BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 1295–1301

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

The phospholipase C inhibitor U-73122 inhibits Ca^{2+} release from the intracellular sarcoplasmic reticulum Ca^{2+} store by inhibiting Ca^{2+} pumps in smooth muscle

D MacMillan and JG McCarron

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

Background and purpose: The sarcoplasmic reticulum (SR) releases Ca^{2+} via inositol 1,4,5-trisphosphate receptors (IP_3R) in response to IP_3-generating agonists. Ca^{2+} release subsequently propagates as Ca^{2+} waves. To clarify the role of IP_3 production in wave generation, the contribution of a key enzyme in the production of IP_3 was examined using a phosphoinositide-specific phospholipase C (PI-PLC) inhibitor, U-73122.
Experimental approach: Single colonic myocytes were voltage-clamped in whole-cell configuration and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) measured using fluo-3. SR Ca^{2+} release was evoked either by activation of IP_3Rs (by carbachol or photolysis of caged IP_3) or ryanodine receptors (RyRs; by caffeine).
Key results: U-73122 inhibited carbachol-evoked $[Ca^{2+}]_{cyto}$ transients. The drug also inhibited $[Ca^{2+}]_{cyto}$ increases, evoked by direct IP_3R activation (by photolysis of caged IP_3) and RyR activation (by caffeine), which do not require PI-PLC activation. U-73122 also increased steady-state $[Ca^{2+}]_{cyto}$ and slowed the rate of Ca^{2+} removal from the cytoplasm. An inactive analogue of U-73122, U-73343, was without effect on either IP_3R- or RyR-mediated Ca^{2+} release.
Conclusions and implications: U-73122 inhibited carbachol-evoked $[Ca^{2+}]_{cyto}$ increases. However, the drug also reduced Ca^{2+} release when evoked by direct activation of IP_3R or RyR, slowed Ca^{2+} removal and increased steady-state $[Ca^{2+}]_{cyto}$. These results suggest U-73122 reduces IP_3-evoked Ca^{2+} transients by inhibiting the SR Ca^{2+} pump to deplete the SR of Ca^{2+} rather than by inhibiting PI-PLC.
British Journal of Pharmacology (2010) **160,** 1295–1301; doi:10.1111/j.1476-5381.2010.00771.x
This article is commented on by Hollywood *et al.*, pp. 1293–1294 of this issue. To view this commentary visit http://dx.doi.org/10.1111/j.1476-5381.2010.00795.x

Keywords: U-73122; phospholipase C; smooth muscle; calcium

Abbreviations: $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; CCh, carbachol; CPA, cyclopiazonic acid; *F*, fluorescence counts; F_0, steady-state fluorescence counts; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphoinositide-specific phospholipase C; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

Introduction

The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$) is critically regulated by the intracellular store (the sarcoplasmic reticulum, SR), which controls Ca^{2+} release (Bootman *et al.*, 2001; McCarron *et al.*, 2006). Two major routes of Ca^{2+} release from the SR exist. The first is the inositol 1,4,5-trisphosphate receptor (IP_3R), the other, the ryanodine receptor (RyR; nomenclature follows Alexander *et al.*, 2009). In several cell types such as smooth muscle and non-excitable cells (e.g. epithelial cells and fibroblasts) IP_3R is the predominant Ca^{2+} release mechanism. IP_3Rs are activated by IP_3 generated via G-protein- or tyrosine kinase-linked receptor-dependent activation of phosphoinositide-specific phospholipase C (PI-PLC) (Bootman *et al.*, 2001). Activation of PI-PLC catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol and IP_3. IP_3 subsequently releases Ca^{2+} from the SR to generate a local Ca^{2+} rise, which may itself induce further synthesis of IP_3 via a Ca^{2+}-dependent positive feedback on PI-PLC (Bartlett *et al.*, 2005). Central therefore to

Correspondence: D MacMillan, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, The John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR, UK. E-mail: d.macmillan@strath.ac.uk
Received 6 November 2009; revised 11 December 2009; accepted 15 January 2010

an understanding of the role of IP_3Rs in Ca^{2+} regulation and wave propagation is an appreciation of the control of the PI-PLC/IP_3 signalling pathway.

Investigation of the PI-PLC pathway in agonist-evoked Ca^{2+} signals has been aided by the development of specific, membrane-permeant, activators and inhibitors. Among several, U-73122 has been established as a selective pharmacological inhibitor of PI-PLC (Bleasdale *et al*., 1990; Smith *et al*., 1990) and is used widely. U-73122 inhibited PI hydrolysis and IP_3 synthesis in broken cell systems and reduced agonist-evoked $[Ca^{2+}]_{cyto}$ rises in intact cells, such as neutrophils (Bleasdale *et al*., 1990; Smith *et al*., 1990), neuroblastoma cells (Thompson *et al*., 1991), acinar cells (Yule and Williams, 1992) and platelets (Bleasdale *et al*., 1990). U-73122 has thus gained general acceptance as a specific PI-PLC inhibitor and inhibition of $[Ca^{2+}]_{cyto}$ rises in intact cells by U-73122 has been interpreted as evidence for a contribution of PI-PLC in the response in a number of studies (Yule and Williams, 1992; Hansen *et al*., 1995; Heemskerk *et al*., 1997), including smooth muscle (Zizzo *et al*., 2008; Frei *et al*., 2009).

In the present study, which examined the role of PI-PLC in IP_3-mediated Ca^{2+} regulation in smooth muscle, U-73122 potently inhibited IP_3-mediated Ca^{2+} release independently of PI-PLC activity.

Methods

Colonic myocyte isolation

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. The colon was immediately removed 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 smooth muscle cells were enzymically isolated (McCarron and Muir, 1999), 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*., 2005). 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 1 M). The Ca^{2+}-free bathing solution additionally contained (mM): $MgCl_2$, 3 (substituted for Ca^{2+}) and EGTA, 1. The pipette solution contained (mM): Cs_2SO4 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 IP_3 trisodium salt 0.025 (pH 7.2 adjusted with CsOH 1 M). Whole-cell currents were amplified by an Axopatch 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 $[Ca^{2+}]_{cyto}$

Cytoplasmic Ca^{2+} concentration was measured as fluorescence using the membrane-impermeable dye fluo-3 (penta-ammonium salt) introduced into the cell via the patch pipette (Bradley *et al*., 2004; MacMillan *et al*., 2008). Fluorescence was quantified using a microfluorimeter that consisted of an inverted microscope (Nikon diaphot, Nikon UK Ltd., Surrey, UK) 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 steady-state (control) values (taken as 1) before stimulation (F_0).

Statistical analysis

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

Materials

Caged IP_3 trisodium salt was purchased from Invitrogen (Paisley, UK). Fluo-3 penta-ammonium salt was purchased from TEF labs (Austin, TX, USA). Cyclopiazonic acid (CPA), U-73122 and its inactive analogue, U-73343, were each purchased from Calbiochem-Novabiochem (Beeston, Nottingham, UK). 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. Carbachol (CCh) (100 μM) and caffeine (10 mM) were each 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. CCh and caffeine were each dissolved in extracellular bathing solution whereas U-73122 and U-73343 were each dissolved in dimethylsulphoxide (final bath concentration of the solvent, 0.05%, was by itself ineffective). U-73122 and U-73343 were each perfused into the solution bathing the cells (~5 mL per min).

Results

Phosphoinositide-specific phospholipase C is a key enzyme in the regulation of IP_3-mediated Ca^{2+} release from the SR. To clarify the role of PI-PLC in IP_3R-mediated Ca^{2+} regulation, the effect of the PI-PLC inhibitor, U-73122, on the IP_3-generating agonist CCh, was examined in voltage-clamped, single colonic myocytes. In these experiments, CCh was applied in a Ca^{2+}-free bath solution to ensure that $[Ca^{2+}]_{cyto}$ rises derived from Ca^{2+} release from the SR. The IP_3-generating muscarinic agonist CCh (100 μM) reproducibly increased $[Ca^{2+}]_{cyto}$ (Figure 1). U-73122 (10 μM; Figure 1) significantly ($P < 0.05$) inhibited CCh-evoked $[Ca^{2+}]_{cyto}$ increases ($\Delta F/F_0$) by 90 ± 3% [from 2.24 ± 0.4 to 0.27 ± 0.1 ($n = 5$)], a result that appears consistent with a requirement for PI-PLC generation of IP_3.

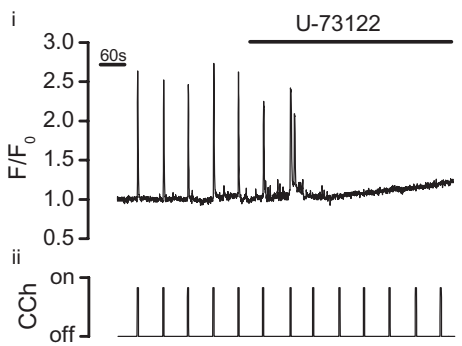

Figure 1 The PI-PLC inhibitor U-73122 decreased carbachol-evoked $[Ca^{2+}]_{cyto}$ increases in voltage-clamped single colonic myocytes. At −70 mV, carbachol (100 μM in the puffer pipette, CCh, ii) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. U-73122 (10 μM, $n = 5$, $P < 0.05$) decreased the carbachol-evoked $[Ca^{2+}]_{cyto}$ transients (i). Carbachol was applied by hydrostatic pressure ejection (see *Methods*) at 1 min intervals (ii). $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, steady-state fluorescence counts; PI-PLC, phosphoinositide-specific phospholipase C.

Unexpectedly, in subsequent experiments, U-73122 appeared to inhibit IP_3-mediated Ca^{2+} release independently of PI-PLC. In these experiments the signal transduction pathway that mediates IP_3 synthesis, that is, PI-PLC, was bypassed by photolysing caged IP_3. U-73122 (1 μM, Figure 2A) significantly ($P < 0.05$) decreased the IP_3-evoked $[Ca^{2+}]_{cyto}$ transient ($\Delta F/F_0$) by 76 ± 6% from 2.78 ± 0.5 to 0.59 ± 0.1 ($n = 5$). U-73122 (10 μM, Figure 2B) significantly ($P < 0.05$) decreased the IP_3-evoked $[Ca^{2+}]_{cyto}$ transient ($\Delta F/F_0$) by 91 ± 2% from 2.44 ± 0.4 to 0.24 ± 0.1 ($n = 5$). Interestingly, the inactive analogue, U-73343 (10 μM) (Bleasdale *et al.*, 1990; Smith *et al.*, 1990) used often as a control compound for U-73122, did not significantly ($P > 0.05$) alter IP_3-mediated $[Ca^{2+}]_{cyto}$ increases evoked by photolysis of caged IP_3. The $[Ca^{2+}]_{cyto}$ increase remained at 105 ± 9% of the control response [$\Delta F/F_0$ from 1.68 ± 0.2 to 1.71 ± 0.2 (Figure 3, $n = 5$)]. As the $[Ca^{2+}]_{cyto}$ increase evoked by photolysis of caged IP_3 does not require IP_3 synthesis, these results suggest that U-73122-evoked inhibition of Ca $^{2+}$ release was not mediated by inhibition of PI-PLC activity.

The inhibition of IP_3-mediated Ca^{2+} release by U-73122 may arise from either a direct effect of the drug on IP_3-mediated Ca^{2+} release or indirectly via a reduction in the store's Ca^{2+} content. To distinguish between these possibilities, the effect of U-73122 on RyR-mediated Ca^{2+} release was examined. If the reduction in IP_3R-mediated Ca^{2+} release arose by depletion of

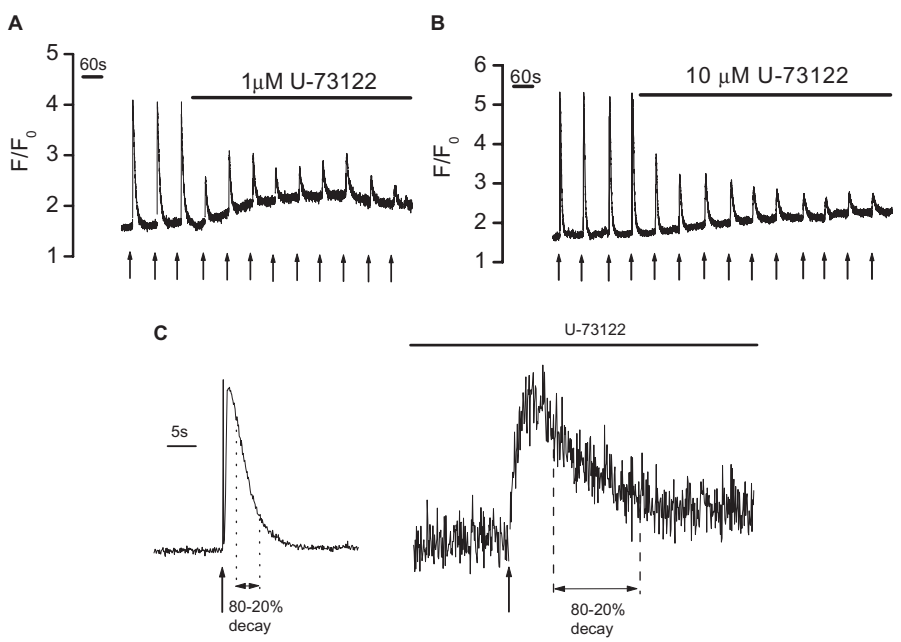

Figure 2 The PI-PLC inhibitor U-73122 decreased $[Ca^{2+}]_{cyto}$ increases produced by photolysed caged IP_3 in voltage-clamped single colonic myocytes. At −70 mV, photolysed caged IP_3 (↑) increased $[Ca^{2+}]_{cyto}$ as indicated by F/F_0. U-73122 [1 μM (A) and 10 μM (B), $n = 5$ and 5, $P < 0.05$] decreased the IP_3-evoked $[Ca^{2+}]_{cyto}$ transients. Representative examples of IP_3-evoked Ca^{2+} transients (from B) show the rate of Ca^{2+} decline before (control) and after U-73122 (C). Ca^{2+} transients in U-73122 have been scaled in amplitude to match the control response. The 80–20% decay interval is shown in each transient. $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, steady-state fluorescence counts; IP_3, inositol 1,4,5-trisphosphate; PI-PLC, phosphoinositide-specific phospholipase C.

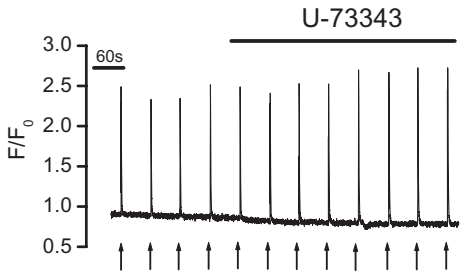

Figure 3 The 'inactive' U-73122 analogue, U-73343, did not significantly ($P > 0.05$) alter IP_3-evoked $[Ca^{2+}]_{cyto}$ increases in voltage-clamped single colonic myocytes. At −70 mV, photolysed caged IP_3 (↑) increased $[Ca^{2+}]_{cyto}$ as indicated by F/F_0. U-73343 (10 μM, $n = 5$, $P > 0.05$) did not inhibit the IP_3-evoked $[Ca^{2+}]_{cyto}$ transients. $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, steady-state fluorescence counts; IP_3, inositol 1,4,5-trisphosphate.

the store of Ca^{2+} then U-73122 should also inhibit RyR-mediated Ca^{2+} release because both IP_3R and RyR access a single common Ca^{2+} store in this smooth muscle preparation (McCarron and Olson, 2008). U-73122 (10 μM, Figure 4A) significantly ($P < 0.05$) decreased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transient $\Delta F/F_0$ by 73 ± 11% from 1.69 ± 0.3 to 0.37 ± 0.1 ($n = 5$). Thus, U-73122 appears to have a general inhibitory effect on Ca^{2+} release from the intracellular SR Ca^{2+} store. Again the inactive analogue, U-73343 (10 μM), was without effect on caffeine-evoked Ca^{2+} release [($\Delta F/F_0$) from 2.05 ± 0.2 to 1.96 ± 0.2 (Figure 4B, $n = 6$, $P > 0.05$)]. These results suggest that the decrease in IP_3R- and RyR-mediated Ca^{2+} release is likely to be explained by a reduction in the store's Ca^{2+} content perhaps by U-73122's inhibition of the SR Ca^{2+} pump and that the control compound, U-73343, is without effect on the pump.

In support of the proposal that U-73122 inhibited the SR Ca^{2+} pump, an increase in steady-state $[Ca^{2+}]_{cyto}$ (measured as fluorescence) was observed following either 1 μM U-73122 [(F/F_0) from 1.26 ± 0.1 to 1.57 ± 0.2 (Figure 2A, $n = 5$, $P < 0.05$)] or 10 μM U-73122 [(F/F_0) from 1.04 ± 0.1 to 1.37 ± 0.1 (Figure 2B, $n = 10$, $P < 0.05$)]. Inhibition of the SR Ca^{2+} pump is known to increase steady-state $[Ca^{2+}]_{cyto}$ (Bradley *et al.*, 2002). The SR Ca^{2+} pump inhibitor CPA was without effect on steady-state $[Ca^{2+}]_{cyto}$ following U-73122. In these experiments U-73122 elevated steady-state $[Ca^{2+}]_{cyto}$ and inhibited caffeine-evoked Ca^{2+} release (Figure 5A) after which CPA (10 μM) failed to further increase steady-state $[Ca^{2+}]_{cyto}$ [(F/F_0) from 1.19 ± 0.03 (U-73122) to 1.2 ± 0.04 (CPA, $n = 4$, $P > 0.05$)]. Similarly, CPA (10 μM) decreased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients and increased steady-state $[Ca^{2+}]_{cyto}$ (Figure 5B) after which U-73122 evoked no further increase in steady-state $[Ca^{2+}]_{cyto}$ [(F/F_0) from 1.21 ± 0.1 (CPA) to 1.19 ± 0.1 (U-73122, $n = 3$, $P > 0.05$)]. These results suggest that U-73122 and CPA each alter Ca^{2+} signals via a common mechanism.

Further support for a U-73122-evoked inhibition of the SR Ca^{2+} pump was found in the observation that the drug significantly slowed the rate of Ca^{2+} removal from the cytoplasm following each of IP_3- or caffeine-evoked Ca^{2+} release (Figures 2C and 4C). The 80–20% decay interval following IP_3- and caffeine-evoked Ca^{2+} release was 3.5 ± 0.7 s and 2.9 ± 0.3 s in controls and 5.9 ± 0.9 s and 4.7 ± 0.4 s in U-73122 ($n = 10$ and 5, $P < 0.05$) respectively. Taken together these data imply that U-73122 reduced the SR Ca^{2+} store content by inhibiting SR Ca^{2+} pump activity, in addition to inhibition of PI-PLC.

Discussion and conclusions

In the present study, our initial experiments found that U-73122 inhibited CCh-evoked $[Ca^{2+}]_{cyto}$ increases, a result that appeared consistent with the proposed mechanism of action of U-73122, that is, inhibiting PI-PLC activity (Bleasdale *et al.*, 1990; Thompson *et al.*, 1991). However, in subsequent experiments, U-73122 also inhibited Ca^{2+} release evoked by photolysed caged IP_3 and hydrostatically applied caffeine. Inhibition of PI-PLC activity is unlikely to account for the inhibition of SR Ca^{2+} release evoked by either photolysed caged IP_3 or caffeine as the former mechanism does not use the signal transduction pathway that mediates IP_3 synthesis and the latter's mechanism of action is independent of PI hydrolysis and IP_3 synthesis. A direct selective inhibitory effect of U-73122 on IP_3R is also unlikely as RyR-mediated Ca^{2+} release was also reduced by U-73122. The decrease in IP_3R- and RyR-mediated Ca^{2+} release can be explained by a reduction in the store's Ca^{2+} content by U-73122's inhibition of the SR Ca^{2+} pump; IP_3R and RyR access a common Ca^{2+} store in this smooth muscle type (McCarron and Olson, 2008). Support for this proposal is found in the observation that the SR Ca^{2+} pump inhibitor, CPA, failed to increase steady-state $[Ca^{2+}]_{cyto}$ after U-73122. U-73122 also failed to increase steady-state $[Ca^{2+}]_{cyto}$ after CPA. These experiments suggest that CPA and U-73122 have a common mechanism of action, that is, SR Ca^{2+} pump inhibition. U-73122 also slowed the rate of Ca^{2+} removal from the cytoplasm, an effect predictable from inhibition of the SR Ca^{2+} pump. Together the results are consistent with U-73122 inhibiting the SR Ca^{2+} pump to reduce IP_3R- and RyR-mediated Ca^{2+} release.

U-73122 is reported to specifically inhibit PI-PLC activity (Bleasdale *et al.*, 1990; Smith *et al.*, 1990). However, a discrepancy between the concentration of U-73122 required to inhibit PI-PLC and those inhibiting Ca^{2+} release was also observed in these initial reports suggesting that U-73122 specifically inhibited PI-PLC activity (Bleasdale *et al.*, 1990; Smith *et al.*, 1990). Importantly, higher concentrations of U-73122 are required to suppress enzyme activity than to inhibit agonist-induced Ca^{2+} release. For example, U-73122 inhibited IP_3-mediated Ca^{2+} release at concentrations of 1–2 μM, while the production of IP_3, used as an indicator of PI-PLC activity, may only be significantly reduced at higher concentrations, for example 10 μM (Alter *et al.*, 1994; Hellberg *et al.*, 1996; Pulcinelli *et al.*, 1998). In platelets, the activity of PI-PLC was resistant to U-73122 up to 50 μM despite the drug inhibiting agonist-induced Ca^{2+} increases at much lower concentrations, for example 1 μM (Muto *et al.*, 1997; Pulcinelli *et al.*, 1998). The reported IC_{50} value of U-73122 for PI-PLC inhibition was 40 μM whereas that for inhibiting the Ca^{2+} transient was 1 μM (Bleasdale *et al.*, 1990). The results in the present study in smooth muscle show that U-73122 inhibited SR Ca^{2+} release at a concentration (1 μM) up to 50-fold lower than concentrations reported necessary to inhibit PI-PLC activity in other cell types (Smith *et al.*, 1990; Muto *et al.*, 1997; Pulcinelli *et al.*, 1998; Hou *et al.*, 2004).

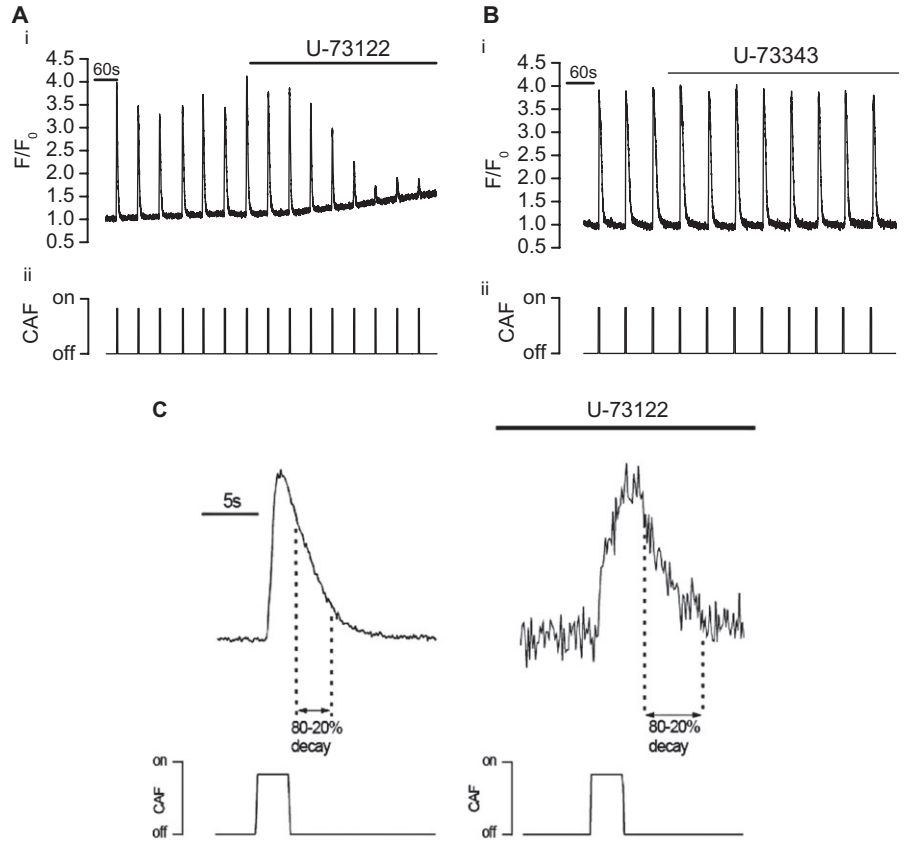

Figure 4 The effect of the PI-PLC inhibitor U-73122 and its inactive analogue, U-73343, on the $[Ca^{2+}]_{cyto}$ rise evoked by caffeine in voltage-clamped single colonic myocytes. (A) At −70 mV, caffeine (CAF, 10 mM, ii) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. U-73122 (10 μM, $n = 5$, $P < 0.05$) decreased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients (i). (B) The inactive U-73122 analogue, U-73343, did not significantly ($P > 0.05$) alter caffeine-evoked $[Ca^{2+}]_{cyto}$ increases. At −70 mV, caffeine (CAF, 10 mM, ii) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. U-73343 (10 μM, $n = 6$, $P > 0.05$) did not alter the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients (i). Representative examples of caffeine-evoked Ca^{2+} transients (from B) show the rate of Ca^{2+} decline before (control) and after U-73122 (C). Ca^{2+} transients in U-73122 have been scaled in amplitude to match the control response. The 80–20% decay interval is shown in each transient. $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; F, fluorescence counts; F_0, steady-state fluorescence counts; PI-PLC, phosphoinositide-specific phospholipase C.

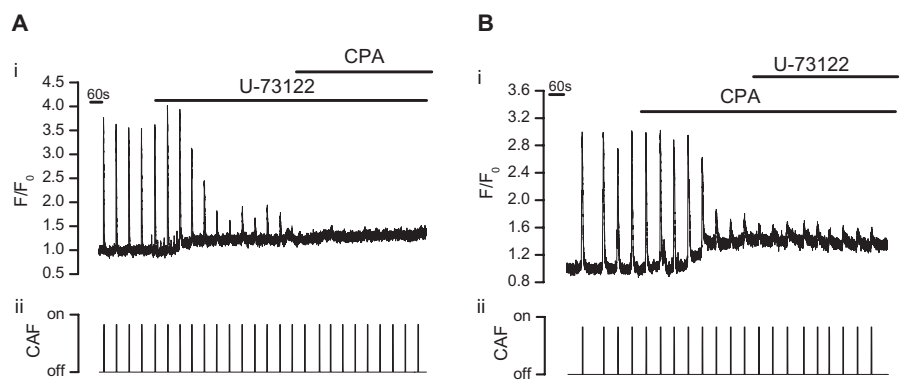

Figure 5 The effect of the SR Ca^{2+} pump inhibitor CPA on caffeine and steady-state $[Ca^{2+}]_{cyto}$ increases before and after U-73122 in single colonic myocytes. Caffeine (CAF, 10 mM, ii) increased $[Ca^{2+}]_{cyto}$ (i) as indicated by F/F_0. U-73122 (10 μM) decreased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients and increased steady-state $[Ca^{2+}]_{cyto}$ (Ai). CPA (10 μM) evoked no further increase in steady-state $[Ca^{2+}]_{cyto}$ [(F/F_0) from 1.19 ± 0.03 (U-73122) to 1.2 ± 0.04 (CPA, $n = 4$, $P > 0.05$)]. Similarly, CPA (10 μM) decreased the caffeine-evoked $[Ca^{2+}]_{cyto}$ transients and increased steady-state $[Ca^{2+}]_{cyto}$ (Bi). U-73122 (10 μM) evoked no further increase in steady-state $[Ca^{2+}]_{cyto}$ [(F/F_0) from 1.21 ± 0.1 (CPA) to 1.19 ± 0.1 (U-73122, $n = 3$, $P > 0.05$))]. $[Ca^{2+}]_{cyto}$, cytoplasmic Ca^{2+} concentration; CPA, cyclopiazonic acid; F, fluorescence counts; F_0, steady-state fluorescence counts; SR, sarcoplasmic reticulum.

The present findings in colonic smooth muscle are in agreement with other observations that suggest U-73122 may inhibit SR Ca^{2+} release independently of PI-PLC inhibition (Berven and Barritt, 1995; Grierson and Meldolesi, 1995; Hellberg *et al*., 1996; Pulcinelli *et al*., 1998). Inhibition of SR Ca^{2+} pump activity in liver microsomes and hepatocytes (De Moel *et al*., 1995) and emptying of the Ca^{2+} stores in PC12 cells by U-73122 have been reported (Clementi *et al*., 1992; Grierson and Meldolesi, 1995). U-73122 has also been shown to increase steady-state $[Ca^{2+}]_{cyto}$ (Smallridge *et al*., 1992) and to slow the rate of recovery of Ca^{2+} to resting levels following release (Grierson and Meldolesi, 1995) in other preparations, although the latter effect was attributed to PI-PLC inhibition (Grierson and Meldolesi, 1995). U-73122 has also been shown to inhibit Ca^{2+} release from RyR in the form of Ca^{2+} sparks and via IP_3R as Ca^{2+} puffs (Bayguinov *et al*., 2000; Liu *et al*., 2007).

The present results are consistent with inhibition of the SR Ca^{2+} pump providing a mechanism by which U-73122 inhibits IP_3-mediated Ca^{2+} release. In some experiments inhibition of IP_3-mediated Ca^{2+} release occurred before an increase in steady-state $[Ca^{2+}]_{cyto}$ was decreased. The latter is a common feature of SR Ca^{2+} pump inhibition. A slow Ca^{2+} leak from the SR by Ca^{2+} pump inhibition may have been initially compensated by Ca^{2+} extrusion from the cytoplasm via the plasma membrane Ca^{2+} pumps so that little change in resting $[Ca^{2+}]_{cyto}$ occurs although the SR Ca^{2+} content has begun to decline (see Figure 2). The reduced SR Ca^{2+} content was apparent when the cell was activated by, for example, IP_3 and so an inhibition of IP_3-mediated Ca^{2+} release occurred prior to an increase in steady-state $[Ca^{2+}]_{cyto}$. As Ca^{2+} is repeatedly released from the store but not subsequently returned via the SR Ca^{2+} pumps (because of inhibition by U-73122), increased steady-state $[Ca^{2+}]_{cyto}$ levels result. Although a direct inhibitory effect of U-73122 on either IP_3R or RyR cannot be fully excluded, reduced IP_3R- and RyR-mediated Ca^{2+} release, an increase in steady-state $[Ca^{2+}]_{cyto}$ and a slowed rate of Ca^{2+} removal are each consistent with SR Ca^{2+} pump inhibition.

In conclusion, U-73122 inhibits SR Ca^{2+} release independently of PI-PLC and via inhibition of the SR Ca^{2+} pump. This additional effect of U-73122 on Ca^{2+} signalling may complicate interpretation of results when the compound is used to define PI-PLC function.

Acknowledgements

This work was funded by the Wellcome Trust (078054/2/05/Z) and the British Heart Foundation (PG/08/066), the support of which is gratefully acknowledged.

Statement of conflicts of interest

None.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC), 4th edn. *Br J Pharmacol* **158**: S1–S254.

Alter CA, Amagasu M, Shah K, Jolly YC, Major C, Wolf BA (1994). U-73122 does not specifically inhibit phospholipase C in rat pancreatic islets and insulin-secreting beta-cell lines. *Life Sci* **54**: PL107–PL112.

Bartlett PJ, Young KW, Nahorski SR, Challiss RA (2005). Single cell analysis and temporal profiling of agonist-mediated inositol 1,4,5-trisphosphate, Ca^{2+}, diacylglycerol, and protein kinase C signaling using fluorescent biosensors. *J Biol Chem* **280**: 21837–21846.

Bayguinov O, Hagen B, Bonev AD, Nelson MT, Sanders KM (2000). Intracellular calcium events activated by ATP in murine colonic myocytes. *Am J Physiol Cell Physiol* **279**: C126–C135.

Berven LA, Barritt GJ (1995). Evidence obtained using single hepatocytes for inhibition by the phospholipase C inhibitor U73122 of store-operated Ca^{2+} inflow. *Biochem Pharmacol* **49**: 1373–1379.

Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ *et al*. (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharmacol Exp Ther* **255**: 756–768.

Bootman MD, Collins TJ, Peppiatt CM, Prothero LS, MacKenzie L, De Smet P *et al*. (2001). Calcium signalling – an overview. *Semin Cell Dev Biol* **12**: 3–10.

Bradley KN, Flynn ER, Muir TC, McCarron JG (2002). Ca^{2+} regulation in guinea-pig colonic smooth muscle: the role of the Na^{+}-Ca^{2+} exchanger and the sarcoplasmic reticulum. *J Physiol* **538**: 465–482.

Bradley KN, Craig JW, Muir TC, McCarron JG (2004). The sarcoplasmic reticulum and sarcolemma together form a passive Ca^{2+} trap in colonic smooth muscle. *Cell Calcium* **36**: 29–41.

Clementi E, Racchetti G, Zacchetti D, Panzeri MC, Meldolesi J (1992). Differential expression of markers and activities in a group of PC12 nerve cell clones. *Eur J Neurosci* **4**: 944–953.

De Moel MP, Van de Put FH, Vermegen TM, De Pont JH, Willems PH (1995). Effect of the aminosteroid, U73122, on Ca^{2+} uptake and release properties of rat liver microsomes. *Eur J Biochem* **234**: 626–631.

Frei E, Hofmann F, Wegener JW (2009). Phospholipase C mediated Ca^{2+} signals in murine urinary bladder smooth muscle. *Eur J Pharmacol* **610**: 106–109.

Grierson JP, Meldolesi J (1995). Calcium homeostasis in mouse fibroblast cells: affected by U-73122, a putative phospholipase C beta blocker, via multiple mechanisms. *Br J Pharmacol* **115**: 11–14.

Hansen M, Boitano S, Dirksen ER, Sanderson MJ (1995). A role for phospholipase C activity but not ryanodine receptors in the initiation and propagation of intercellular calcium waves. *J Cell Sci* **108**: 2583–2590.

Heemskerk JW, Farndale RW, Sage SO (1997). Effects of U73122 and U73343 on human platelet calcium signalling and protein tyrosine phosphorylation. *Biochim Biophys Acta* **1355**: 81–88.

Hellberg C, Molony L, Zheng L, Andersson T (1996). Ca^{2+} signalling mechanisms of the beta 2 integrin on neutrophils: involvement of phospholipase C gamma 2 and $Ins(1,4,5)P_3$. *Biochem J* **317**: 403–409.

Hou C, Kirchner T, Singer M, Matheis M, Argentieri D, Cavender D (2004). *In vivo* activity of a phospholipase C inhibitor, 1-(6-((17beta-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122), in acute and chronic inflammatory reactions. *J Pharmacol Exp Ther* **309**: 697–704.

Liu QH, Zheng YM, Wang YX (2007). Two distinct signaling pathways for regulation of spontaneous local Ca^{2+} release by phospholipase C in airway smooth muscle cells. *Pflugers Arch* **453**: 531–541.

McCarron JG, Muir TC (1999). Mitochondrial regulation of the cytosolic Ca^{2+} concentration and the $InsP_3$-sensitive Ca^{2+} store in guinea-pig colonic smooth muscle. *J Physiol* **516**: 149–161.

McCarron JG, Olson ML (2008). A single luminally continuous sarcoplasmic reticulum with apparently separate Ca^{2+} stores in smooth muscle. *J Biol Chem* **283**: 7206–7218.

McCarron JG, Chalmers S, Bradley KN, MacMillan D, Muir TC (2006). Ca^{2+} microdomains in smooth muscle. *Cell Calcium* **40**: 461–493.

MacMillan D, Currie S, Bradley KN, Muir TC, McCarron JG (2005). In smooth muscle, FK506-binding protein modulates IP_3 receptor-evoked Ca^{2+} release by mTOR and calcineurin. *J Cell Sci* **118**: 5443–5451.

MacMillan D, Currie S, McCarron JG (2008). FK506-binding protein (FKBP12) regulates ryanodine receptor-evoked Ca^{2+} release in colonic but not aortic smooth muscle. *Cell Calcium* **43**: 539–549.

Muto Y, Nagao T, Urushidani T (1997). The putative phospholipase C inhibitor U73122 and its negative control, U73343, elicit unexpected effects on the rabbit parietal cell. *J Pharmacol Exp Ther* **282**: 1379–1388.

Pulcinelli FM, Gresele P, Bonuglia M, Gazzaniga PP (1998). Evidence for separate effects of U73122 on phospholipase C and calcium channels in human platelets. *Biochem Pharmacol* **56**: 1481–1484.

Smallridge RC, Kiang JG, Gist ID, Fein HG, Galloway RJ (1992). U-73122, an aminosteroid phospholipase C antagonist, noncompetitively inhibits thyrotropin-releasing hormone effects in GH3 rat pituitary cells. *Endocrinology* **131**: 1883–1888.

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, Bleasdale JE (1990). Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J Pharmacol Exp Ther* **253**: 688–697.

Thompson AK, Mostafapour SP, Denlinger LC, Bleasdale JE, Fisher SK (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for Gp in receptor compartmentation. *J Biol Chem* **266**: 23856–23862.

Yule DI, Williams JA (1992). U73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J Biol Chem* **267**: 13830–13835.

Zizzo MG, Mule F, Serio R (2008). Activation of P2Y receptors by ATP and by its analogue, ADPbetaS, triggers two calcium signal pathways in the longitudinal muscle of mouse distal colon. *Eur J Pharmacol* **595**: 84–89.