C99

The role of protein kinase C and Rho kinase in cGMP-mediated calcium desensitisation in rat intrapulmonary arteries

A. De Silva, P.I. Aaronson and J.P. Ward

Asthma Allergy and Respiratory Science, King's College London, London, UK

Calcium desensitisation is defined by the ability of a mediator to cause smooth muscle relaxation independent of a reduction in intracellular [Ca^{2+}]. One such mediator is cGMP and cGMP-dependent protein kinase G (PKG). Several protein kinases have been implicated in the above process, and the present study was aimed at investigating the role of protein kinase C and Rho Kinase in calcium desensitisation induced in rat arteries by 8-bromo-cGMP (8-Br-cGMP), a cGMP analogue. Small intrapulmonary arteries (IPA, internal diameter ~200-500 μm) were mounted on a wire myograph, and permeabilised with 60μg/ml alpha toxin in pCa 6.5. IPA were bathed in PIPES-buffered solution (pH 7.1), gassed with 100% air and incubated at 26°C. In all preparations 1μM thapsigargin was present to prevent involvement of intracellular Ca^{2+} stores. Ca^{2+} concentration was regulated by adjusting the ratio of K_2EGTA to CaEGTA. Values are given as mean ± SEM; tests for significance were performed on -log concentrations, using Student's unpaired t test. The relaxation induced by 8-Br-cGMP (10μM) was found to be Ca^{2+} dependent, being 60 ± 6% at pCa 6.7 (n=4) and 38 ± 12% at pCa 6.4 (n=5), with an IC_{50} for 8-Br-cGMP of ~25nM. At a pCa 6.4 the Rho kinase inhibitor Y-27632 (10μM) if anything increased relaxation to 8-Br-cGMP (45 ± 13%, n=6), whereas the PKC inhibitor Ro-31-8220 decreased it (18 ± 8%, n=5), though neither reached significance. At a pCa of 6.7, however, Ro-31-8220 caused a significant inhibition of relaxation to 10 μM 8-Br-cGMP (12 ± 13%, n = 7, p<0.01). Due to the degree of depression of tension at pCa 6.7 by Y-27632, its effect on 8-Br-cGMP could not be evaluated, so further experiments were performed on Ca^{2+} response curves. 8-Br-cGMP (100nM) caused a significant rightward shift of the Ca^{2+} response curve (EC_{50} Control: 157 ± 23 nM; 8-Br-cGMP: 252 ± 47 nM; p=<0.001; n=14). Y-27632 (10μM) alone caused a significant rightward shift of the Ca^{2+} response curve (204 ± 26nM, p<0.05, n=8), but in the presence of Y-27632, 8-Br-cGMP caused a further significant shift to the right (8-Br-cGMP+Y-27632: 306 ± 45nM, p<0.001 vs. Y-27632 alone, n=6). Ro-31-8220 alone also shifted the curve to the right (224 ± 5nM), but in the presence of Ro-31-8220 8-Br-cGMP had no further effect (8-Br-cGMP+Ro-31-8220: 285 ± 18nM (n=4). These results suggest a role for PKC but not Rho kinase in 8-Br-cGMP-induced Ca^{2+} desensitization in rat IPA.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C100

Effects of C-natriuretic peptide in resistance and conduit arteries of the rat

B. Teague[1], T.H. Lewis[2], T.P. Robertson[2], G. Knock[1], J.P. Ward[1] and P.I. Aaronson[1]

[1]Asthma, Allergy and Respiratory Science, King's College London, London, UK and [2]Department of Physiology and Pharmacology, University of Georgia, Athens, GA, USA

C-type natriuretic peptide (CNP) is an endothelium-derived factor which causes vascular smooth muscle relaxation. The mechanism by which CNP acts is controversial. Most reports indicate that CNP acts on NPR-B receptors to increase smooth muscle cyclic GMP levels. However, it has recently been proposed that CNP instead stimulates NPR-C receptors to activate inwardly rectifying K^+ channels, and that CNP represents a major endothelium dependent hyperpolarizing factor released by acetylcholine in rat mesenteric resistance arteries. We therefore examined the properties and mechanism of the response to CNP in rat mesenteric resistance arteries (RMA), superior mesenteric arteries (SMA) and isolated coronary arteries.

CNP (1-1000nM) failed to induce a relaxation in the RMA and coronary arteries but caused a substantial transient relaxation in the SMA (1μM: 77±3% relaxation, n=6). This relaxation was insensitive to the combination of TRAM-34 (1μM) and apamin (100nM) (63±5% relaxation, n=6) and pre-treatment with pertussis toxin (400ng/ml, 77±4% relaxation, n=6) but was antagonized by 25mM K^+ (-3±3% relaxation, n=6, P<0.001), and the combination of Ba^{2+} (10μM) and ouabain (100μM) (14±3% relaxation, n=6, P<0.01). Relaxation was also prevented by the BK_{Ca} channel inhibitor iberiotoxin (100nM, -10±3% relaxation, n=6, P<0.001), which however had no effect on acetylcholine-induced relaxations. The NPR-C agonist cANF (1μM) had no effect in either the RMA or SMA (0±1%, n=6), suggesting that CNP was acting through the NPR-B receptor in the SMA. Relaxation was insensitive to the protein kinase G inhibitor KT5823 (2μM, 78±3% relaxation, n=6) but was inhibited by milrinone (30μM, 19±5% relaxation, n=6, P<0.01), a drug which selectively inhibits cyclic GMP-dependent phosphodiesterase, (PDE3) and by H-89 (30μM, 2±2% relaxation, n=5, P<0.01), which inhibits protein kinase. Relaxation was also abolished by the phospholipase C antagonist U73122 (5μM, 11±1% relaxation, n=4, P<0.001) and by $[Ca^{2+}]_i$ store-releasing agent caffeine (0.5mM, 6±3% relaxation, n=6, P<0.001)). Relaxation of the SMA to 8-Br-cyclic GMP (200μM, 83±4% relaxation, n=6) was also inhibited by U73122 and Milrinone (5±5% relaxation, n=5, P<0.01), but not KT5823.

The results indicate that CNP relaxes the SMA via the NPR-B receptor, which would be expected to raise levels of cyclic GMP. We propose that then inhibits PDE3, leading to a rise in intracellular cyclic AMP levels and the accumulation of Ca^{2+} in the sarcoplasmic reticulum. This may result in an increase in vectorial Ca^{2+} release and the opening of BK_{Ca} channels, leading to membrane hyperpolarization and relaxation. The results also demonstrate that CNP is unlikely to be an EDHF in the resistance vasculature, at least in the rat.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

C101

2-Aminoethoxydiphenyl borate augments contraction in mouse aortic smooth muscle

S.K. Blackman, A. Gibson and I. McFadzean

Pharmaceutical Science Research Division, King's College London, London, UK

2-Aminoethoxydiphenyl borate (2-APB) has been widely used as a pharmacological tool in the investigation of calcium signalling mechanisms. Initially described as an IP_3 receptor antagonist, the drug has since been reported to have complex effects on calcium responses in single cells. Of note are its actions on store-operated calcium entry (SOCE); low concentrations (<50μM) enhance, whilst higher concentrations inhibit, SOCE in DT40 cells [1]. To determine if these actions of 2-APB translate to functional responses we studied its effects on contractions of mouse aorta, a tissue in which SOCE has been shown to be important [2].

Aortic rings (3-4mm) from male C57BL6 mice were set up for recording isometric tension changes (resting tension 0.5-0.7g) in Krebs' solution. Nω-Nitro-L-arginine methyl ester hydrochloride (300μM) and indomethacin (3μM) were included in the Krebs' solution to inhibit the relaxant effects of nitric oxide and prostanoids respectively. Where thapsigargin (Tg) or noradrenaline (NA) were used as contractile agents the Krebs' contained verapamil (3μM) to inhibit voltage-operated calcium channels. After 1hr rest (tension adjusted as necessary) rings were contracted by a 3min application of KCl (40mM), repeated every 30min until consistent responses were obtained. Contractions are expressed as mean±SEM (n in parentheses) of the KCl response. Data were analysed by Student's t test, with $p<0.05$ considered significant.

When increasing concentrations of 2-APB were applied cumulatively to tissues pre-contracted with NA (10μM; n=4), concentrations in the range 0.1 to 3μM produced an increase in tension; concentrations of ≥30μM caused relaxations, with 100μM abolishing the contractile response to NA. Similar results were obtained using Tg (4μM; n=4) as the pre-contractile agent, but with two key differences; first, less than 1μM 2-APB had no effect; second, whilst 100μM 2-APB produced relaxant effects, it did not inhibit the contraction to Tg, but only the enhancement produced by lower concentrations of the drug. 2-APB (1–100μM) had no effect on contractions to KCl (40mM; n=4).

In another set of experiments cumulative concentration response curves to NA (1nM–10μM) were constructed before and after treatment with 2-APB (1μM or 100μM). At the lower concentration, 2-APB (n=5) increase both the potency (EC_{50} control; 52±6.7nM; EC_{50} in 2-APB 24±4.2nM) and maximum response (control maximum 109.9±14.8%; in 2-APB 133.3±12.7%) to NA. At the higher concentration 2-APB reduced the maximum response to NA (control 81.8±1.85%; in 2-APB 28.7±10.0%) with no change in the EC_{50} (n=4).

These results show that the complex actions of 2-APB in single cells are mirrored by its effects on functional responses in smooth muscle.

Ma HT *et al.* (2002). J Biol Chem **277**, 6915-6922.

Trepakova ES *et al.* (2001). J Biol Chem **276**, 7782-7790.

S.K.B. is the recipient of a BHF studentship.

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C102

Sub-contractile concentrations of sphingosylphosphorylcholine strongly potentiate vasoreactivity of rat intrapulmonary arteries via PKC-dependent enhancement of calcium entry mechanisms

G.D. Thomas, V.A. Snetkov, B. Teague, P.I. Aaronson and J.P. Ward

Asthma, Allergy & Respiratory Science, King's College London, London, UK

Sphingolipids are important modulators of vascular tone, and we have reported that sphingosylphosphorylcholine (SPC) acts via both Rho kinase-mediated calcium sensitisation and elevation of intracellular calcium due to activation of receptor operated non-selective cation channels in rat small intrapulmonary arteries (IPA) (Thomas et al. 2005). However, the concentrations of sphingolipids required to elicit contraction in most isolated artery preparations (10-100μM) are much higher that those reported in vivo. We therefore examined whether sub-contractile concentrations of SPC affected vasoreactivity of small IPA of the rat to other stimuli. Small (300-500μm i.d.) IPA were mounted on a myograph for measurement of tension; in some experiments IPA were loaded with Fura PE-3 for measurement of intracellular calcium. Data are given as mean ± SEM, and tested for significance using either Student's t test or ANOVA as appropriate.

SPC (1μM) alone caused no contraction and either no or a very small increase in intracellular calcium. However, the concentration-response relationships for depolarising potassium concentrations and prostaglandin F2α (PGF) were significantly shifted to the left (EC_{50} potassium, control: 41 ± 4mM, SPC: 31 ± 2mM, n=9, $p<0.05$; PGF control: 18 ± 7μM, SPC: 9 ± 1μM, n=11, $p<0.05$). In separate experiments, pre-treatment with 1 μM SPC potentiated constriction induced by 23mM potassium by 460 ± 57% after 30min (n=11, $p<0.01$). This potentiation was not significantly affected by removal of the endothelium, inhibition of Rho kinase with Y-27632 (10μM), or blockade of non-selective cation channels with 10μM lanthanum or 75μM 2-aminophenylborate (2-APB) (n=5-6). Following inhibition with the PKC inhibitor Ro 31-8220 (3μM), however, the potentiation was reduced and was no longer significant (160 ± 54%, n=5). Addition of 1μM SPC to PGF-preconstricted IPA caused a smaller but significant increase in tension (40 ± 7%, n=10, $p<0.01$), which was also resistant to Y-27632 (n=6) but suppressed by Ro 31-8220 (6 ± 4%, n=6, $p<0.001$). The potentiation was halved by diltiazem (10μM; 21 ± 3%, n=4, $p<0.05$), but abolished by 2-APB (4 ± 4%, n=4, $p<0.001$). Further experiments showed that 1μM SPC substantially increased the rise in intracellular calcium induced by either ~23mM potassium or

100nM U-46619 (a TP agonist), and that these increases were suppressed by PKC inhibition (n=4-5). We propose that sub-contractile concentrations of SPC increase vasoreactivity in IPA via a PKC-dependent mechanism that enhances calcium entry via both voltage dependent L-type channels, and non-selective, receptor operated channels. This may have implications for the physiological relevance of SPC.

Thomas GD, Snetkov VA, Patel R, Leach RM, Aaronson PI, & Ward JPT (2005). Cardiovasc Res 68, 56-64.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC108

The effect of 5-HT_{2C} receptor agonist Ro 60-0175 in the control of the urethra and micturition in anaesthetised female rats

M. Yvonne[1], J. Gardiner[2], G. McMurray[2] and R. Andrew[1]

[1]Pharmacology, UCL, London, UK and [2]Discovery Biology, Pfizer Global Research and Development, Kent, UK

In rats, stimulation of 5-HT_{2C} receptors caused inhibition of the micturition reflex, while stimulation of 5-HT_{2A} receptors caused activation of the external urethral sphincter (EUS); (Mbaki *et al.* 2005). However, in the guinea-pig using the selective 5-HT_{2C} receptor agonist Ro 60-0175, it was shown that stimulation of 5-HT_{2C} receptors caused activation of the EUS (McMurray & Miner, 2005). The present experiments were carried out to further investigate the effects of Ro 60-0175 on EUS-EMG activity, urethral pressure and the micturition reflex in the rat.

Experiments were performed on spontaneously breathing female Sprague-Dawley rats (250-300g) anaesthetized with isofluorane (5% in 100% oxygen) and maintained with urethane (1.2 g kg^{-1}, i.v.). Simultaneous recordings of EUS-EMG activity, urethral and bladder pressures, and carotid arterial blood pressure were made. Micturition reflexes were evoked by saline infusion (0.1 ml min^{-1}) into the bladder. All substances were given as i.v. bolus doses. Changes were compared with vehicle controls for baseline EUS-EMG activity and urethral pressure by two-way ANOVA and the micturition reflex by unpaired Student's *t* test. All values are expressed as means ± S.E.M. $P < 0.05$ was considered to be significant.

Ro 60-0715 (300 μg kg^{-1}, n=5) caused a significant increase in baseline EUS-EMG activity and urethral pressure (22 ± 3 vs. 1 ± 1 V and 2 ± 0.2 vs. 0.1 ± 0.1 mmHg, respectively). The selective 5-HT_{2C} receptor antagonist SB 242084 (30 μg kg^{-1}, n=5) blocked the effects of Ro 60-0175 on EUS-EMG activity but failed to block the increase in urethral pressure. The 5-HT_{2A} receptor antagonists MDL 100907 and ketanserin (30 μg kg^{-1}, n=5) also blocked the effects of Ro 60-0175 on EUS-EMG activity but failed to block the increase in urethral pressure. Ro 60-0175 significantly increased bladder threshold pressure (5.7 ± 1.6 vs. 0.5 ± 0.9 mmHg), residual volume (0.2 ± 0.04 vs. 0.01 ± 0.01 ml) and volume threshold (0.4 ± 0.1 vs. 0.2 ± 0.02 ml). SB 242084 significantly decreased the effects of RO 60-0175 on bladder threshold pressure and volume threshold. MDL 100907 and ketanserin failed to block the effects of Ro 60-0175 on bladder threshold pressure, residual volume and volume threshold.

These data indicate that activation of either 5-HT_{2A} or 5-HT_{2C} receptors can cause excitation of the EUS in the rat. The data above also confirms an inhibitory role of 5-HT_{2C} receptors in the control of micturition. However, the receptor subtype by which 5 HT_2 receptor agonists cause an increase in urethral pressure (Mbaki *et al.* 2005) remains to be determined. Interestingly in guinea-pigs and dogs it has been shown that 5-HT_{2C} receptor activation increases urethral pressure, which is more predictive for humans (Conlon et al. 2005).

McMurray & Miner (2005). FASEB J **19**, A536.

Mbaki et al. (2005). PA2onlineVol3Issue4abst065P.

Conlon et al. (2005). Soc for Neurosci Online abst 48.14.

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PC109

Arginine vasopressin enhances Ca^{2+} store loading in pig retinal arterioles

G. McGeown[1], O. Jeffries[1], N. Scholfield[1] and T. Curtis[2]

[1]Vascular Biology Group, Queen's University of Belfast, Belfast, UK and [2]Centre for Vision Science, Queen's University of Belfast, Belfast, UK

In a study into possible mechanisms of desensitisation to arginine vasopressin (AVP), it was discovered serendipitously that $[Ca^{2+}]_i$ increases in response to caffeine were enhanced following exposure to AVP. We now report investigations designed to characterize this effect and the signalling pathway responsible for it.

Eyes were removed from freshly killed pigs at the local abattoir. Arterioles were mechanically dispersed from the retinas using a fire-polished Pasteur pipette and microvascular smooth muscle (MVSM) cells were loaded with 10mM Fura-2AM for 2 hours. Changes in $[Ca^{2+}]_i$ were recorded from fragments of intact arteriole using ratiometric microfluorimetry. Caffeine (10 mM) was used to release stored Ca^{2+} and the resulting increase in the fluorescence ratio used as a measure of the increase in $[Ca^{2+}]_i$. Caffeine responses were compared in the same vessels before and after treatment with AVP or other agents under test, and the statistical significance of any changes assessed ANOVA with the Tukey Kramer post-hoc test unless otherwise indicated.

Caffeine responses were increased by 52±20% (mean±SEM) 10 min after exposure to AVP (10nM) ($P<0.005$, paired t test, n=17). When caffeine was applied a third time, some 90 min after washout of AVP, the response was still 55±23% greater than the control caffeine response before AVP in the same vessels ($P<0.05$, n=6). There were no significant changes in caffeine responses when the drug was applied repeatedly in the absence of AVP (n=8). Previous studies in vascular smooth muscle have shown that cAMP can promote store filling (Porter et al. 1998). Experiments were carried out to determine whether this was the possible signalling pathway responsible in this case. Ten minutes exposure to forskolin, which elevates cAMP levels, increased caffeine responses by 30±10%, ($P<0.05$, n=12). When tissues were pre-incubated for 1-2 h with Rp-8-HA-cAMPS (10mM), a cell permeant inhibitor of protein kinase A, control caffeine responses remained brisk, but there was no enhancement of these responses after treatment with either AVP (n=19) or forskolin (n=12).

We conclude that AVP, a physiological agonist in retinal microvessels, also stimulates enhancement of store loading via a cAMP, PKA-dependent mechanism in these vessels.

Porter VA et al. (1998). Am J Physiol 274, C1346-55.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC110

Induction of Ca^{2+} oscillations by endothelin in rat retinal arteriolar smooth muscle cells

J. Tumelty[2], T. Curtis[2], N. Scholfield[1] and G. McGeown[1]

[1]*Vascular Biology Group, Queen's University Belfast, Belfast, UK and* [2]*Centre for Vision Science, Queen's University Belfast, Belfast, UK*

Endothelin is an endogenous vasoconstrictor likely to modulate the tone of retinal arterioles. We have previously demonstrated that this agonist can induce cyclical, inwardly directed Ca^{2+}-activated Cl^- currents in ocular blood vessels (Curtis & Scholfield, 2000). In this study the effects of endothelin on Ca^{2+} signalling in arteriolar smooth muscle were examined at the cellular level using high speed Ca^{2+} imaging.

Arterioles removed post mortem from Sprague Dawley rats (200-300g) were mechanically dispersed from fresh retinae using a fire-polished Pasteur pipette and microvascular smooth muscle (MVSM) cells loaded with 10μM Fluo-4AM for 2 hours. Changes in $[Ca^{2+}]_i$ were imaged in MVSM cell arrays (9-15 cells) using confocal scanning laser microscopy in line scan mode (500 scans/s). The scanline was oriented parallel with the vessel lumen, so that $[Ca^{2+}]_i$ was imaged simultaneously across the width of adjacent cells. Raw fluorescence data were extracted for graphical presentation using Image J (NIH), and $[Ca^{2+}]_i$ events were detected and analysed using custom-designed software.

Although high concentrations of endothelin-1 produced synchronised increases in global $[Ca^{2+}]_i$ in adjacent MVSM cells, lower concentrations (10nM) induced rhythmical $[Ca^{2+}]_i$ oscillations (Fig. 1). These were often very regular in frequency within a given cell. The effects of endothelin were blocked by pre-exposure to BQ123 (100nM), an inhibitor of endothelin A-type receptors (n=5). Endothelin-1-induced $[Ca^{2+}]_i$ oscillations could also be reversed by subsequent application of tetracaine (100μM, n=6). They were also inhibited by the inositol 1,4,5-trisphosphate (IP_3) receptor blockers xestospongin C (10μM, n=7; and 50μM, n=8) and 2-aminoethoxydiphenyl borate (100μM, n=5). We conclude that endothelin-1, a physiological agonist of retinal MVSM cells, stimulates rhythmical Ca^{2+} oscillations in these microvessels via activation of endothelin A receptors. The response seems to be dependent on release of Ca^{2+} stores from the sarcoplasmic reticulum via both ryanodine receptors and IP_3 receptors, since inhibition of either pathway reversed the effects seen. This endothelin-induced activity may explain the Ca^{2+}-activated Cl^- currents previously observed in the presence of this agonist (Curtis & Scholfield, 2000).

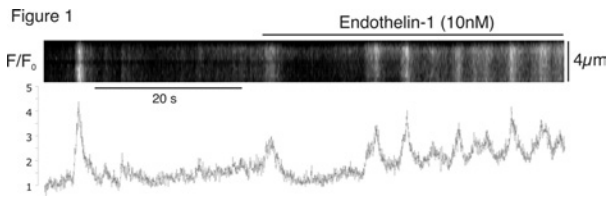

Figure 1: Linescan image showing the effects of endothelin on fluo-4 fluorescence in a smooth muscle cell in the wall of a rat retinal arteriole. The average fluorescence (normalised to the resting fluorescence, F_0) is plotted below the image panel.

Curtis TM & Scholfield CN (2000). Invest Ophthalmol Vis Sci 41, 2279-2285.

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Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC111

Interstitial cells in the rabbit urethra: structural relationships with nerves and smooth muscle

A.D. Lyons, T. Gardiner and K.D. McCloskey

Physiology, Queen's University, Belfast, Belfast, Northern Ireland, UK

Studies of enzymatically isolated cells from the rabbit urethra have established that there is a population of specialised cells which display spontaneous electrical activity and have morphological properties of interstitial cells (Sergeant et al. 2000). The aim of the present study was to identify such cells within the wall of the rabbit urethra using antibodies to the Kit receptor and examine their location, morphology and relationships with nerves and smooth muscle.

Urethras were obtained post mortem from rabbits. Tissues were labelled with antibodies and examined with confocal microscopy. Some specimens were embedded in paraffin and processed for histological staining.

Histological sections from the most proximal third and mid-third region of rabbit urethra were stained with Casson's Trichrome to reveal their cellular arrangement. Sections from both regions had outer longitudinal and inner circular layers of smooth muscle and a lamina propria containing connective tissue and blood vessels. The lumen was lined with urothelial cells. The mid-third region had a better developed circular smooth muscle layer compared to the most-proximal samples and had extensive inner longitudinal smooth muscle bundles in the lamina propria.

Labelling with anti-c-Kit revealed a population of Kit-positive cells within the wall of the rabbit urethra (n=15). These were found in the circular and longitudinal layers of the muscularis. Double-labelling with anti-smooth muscle myosin (n=13) showed Kit-positive cells on the boundary of the smooth muscle bundles, orientated in parallel with the axis of the bundles. Others were found in the spaces between the bundles and often made contact with each other, forming small network areas. The Kit-positive cells had a number of morphologies: elongated with several lateral branches or stellate with branches coming from a central nuclear region. Relationships with intramural nerves was examined by co-labelling with anti-neurofilament (n=5). Frequent points of contact were observed between the Kit-positive cells and nerves. Similar observations were made with co-labelling with anti-neuronal nitric oxide synthase (n=5).

In conclusion, Kit-positive interstitial cells are found within the smooth muscle layers of the rabbit urethra. These are located on the edge of smooth muscle bundles and in the spaces between the bundles and make structural associations with nerves. The contact with nNOS containing neurons might imply participation of the interstitial cells in the nitrergic inhibitory neurotransmission of the urethra.

Sergeant GP, Hollywood MA, McCloskey KD, Thornbury KD & McHale G (2000). J Physiol 52, 359-366.

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PC112

Cholinergic-induced Ca^{2+} responses in interstitial cells isolated from the guinea-pig bladder

L. Johnston and K.D. McCloskey

Physiology, Queen's University, Belfast, Belfast, Northern Ireland, UK

There is an increasing body of evidence that the urinary bladder contains, in addition to bulk smooth muscle, a population of cells with many characteristics of interstitial cells. These cells can be identified using antibodies to the Kit receptor both in fixed tissue preparations and when enzymatically dispersed [1]. The physiological roles of bladder interstitial cells has yet to be established; however, information on their electrophysiological and Ca^{2+} signalling characteristics is beginning to emerge [2-4]. The major excitatory innervation to the bladder is cholinergic and it was therefore of interest to investigate the response of freshly-dispersed interstitial cells to cholinergic stimulation.

Bladders were removed post mortem from guinea-pigs of either sex; they were opened longitudinally and the mucosa removed. Cells were isolated from the detrusor region as previously described (McCloskey & Gurney, 2002), loaded with 1μM Fluo 4AM and studied using confocal microscopy.

Application of 1 or 10μM carbachol induced an increase in intracellular Ca^{2+}. These responses were repeatable after 70s and comprised large transients lasting several seconds. The carbachol-induced transients were not blocked by 1μM nifedipine (n=7). The participation of Ca^{2+} release from intracellular stores was initially investigated using 2-APB, often used to block release from IP3-sensitive stores. The effect with this drug on the carbachol responses was variable with no effect seen in 7 cells and a reduction seen in 4 others; however, xestospongin-C, regarded as a better blocker of IP_3 receptors did block the responses (n=6) suggesting that release of Ca^{2+} from IP_3-sensitive stores was a major component of the Ca^{2+} transients. Previous work had shown that the carbachol responses were sensitive to the muscarinic blocker atropine, so pharmacological blockers of the M2 and M3 receptors were used to examine this further. Methoctramine, an M2 receptor antagonist had little effect on the carbachol responses (n=4) whereas the M3 receptor antagonist, 4-DAMP inhibited the responses (n=2).

In summary, detrusor interstitial cells respond to cholinergic stimulation by firing Ca^{2+} transients. These appear to be mediated via M3 muscarinic receptors as they were blocked by atropine and 4-DAMP but not methoctramine. A major source of Ca^{2+} appears to be via the IP_3-sensitive store as the responses were sensitive to xestospongin-C but not nifedipine. The findings of this study suggest that detrusor interstitial cells could participate in cholinergic neurotransmission in the normal bladder.

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PC113

Effects of PKC activation on Ca^{2+} transients and phasic contractions in ureteric smooth muscle

W. Hue, S. Shabir, R. Floyd, L. Borisova, S. Kenny and T. Burdyga

The Physiological Laboratory, University of Liverpool, Liverpool, UK

In the present study the effects of PKC activation and inhibition on Ca^{2+} transients and phasic contractions evoked by electrical field stimulation (EFS) *in vitro* in ureteric smooth muscle have been investigated. Simultaneous measurements of Ca^{2+} and force was done using Indo-1-loaded strips of ureteric muscles. Western blotting and immuno-histochemistry have been used to define the presence of PKCα in ureter muscle. Using Western blotting and immuno-histochemistry we found that PKCα was expressed in both guinea-pig and rat ureteric smooth muscles. In small bundles of ureter activation of PKC by low concentrations of PDBU (100nM) produced a marked increase in the duration of the Ca^{2+} transient (3.2±0.4 times, n=7, recorded using Indo-1), and the amplitude and duration of the phasic contractions (1.9±0.1 times and 3.4±0.3 times, respectively, n=7). These effects of PDBU were blocked by two inhibitors of the PKC, GF109203X (5μM) or RO-32-0432 (5μM) (n=5). Neither of these PKC inhibitors had any significant effects on the Ca^{2+} transients and phasic contractions evoked by EFS in the absence of PDBU (n=7). Inhibition of the SR Ca^{2+}-ATPase by cyclopiazonic acid (20μM) (n=4) or inhibition of BK_{Ca} channels by low concentrations of TEA (1mM) (n=6), did not prevent the stimulant action of PDBU on the Ca^{2+} transients and phasic contractions. These data therefore suggest that the stimulatory action of PDBU on guinea-pig ureteric smooth muscle can not be explained by inhibition of a Ca^{2+} sparks/STOCs (spontaneous transient outward currents via BK channels) coupling mechanism reported for vascular smooth muscle (1). Pretreatment of the ureteric smooth muscle cells with the L-type Ca^{2+} channel agonist BayK 8644 (1μM) strongly inhibited the stimulant action of PDBU on ureteric smooth muscle suggesting that L-type Ca^{2+} channels are likely to be targeted by PKC (n=3). The stimulation of PDBU on the ureteric cells was strongly inhibited by intracellular acidification and potentiated by intracellular alkalinization in the guinea pig ureter (n=5).

These data suggest that PKC can play an important role in control of phasic contractions in ureter muscle mediated by possible modulation of the L-type Ca^{2+} channels and that these effects of PKC are critically dependent on the intracellular pH.

Bonev AD, Jaggar JH, Rubart M & Nelson MT (1997). Am J Physiol 273, C2090-2095.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC114

The protective effect of intracellular acidosis on contraction in corpus cavernosum

P. Kumar, S. Minhas, D. Ralph and C. Fry

Institute of Urology, UCL, London, UK

Priapism is defined as a pathological condition where penile erection persists beyond, or is unrelated to sexual stimulation. In patients with ischaemic priapism, corporal blood aspirates show evidence of hypoxia, acidosis and glucopenia. One hypothesis for ischaemic priapism is reduced contractile function of cavernosal smooth muscle (CSM) secondary to these metabolic changes. This study aims to determine the effect of simulated ischaemia on contraction in CSM and which components of ischaemia are central to these changes.

Isometric contractions were recorded from strips of guinea pig corpus cavernosum in response to electrical field stimulation (EFS at 60 Hz, sensitive to 1 μM tetrodotoxin) or exposure to 15 μM phenylephrine (PE). Strips were superfused at 37°C with a HCO_3^-/CO_2 buffered solution (pH 7.39). Hypoxia was generated by substituting 95% O_2 for 95% N_2 in the superfusing gas mixture. Glucopenia was achieved by omitting glucose and Na pyruvate. Acidosis in both the intra and extracellular compartments was generated by increasing the gas mixture CO_2 from 5% to 10% (pH 6.99). [HCO_3^-/CO_2] was reduced in the superfusate to create an extracellular acidosis (pH 6.97). Intracellular acidosis was generated by increasing the CO_2 percentage and superfusate [HCO_3^-/CO_2] in proportion (pH 7.44). Superfusate [$CaCl_2$] was altered appropriately to maintain Ca^{2+} activity (Wu & Fry, 1998). The effects of these interventions were recorded in isolation and in combination on both nerve-mediated and agonist-induced contractions. Data are mean ± s.d. Statistical differences ($p<0.05$) between data sets were examined with Student's t tests.

A combination of hypoxia, glucopenia and acidosis severely affects contractile function. This effect is irreversible when the muscle is activated via the motor nerves but not when the muscle is directly activated. Intracellular acidosis conferred some protection against the effect of this metabolic depletion when the muscle was activated directly with an agonist, but not when nerve-mediated stimulation was used. The mechanisms by which intracellular acidosis exerts this protective effect on the muscle needs to be assessed.

Table 1. Effect of interventions on CSM contraction

Interventions	30 min EFS (% control)	Recovery EFS (% control)	30 min PE (% control)	Recovery PE (% control)
HIAG	41±25 △	37±30 △	42±31 △	94±21 §
HG	15±10 △	27±16 △	50±19 △	86±7 §
HAG	26±20 △	51±27 △	27±9 △	94±10 §
HIG	18±23 △	28±29 △	96±51 $	99±22 §

Interventions: hypoxia, H; glucopenia, G; acidosis, IA; intracellular acidosis, I; extracellular acidosis, A. △ Significant effect of intervention vs. control; § significant difference in recovery of PE vs. recovery of EFS; $ significant difference in effect on PE contracture of HIS vs. HIAS, HS and HAS.

Berger R et al. (2001). Int J Impot Res 13, S39-43.

Wu C & Fry CH (1998). J Physiol 508, 131-143.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

PC115

The contractile properties of human paediatric detrusor smooth muscle in congenital bladder anomalies

N. Johal, P. Cuckow and C. Fry

Institute of Urology, London, UK

Children born with congenital bladder anomalies undergo abnormal bladder development. They often need multiple reconstructive surgery to improve bladder capacity, empyting, continence and to protect the upper urinary tract. Despite this these children continue to display symptoms of urinary incontinence, frequency, and urinary tract infection (Gearhart & Jeffs, 1998). Abnormal connective tissue and poor detrusor smooth muscle function have both been implicated (Thiruchelvam et al. 2003). The contractile properties of detrusor muscle in this population are unknown. We aimed to examine them the in vitro using human paediatric detrusor from normal patients and those with congenital bladder anomalies. Pathological bladder conditions included children with neuropathic bladder, bladder exstrophy and cloacal exstrophy. Full thickness bladder samples were obtained during open surgery. Normal tissue was obtained from those with urachal anomalies or those undergoing ureteric reimplantation. Detrusor strips (≈1mm diameter) were superfused at 37°C with a HCO_3^-/CO_2 buffered physiological solution. Nerve-mediated responses were elicited by electrical field stimulation (1-60Hz, sensitive to 1μM tetrodotoxin) in the absence and presence of 10μM atropine. Agonist-induced responses were generated by carbachol (0.1-30μM) and α,βmethylene-ATP (ABMA 1μM). Tension was normalised to unit cross-sectional area (mN mm^{-2}). Data are mean±s.d. and significance of differences ($p<0.05$) between sets were examined by Student's t test. Nerve-mediated contractions were significantly less in samples from patients with pathological bladders compared to normals. The estimated maximum tensions from force-frequency plots were 1.8±3.1mN mm^{-2} and 8.1±8.7mN (n=52,18, pathology vs normal). The frequency required to generate half-maximum tension was not different in the pathological state (15.1±11.0 vs 17.8±10.5Hz, respectively). Atropine-resistant contractions were recorded in all preparations but they were a significantly greater proportion of total nerve-mediated responses in the normal group. Contractile responses to carbachol and ABMA were also significantly less in the pathological group. For carbachol the tension at the highest concentrations from dose-response curves were 6.8±7.0 vs 30.9±22.2mN mm^{-2}, respectively. The carbachol EC_{50} was reduced in the pathological group (EC_{50}s; 1.8±1.6 vs 3.2±1.2, n=45,16, respectively). Responses to 1μM ABMA were 1.5±2.1 vs 11.2±8.3mN mm^{-2}, respectively. The data shows that detrusor from patients with pathological bladders exhibited

reduced contractility, whether elicited by excitatory nerves or agonists. This was offset by an increase in the sensitivity to carbachol in the pathological group.

Gearhart JP & Jeffs RD (1998). Exstrophy – epispadias complex and bladder anomalies. In Campbells Urology, 7th edn, ed. Walsh et al. Saunders, New York, NY.

Thiruchelvam N, Wu C, David A, Woolf AS, Cuckow PM & Fry CH (2003). Am J Physiol 284, R1296–R1305.

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PC116

Functional muscarinic receptor subtypes in guinea-pig corpus cavernosal smooth muscle

C. Li, S. Minhas, D.J. Ralph and C.H. Fry

Institute of Urology, London, UK

Binding and molecular studies using corporal smooth muscle (CSM) have shown that M2 and M3 muscarinic receptors are present (Traish et al. 1990, 1995). However, the functional subtypes are not known and this was the aim of this study.

Guinea-pig CSM strips (~1 mm diameter) were superfused with Tyrode solution (5% CO_2, 24mM $NaHCO_3$, pH7.4) and attached to an isometric force transducer. Contractions were elicited either by nerve-mediated, electrical field stimulation (EFS: abolished by 1μM tetrodotoxin), or by application of 1.5μM phenylephrine. Relaxation was elicited in pre-contracted strips by 0.3μM carbachol. EFS-induced relaxations were elicited in strips pre-contracted with 15μM phenylephrine. Atropine (1μM), selective muscarinic antagonists pirenzepine (M1), gallamine (M2), 4-DAMP (4-diphenylacetoxy-N-methyl-piperidine methiodide, M3) and L-NAME (100μM) were used to characterise cholinergic and nitric oxide (NO)-mediated relaxation. Contractions are expressed as mN/mm^2 and muscarinic relaxation as a percentage of the previously-induced contraction. Data are mean ± S.D., differences between means ($p<0.05$) were examined using Student's t tests.

L-NAME enhanced EFS-induced responses by 60±37% (n=12) at 32Hz stimulation. When strips were pre-treated with atropine, the enhancement by L-NAME was reduced significantly to 21±18% (n=5), implying a component of the NO effect is mediated by a cholinergic pathway. EFS-induced contractions were unaffected by pirenzepine or gallamine (1μM, n=7). However, 4-DAMP (1μM, n=6) enhanced the responses, although there was a large variability of responses (mean percentage increase 46±44%). Carbachol induced a dose-dependent relaxation of the phenylephrine contracture. L-NAME, atropine (n=6) and 4-DAMP (1nM-10μM, n=8) completely reversed this relaxation. Pirenzepine (3nM-100μM, n=8) and gallamine (1nM-100μM, n=8) had no significant effect. EFS also induced a frequency-dependant relaxation of the phenylephrine contracture. The frequency for half-maximal effect (3.4±1.6Hz) was significantly less than that required to induce EFS-contractions (33±2Hz). These relaxations were partially reversed by 4-DAMP (1μM, n=4) at 4Hz stimulation (to 52±16% of control). In turn this reversal was partially offset by subsequent addition of gallamine (1μM, n=4, to 64±9% of control).

In guinea-pig CSM, smooth muscle relaxation is mediated by M3 muscarinic receptors via NO release. An M2 muscarinic receptor antagonist (gallamine) modified this response.

Traish AM et al. (1990). J Urol 144, 1036-1040.

Traish AM et al. (1995). Receptor 5, 159-176.

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PC117

Role of src-family kinases in changes in protein tyrosine phosphorylation, force generation and $[Ca^{2+}]_i$, induced by prostaglandin $F_{2\alpha}$ in rat intrapulmonary arteries

G.A. Knock, V.A. Snetkov, J.P. Ward and P. Aaronson

King's College London, London, UK

Little is known about the involvement of receptor or non-receptor tyrosine kinases in contractile responses of pulmonary arteries. Intrapulmonary arteries (IPA) were obtained from the lungs of male Wistar rats. IPA were either treated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 20μM) and then snap-frozen for SDS-PAGE Western blot analysis (first and second order branches), or mounted on a wire myograph for measurement of isometric tension (second or third order branches). Experiments were performed at 37°C in bicarbonate-buffered physiological salt solution, pH 7.4, gassed with 5% CO_2/balance air. P values are derived from paired Student's t test, unless otherwise stated.

Using anti-phospho-tyrosine antibody (Cell Signalling) multiple protein bands were visualised, including ones at approximately 120, 90, 75 and 65 kD. Band intensities from $PGF_{2\alpha}$-treated IPA were expressed as a percentage of those from paired untreated IPA. $PGF_{2\alpha}$ induced a 60-190% increase in tyrosine phosporylation. These increases were statistically significant ($P<0.05$ by analysis of 95% confidence interval, n = 8). All increases were reversed by the src-family kinase inhibitor PP2 (30μM, n = 5).

PP2 (30μM) produced concentration-dependent relaxation of myograph-mounted IPA pre-contracted with 20 μM $PGF_{2\alpha}$ (45 ± 4% block, $P< 0.01$, n = 9), as did 10μM of the epidermal-growth-factor-receptor kinase inhibitor PD-174265 (60 ± 4% block, $P< 0.001$, n = 9). In FURA-PE3-loaded IPA, 30μM PP2 significantly inhibited the rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by 0.1μM U46619 (51 ± 7% block, $P<0.05$, n = 7), but not that induced by 0.1μM $PGF_{2\alpha}$ (9 ± 12% block, N.S., n = 4). In addition, 30μM PP2 also relaxed $PGF_{2\alpha}$ contracted α-toxin permeabilised IPA, where $[Ca^{2+}]_i$ was clamped at pCa 6.8 (26 ± 2%, $P< 0.05$, n = 3).

In conclusion, $PGF_{2\alpha}$-mediated contraction in rat IPA is associated with tyrosine phosphorylation of multiple protein targets. Src-family kinase and EGFR-kinase activity are implicated. src-family kinases appear to influence contraction via both $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent pathways.

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PC118

Effects of agents affecting sarcoplasmic reticulum and plasmalemmel Ca^{2+} homeostasis on contractions induced by hypoxia and FCCP in rat small intrapulmonary arteries

S. Becker, L.M. Baxter, V.A. Snetkov, J.P. Ward and P.I. Aaronson

Asthma, Allergy & Lung Biology, King's College London, London, UK

The pulmonary circulation responds to acute moderate hypoxia with an immediate vasoconstriction (HPV), which helps to match pulmonary ventilation and perfusion and optimises gas exchange in the lung. In this study we have investigated the potential role of mitochondrial depolarisation in HPV using FCCP.

Small intrapulmonary arteries (IPA) from male Wistar rats (200-300g) were dissected free and mounted on a Mulvany-Halpern myograph, bathed in Krebs solution and gassed with 5% CO_2, balance air. In order to evoke HPV vessels were preconstricted with $PGF_{2\alpha}$ and subsequently exposed to 5% CO_2, balance nitrogen. This evoked a biphasic contraction of the pulmonary arteries with a rapidly developing transient and a slow sustained (phase 2) contraction.

Phase 2 HPV was nearly abolished by preincubation with the ryanodine receptor blockers dantrolene (50 μM; 62% inhibition, $P<0.001$, n=10) and ryanodine (100 μM; complete inhibition, $P<0.001$, n=3) and the SOCC inhibitor 2-APB (75 μM; 60% inhibition, $P<0.001$, n=5), indicating an important contribution of intracellular Ca^{2+} release via ryanodine receptors and Ca^{2+} influx via a non-voltage-dependent pathway.

FCCP evoked a biphasic contractile response in $PGF_{2\alpha}$–preconstricted pulmonary arteries, which strongly resembled HPV in size and time course. In mesenteric resistance arteries FCCP produced a transient, but not a sustained contraction (n=8). In analogy to HPV, the FCCP–induced contraction in IPA was attenuated by preincubation with dantrolene (74% inhibition, P=0.03, n=3) or 2-APB (complete inhibition, p=0.02, n=4). Moreover, preincubation of IPA with FCCP strongly attenuated the development of the sustained contraction during hypoxia (58% inhibition, $P<0.01$, n=5). However, the FCCP response differed from that to hypoxia in that it was strongly inhibited by 10 μM diltiazem (64% inhibition, P=0.04, n=4) and also by 30 μM cyclopiazonic acid (73% inhibition, P=0.01, n=5), neither of which inhibited sustained HPV.

Therefore, although FCCP inhibited phase 2 HPV, and caused a contraction in IPA which in some ways mimicked this response, the results suggest that mitochondrial depolarisation is unlikely to be a major factor in causing HPV. The results indicate also that mitochondrial Ca^{2+} content in IPA is strongly influenced by plasmalemmel and sarcoplasmic reticulum Ca^{2+} homeostasis.

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