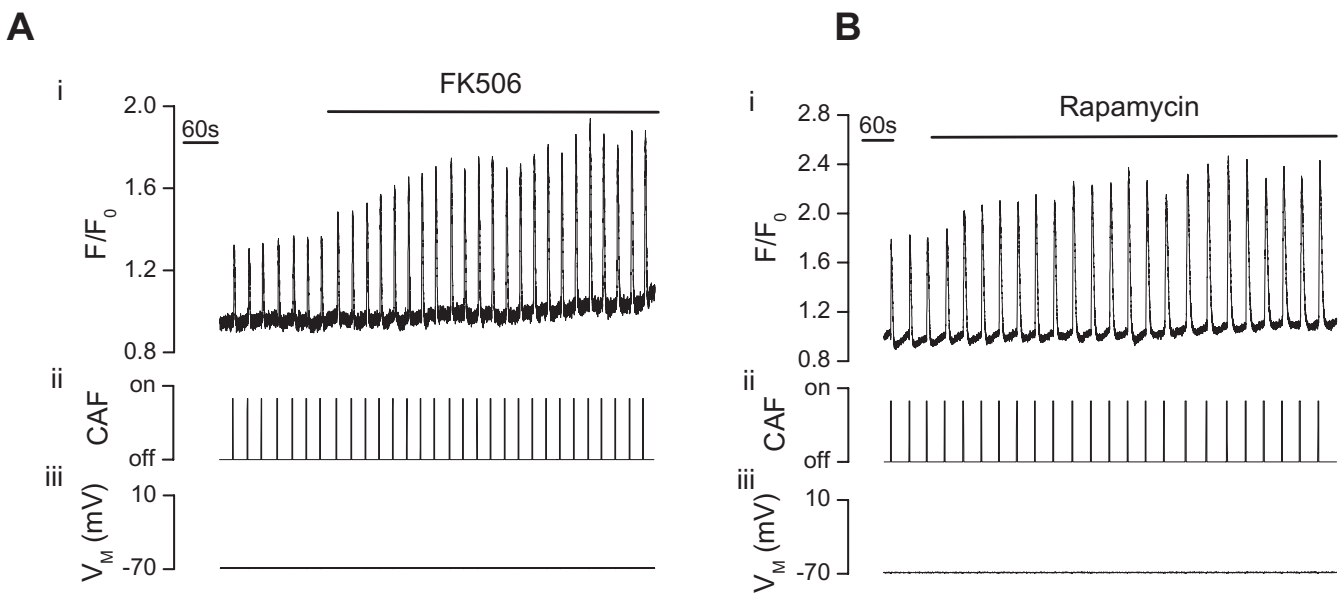

Figure 1 FK506 or rapamycin increased the $[Ca^{2+}]_{cyto}$ rise evoked by caffeine in voltage clamped single portal vein myocytes. At –70 mV (iii) caffeine (CAF, 10 mM, ii) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. FK506 (10 μM, $n = 3$, $P < 0.05$, A) and rapamycin (10 μM, $n = 7$, $P < 0.05$, B) each significantly increased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients (i). $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, baseline fluorescence counts.

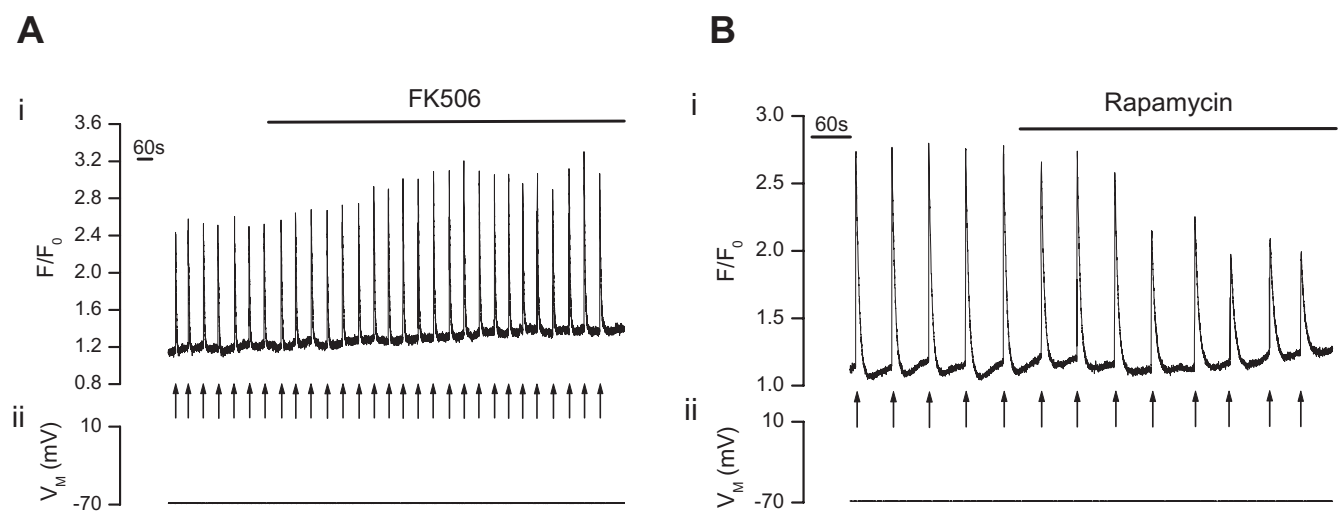

Figure 2 FK506 increased but rapamycin decreased IP_3-evoked Ca^{2+} increases in voltage clamped single portal vein myocytes. At –70 mV (ii) photolysed caged IP_3 (↑) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. FK506 (10 μM, $n = 3$, $P < 0.05$, A) increased but rapamycin (10 μM, $n = 3$, $P < 0.05$, B) decreased the $[Ca^{2+}]_{cyto}$ transients (i). $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, baseline fluorescence counts; IP_3, inositol 1,4,5-trisphosphate.

Figure 1B) each significantly ($P < 0.05$) increased caffeine-induced Ca^{2+} release ($\Delta F/F_0$) by 70 ± 11% and 59 ± 7% respectively [from 0.4 ± 0.02 (control) to 0.68 ± 0.08 (FK506, $n = 3$, Figure 1A) and 0.66 ± 0.11 (control) to 1.02 ± 0.16 (rapamycin, $n = 7$, Figure 1B)]. FK506 (10 μM; Figure 2A) also increased IP_3-induced Ca^{2+} release by 30 ± 1% ($\Delta F/F_0$ from 0.93 ± 0.2 to 1.23 ± 0.2, $n = 3$). Rapamycin (10 μM; Figure 2B) on the other hand, significantly ($P < 0.05$) decreased the IP_3-evoked Ca^{2+} transient ($\Delta F/F_0$) by 55 ± 8% from 1.78 ± 0.5 to 0.74 ± 0.14 ($n = 3$).

FK506 and rapamycin each form a complex with FKBP to disrupt binding of FKBP to IP_3R and RyR. Disrupting FKBP binding to the channels is thus a feature which is common to the mechanism of action of both drugs. However, thereafter the action of the drug differs. The FK506-FKBP complex inhibits calcineurin whereas the rapamycin-FKBP complex inhibits mTOR. Therefore, the effects of FK506 and rapamycin on Ca^{2+} release may be either mediated by calcineurin or mTOR inhibition respectively, or by the removal of FKBP from the receptor.

Role of calcineurin in FK506-evoked potentiation of Ca^{2+} release

If the potentiation of RyR activity by FK506 was explained by inhibition of calcineurin, then the potentiation should be mimicked by calcineurin inhibitors. However, drugs which can inhibit calcineurin, cypermethrin (10 μM; Figure 3A) or okadaic acid (5 μM; Figure 3B), failed to alter caffeine-evoked Ca^{2+} transients [($\Delta F/F_0$) from 1.22 ± 0.25 (control) to 1.22 ± 0.26 (cypermethrin, $n = 8$, $P > 0.05$) and 0.65 ± 0.08 (control) to 0.65 ± 0.07 (okadaic acid, $n = 5$, $P > 0.05$) respectively]. Neither did the CiP prevent FK506 potentiation of caffeine-evoked Ca^{2+} release. CiP was administered into the cell via the pipette solution (because it is impermeant) and FK506 remained effective in increasing caffeine-evoked Ca^{2+} transients [(F/F_0) from 0.9 ± 0.09 (control) to 1.33 ± 0.09 (FK506, $n = 7$, $P < 0.05$); Figure 3C].

If the potentiation of IP_3-mediated Ca^{2+} release by FK506 arose by inhibition of calcineurin, then inhibitors of the phosphatase should increase IP_3-mediated Ca^{2+} release. However, again, neither cypermethrin (10 μM; Figure 4A) nor okadaic acid (5 μM; Figure 4B) increased IP_3-mediated Ca^{2+} release [($\Delta F/F_0$) (from 2.02 ± 0.21 (control) to 2.03 ± 0.21 (cypermethrin, $n = 5$, $P > 0.05$) and 1.21 ± 0.19 (control) to 1.16 ± 0.19 (okadaic acid, $n = 6$, $P > 0.05$)]. Following calcineurin inhibition with CiP (Figure 4C), FK506 also remained effective in increasing IP_3-evoked Ca^{2+} transients [(F/F_0) from 1.39 ± 0.12 (control) to 2.00 ± 0.15 (FK506, $n = 6$, $P < 0.05$)].

Role of mTOR in rapamycin-evoked suppression of IP_3-mediated Ca^{2+} release

Interestingly, removal of FKBP by rapamycin decreased IP_3-mediated Ca^{2+} release. The decrease is unlikely to be explained by a reduction in the store's Ca^{2+} content by rapamycin's inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA; Bultynck *et al.*, 2000; Loughrey *et al.*, 2007), because the Ca^{2+} transient in response to RyR activation with caffeine was significantly ($P < 0.05$) increased by the drug (Figure 1B). Had rapamycin inhibited SERCA the caffeine-evoked $[Ca^{2+}]_{cyto}$ rise would also have been reduced. Furthermore, inhibition of SERCA pump activity should increase steady-state $[Ca^{2+}]_{cyto}$ (Bultynck *et al.*, 2000). No increase in steady-state $[Ca^{2+}]_{cyto}$ (measured as fluorescence) was observed either following FK506 [(F/F_0) from 1.06 ± 0.05 (control) to 1.09 ± 0.07 (FK506, $n = 6$, $P > 0.05$)] or rapamycin [(F/F_0) from 1.07 ± 0.04 (control) to 1.08 ± 0.04 (rapamycin, $n = 10$, $P > 0.05$)]. Moreover, neither FK506 nor rapamycin significantly altered the rate of Ca^{2+} removal from the cytoplasm following each of IP_3- or caffeine-evoked Ca^{2+} release. The 80–20% decay interval following IP_3- and caffeine-evoked Ca^{2+} release was 4.0 ± 1.0 s and 6.2 ± 1.4 s in controls and 4.7 ± 1.2 s and 6.3 ± 1.5 s in FK506, (each $n = 3$, $P > 0.05$) respectively. Similarly, the 80–20% decay interval following IP_3- and caffeine-evoked Ca^{2+} release was 5.8 ± 0.2 s and 6.2 ± 0.6 s in controls and 6.5 ± 0.9 s and 6.4 ± 0.5 s in rapamycin, ($n = 3$ and 7, $P > 0.05$) respectively.

Rapamycin-FKBP-mediated inhibition of mTOR may explain the inhibition of IP_3-mediated Ca^{2+} release. To test this, the effect of the mTOR inhibitor LY294002 (inhibits mTOR without first binding to FKBP) was examined on IP_3-evoked Ca^{2+} release. If the rapamycin-induced decrease in IP_3-mediated Ca^{2+} release arose by inhibition of mTOR, then an inhibitor of the kinase should also decrease IP_3-mediated Ca^{2+} release. LY294002 (20 μM; Figure 5) significantly ($P < 0.05$) decreased the IP_3-evoked Ca^{2+} transient ($\Delta F/F_0$) by 52 ± 12% from 2.64 ± 0.4 to 1.24 ± 0.3 ($n = 4$).

Discussion and conclusions

FK506 and rapamycin exert multiple effects on intracellular signaling via their effects on FKBP binding to RyRs and IP_3Rs, and by regulating the phosphatase calcineurin and the kinase mTOR. The multiple effects of the drugs explain the present results and account for apparently contradictory findings which exist in the literature on the role of FKBPs in Ca^{2+} signaling. The present study, in portal vein myocytes, suggests RyR is modulated directly by FKBP to decrease Ca^{2+} release via the channel. Neither calcineurin nor mTOR are required for FKBP modulation of RyR activity to occur. IP_3R activity, like RyR, is modulated directly by FKBP but, unlike RyR, also indirectly via the kinase mTOR. FKBP binding to IP_3R decreases Ca^{2+} release while inhibition of mTOR, by the FKBP-rapamycin complex, additionally decreases IP_3-mediated Ca^{2+} release.

An association between FKBPs and either RyR or IP_3R to regulate the activities of the channels has been reported in various cell types (e.g. Carmody *et al.*, 2001; MacMillan *et al.*, 2005b; 2008) including vascular smooth muscle (Tang *et al.*, 2002; Zheng *et al.*, 2004). However, FKBPs may increase (Dargan *et al.*, 2002; Su *et al.*, 2003; MacMillan *et al.*, 2005b), decrease (Cameron *et al.*, 1995b; Zheng *et al.*, 2004; MacMillan *et al.*, 2008) or have no effect on channel activity (Barg *et al.*, 1997; duBell *et al.*, 1997; Kanoh *et al.*, 1999; Yasutsune *et al.*, 1999; Bultynck *et al.*, 2000). The drugs FK506 and rapamycin each inhibit FKBP association with IP_3R and RyR but differ in that the FK506-FKBP complex inhibits calcineurin (Liu *et al.*, 1991) whereas the rapamycin-FKBP complex inhibits mTOR (Brown *et al.*, 1995). Therefore, the effects of

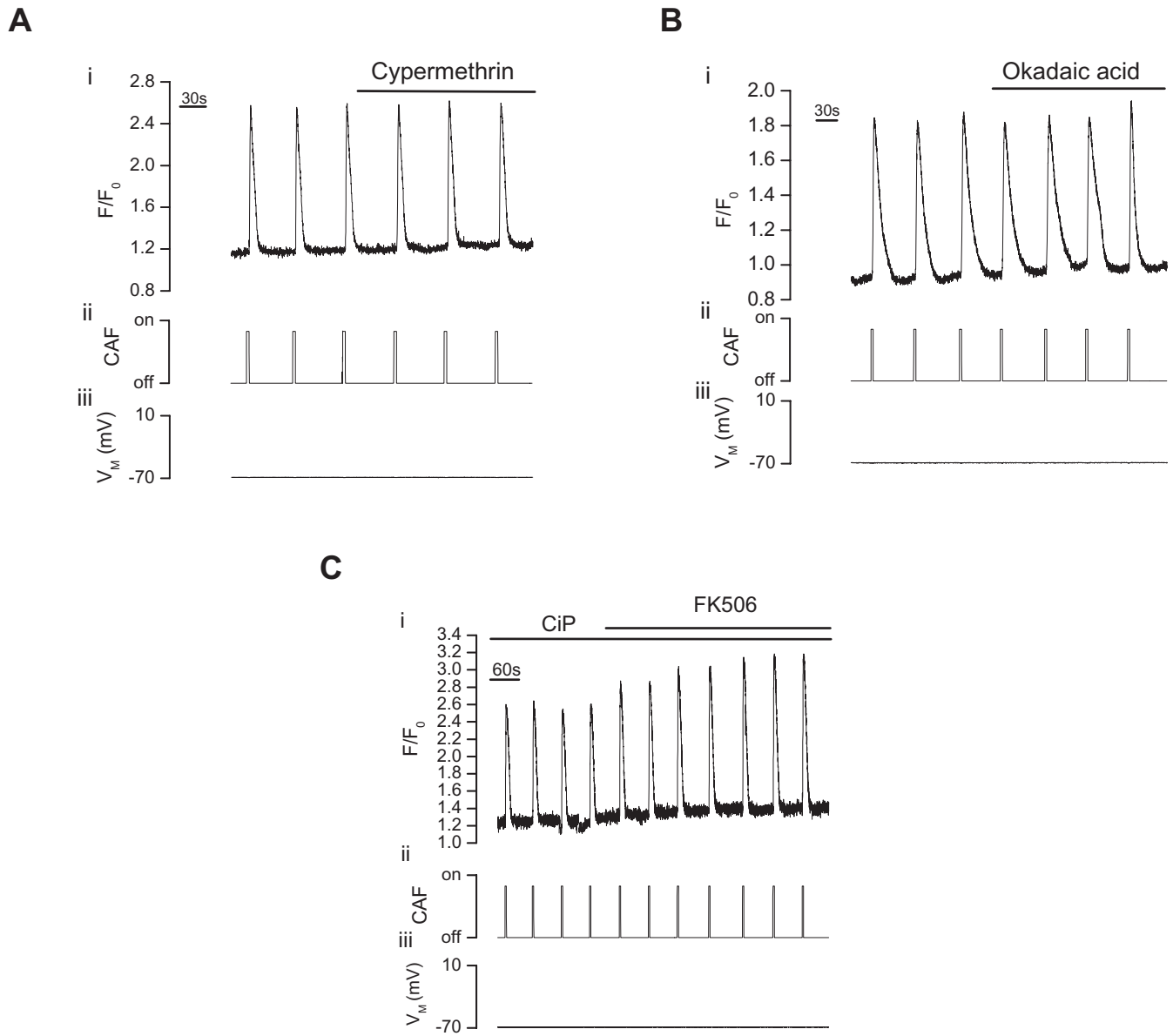

Figure 3 Effect of calcineurin inhibition on caffeine-evoked Ca^{2+} increases in voltage-clamped single portal vein myocytes. Caffeine (CAF, 10 mM, ii) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. Neither cypermethrin (10 μM, $n = 8$, A) nor okadaic acid (5 μM, $n = 5$, B) significantly ($P > 0.05$) altered the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients (V_M −70 mV, iii). Following pretreatment with the calcineurin inhibitor CiP (100 μM, C) via the patch pipette filling solution, FK506 (10 μM) still significantly ($P < 0.05$) increased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients (V_M −70 mV, iii, $n = 7$). $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; CiP, calcineurin inhibitory peptide; F, fluorescence counts; F_0, baseline fluorescence counts.

FK506 and rapamycin may be either mediated by calcineurin or mTOR inhibition respectively, or by the removal of FKBP from the channels.

In the present study while FK506 increased, drugs which can inhibit calcineurin (cypermethrin or okadaic acid) failed to alter caffeine- or IP_3-evoked Ca^{2+} release. FK506 also remained effective in increasing the caffeine- and IP_3-evoked Ca^{2+} transients in the presence of CiP. These results suggest that calcineurin is unlikely to contribute to the FK506-induced potentiation of caffeine- or IP_3-evoked Ca^{2+} increases in portal vein. Had it done so the calcineurin inhibitors would have increased Ca^{2+} release and the effect of FK506 would have been inhibited. As FK506 and rapamycin each increased caffeine-induced Ca^{2+} release, a mechanism common to the action of both drugs, i.e. FKBP removal from the FKBP-RyR complex, may explain the increased Ca^{2+} release in the present study. This finding is consistent with our own previous results in colonic smooth muscle (MacMillan *et al.*, 2008) and those of other investigators in various tissues which suggest that FK506 and rapamycin each increase the activity of RyR by disrupting FKBP-RyR association (Brillantes *et al.*, 1994; Kaftan *et al.*, 1996; Bultynck *et al.*, 2000; Weidelt and Isenberg, 2000; Xiao *et al.* 2007).

On the other hand, while FK506 increased, rapamycin decreased IP_3-mediated Ca^{2+} release. The target for the rapamycin-FKBP12 complex has been identified as the kinase

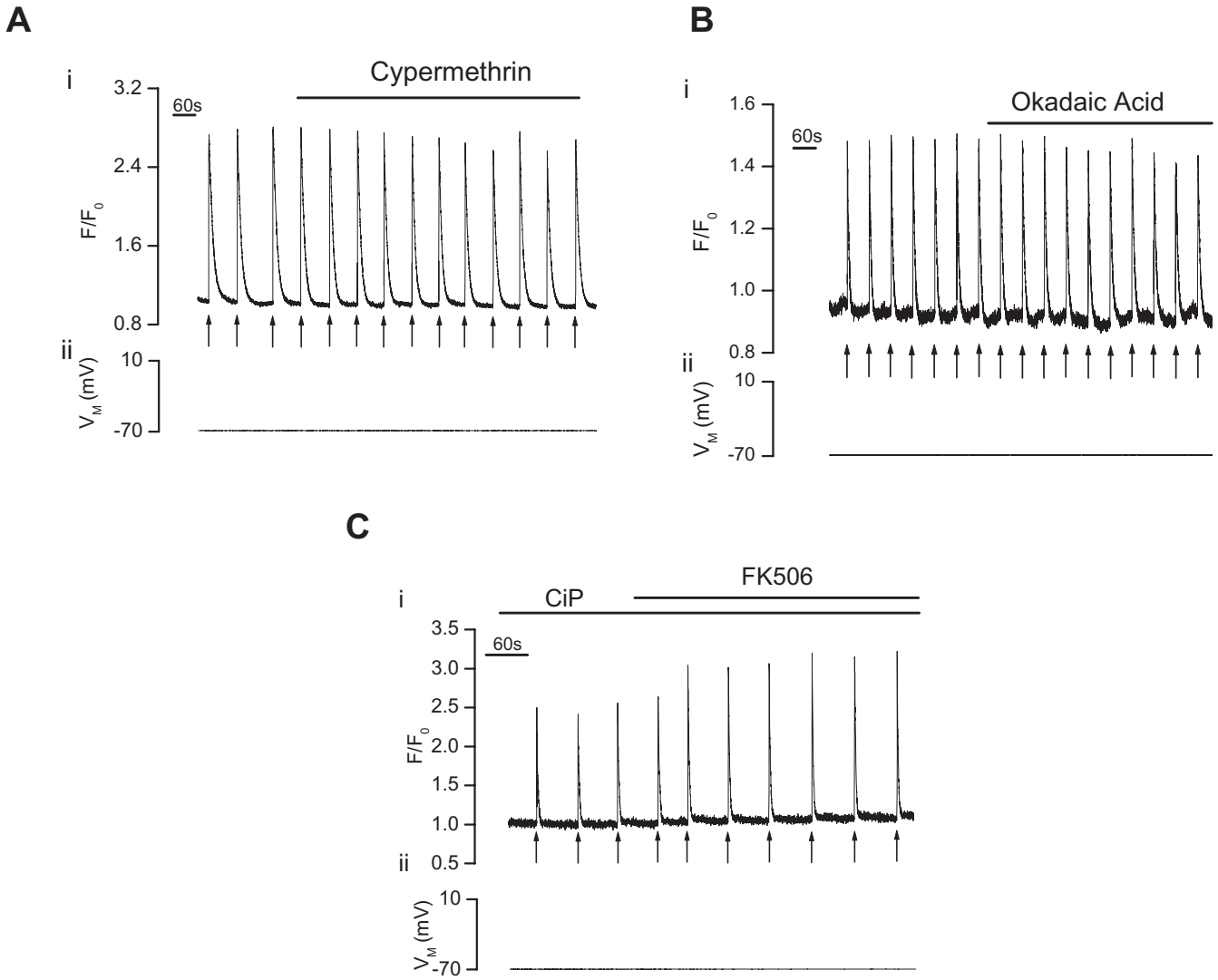

Figure 4 Effect of calcineurin inhibition on IP_3-evoked Ca^{2+} increases in voltage-clamped single portal vein myocytes. Photolysed caged IP_3 (↑) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. Neither cypermethrin (10 μM, $n = 5$, A) nor okadaic acid (5 μM, $n = 6$, B) significantly ($P > 0.05$) altered the IP_3-evoked $[Ca^{2+}]_{cyto}$ transients (V_M −70 mV, ii). Following pretreatment with the calcineurin inhibitor CiP (100 μM, C), via the patch pipette filling solution, FK506 (10 μM) still significantly ($P < 0.05$) increased the IP_3-evoked $[Ca^{2+}]_{cyto}$ transients produced by photolysed caged IP_3 (V_M −70 mV, ii, $n = 6$). $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; CiP, calcineurin inhibitory peptide; F, fluorescence counts; F_0, baseline fluorescence counts; IP_3, inositol 1,4,5-trisphosphate.

mTOR (Sabatini *et al.*, 1994; Brown *et al.*, 1995). The rapamycin-induced reduction in IP_3-mediated Ca^{2+} release in the present study may be mediated by inhibition of mTOR. In support, the inhibitor LY294002 which inhibits PI-3 kinase and mTOR (Brunn *et al.*, 1996), but does not remove FKBP from the channel, reduced the IP_3-mediated Ca^{2+} release. As LY294002 mimicked the effects of rapamycin, inhibition of IP_3-mediated Ca^{2+} release by rapamycin may be mediated by mTOR. Thus, in addition to regulating vascular smooth muscle cell differentiation, proliferation and migration our study suggests mTOR also regulates Ca^{2+} release in vascular (portal vein) smooth muscle.

In addition to mTOR regulation of IP_3-mediated Ca^{2+} release, the present study has also shown that FKBP may modulate IP_3R directly in portal vein myocytes. This conclusion is derived from the finding that FK506 but not calcineurin inhibitors increased IP_3-mediated Ca^{2+} release. In our previous study in colonic smooth muscle, FKBP had little direct effect on IP_3-mediated Ca^{2+} release but indirectly modulated Ca^{2+} release through mTOR, to increase, and calcineurin, to inhibit, Ca^{2+} release (MacMillan *et al.*, 2005b). Apart from the differences in the tissues used in the two studies, experimental conditions were otherwise identical. FKBPs, calcineurin and mTOR may each regulate RyR and IP_3R differently in various tissues and calcineurin appears to regulate neither IP_3R or RyR in portal vein. Similar findings were obtained in other vascular tissues. Calcineurin did not alter $[Ca^{2+}]_{cyto}$ in coronary (Ashizawa *et al.*, 1989; Hirano *et al.*, 1989) and pulmonary (Zheng *et al.*, 2004) arteries nor SR $[Ca^{2+}]$ in aorta myocytes (Avdonin *et al.*, 1999). Yet, the differences in IP_3R regulation by FKBP and calcineurin between colonic and portal vein myocytes, are not explained simply

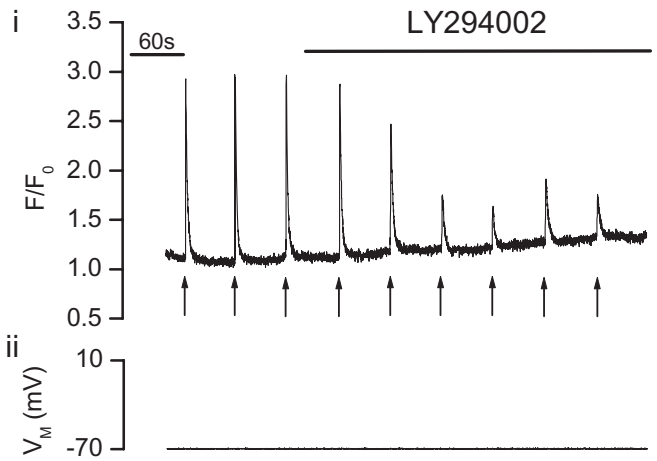

Figure 5 The mTOR inhibitor LY294002 decreased IP_3-evoked Ca^{2+} increases in voltage clamped single portal vein myocytes. Photolysed caged IP_3 (↑) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. Addition of LY294002 (20 μM, $n = 4$, $P < 0.05$) decreased the IP_3-evoked $[Ca^{2+}]_{cyto}$ transients (i) (V_M −70 mV, ii). $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, baseline fluorescence counts; IP_3, inositol 1,4,5-trisphosphate; mTOR, mammalian target of rapamycin.

by peculiarities of vascular and gastrointestinal smooth muscle. We have investigated RyR and IP_3R regulation in two types of vascular smooth muscle and, again, interesting differences exist. While FKBP modulated RyR and IP_3R in portal vein, it neither modulated Ca^{2+} release or even associated with either RyR or IP_3R in aorta (MacMillan *et al.*, 2005b; 2008). Clearly, substantial tissue to tissue variation exists in the regulation of RyR and IP_3R by FKBPs and the effects of compounds such as FK506 and rapamycin.

In summary, the present study, in portal vein myocytes, suggest RyR-mediated Ca^{2+} release is modulated directly by FKBP as a result of the binding of the accessory protein to the channel. IP_3-mediated Ca^{2+} release may be modulated by both FKBP binding to IP_3R and also indirectly via the kinase mTOR. FK506 increased RyR and IP_3R channel activity whereas rapamycin decreased IP_3R activity. The opposing effects of FK506 and rapamycin on IP_3-mediated Ca^{2+} release suggest different roles for FKBPs and mTOR in IP_3R-mediated Ca^{2+} release in portal vein myocytes; FKBPs decrease RyR and IP_3R activity whereas mTOR increases IP_3R activity.

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Conflicts of interest

None.

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