Actions of cAMP on calcium sensitization in human detrusor smooth muscle contraction

林, 摩耶

Received Date : 27-Nov-2014
Revised Date : 31-Mar-2015
Accepted Date : 01-May-2015
Article type : Original Article
Section : Functional Urology

Actions of cyclic 3'5'-adenosine monophosphate (cAMP) on calcium sensitisation in human detrusor smooth muscle contraction.

By

Maya Hayashi, Shunichi Kajioka, Momoe Itsumi, Ryosuke Takahashi, Nouval Shahab, Takao Ishigami, Masahiro Takeda, Noriyuki Masuda, Akito Yamaguchi & Seiji Naito

from

Department of Urology, Graduate School of Medical Sciences, Kyushu University (M.H., S.K., M.I., R.T., S.N.)

Department of Urology, Faculty of Medicine and Health Sciences, Syarif Hidayaullah Jakarta State Islamic Univesrity (N.S.)

Urology, Research Unit, Drug Discovery Research, Drug Discovery Research, Astellas Pharma Inc. (T.I., M.T.)

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bju.13180

Innovation & Research Portfolio Planning, Drug Discovery Research, Astellas Pharma Inc. (N.M.)

Division of Urology, Harasanshin Hospital, Fukuoka, Japan (A.Y.)

Running title: Effect of cAMP on Calcium Sensitisation in Human Detrusor Muscle

Corresponding Author: Shunichi Kajioka, MD, DDS, PhD, D Phil.

Department of Urology Graduate School of Medical Sciences Kyushu University

3-1-1 Maidashi, Higashiku, Fukuoka, 812-8582 Japan

Tel: +81(0)92-642-5603, **Fax**: +81(0)92-642-5618

E-mail: kajioka@uro.med.kyushu-u.ac.jp

Abbreviations: cAMP, cyclic adenosine monophosphate; CCh, carbachol; CPA, cyclopiazonic acid; IP_3, inositol 1,4,5-triphosphate; ROK, rho kinase; PKC, protein kinase C; SPC, sphingosylphosphorylcholine; PDBu, phorbol 12,13 dibutyrate; DSM, detrusor smooth muscle.

Keywords: Human detrusor smooth muscle, permeabilization, α-toxin, cAMP, calcium sensitisation, rho kinase

ABSTRACT

Objectives: To clarify the effect of cyclic adenosine monophosphate (cAMP) on the Ca^{2+}-sensitised smooth muscle contraction in human detrusor, as well as the role of novel exchange protein directly activated by cAMP (Epac) in cAMP-mediated relaxation.

Materials and Methods: All experimental protocols to record isometric tension force were performed using α-toxin-permeabilized human detrusor smooth muscle strips. The mechanisms of cAMP-mediated suppression of Ca^{2+} sensitisation activated by 10 μM carbachol (CCh) and 100 μM guanosine-5'-triphosphate (GTP) were studied using a selective rho kinase (ROK) inhibitor, Y-27632, and a selective protein kinase C (PKC) inhibitor, GF-109203X. The relaxation mechanisms were further probed using a selective protein kinase A (PKA) activator, 6-Bnz-cAMP, and selective Epac activator, 8-pCPT-2'-*O*-Me-cAMP.

Results: CCh-induced Ca^{2+} sensitisation was inhibited by cAMP in a concentration-dependent manner. GF109203X (10 μM) but not Y-27632 (10 μM) significantly enhanced the relaxation effect induced by cAMP (100 μM). 6-Bnz-cAMP (100 μM) predominantly decreased the tension force in comparison with 8-pCPT-2'-*O*-Me-cAMP (100 μM).

Conclusions: We demonstrated that cAMP predominantly inhibited the ROK pathway but not the PKC pathway. The PKA-dependent pathway is dominant, while Epac plays a minor role in human DSM Ca^{2+} sensitisation.

INRODUCTION

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are ubiquitous second messengers that control multiple physiological processes, including smooth muscle tone regulation. Since their discovery, numerous studies have addressed the mechanisms underlying cyclic nucleotide-induced smooth muscle relaxation. At least two major mechanisms are currently known: (i) decreased cytosolic calcium concentration ($[Ca^{2+}]_i$) involving the activation of plasmalemmal and sarcoplasmic Ca^{2+}-ATPases[1, 2], or inactivation of voltage-dependent Ca^{2+} channels caused by hyperpolarisation via the activation of certain K^+ channels [3, 4]; and (ii) decreased contractile protein Ca^{2+} sensitivity, even at constant $[Ca^{2+}]_i$, (i.e. inhibition of Ca^{2+} sensitisation that modulates the activity of myosin light chain kinase [MLCK] or myosin light chain phosphatase [MLCP]) [5, 6]. Since these two pathways have great effect on each other complicatedly leading to smooth muscle relaxation, it is hard to distinguish these two pathways. Above all, as representative Ca^{2+} sensitisation, it has been reported that activation of rho kinase (ROK) and protein kinase C (PKC) inhibit MLCP activity, which, in turn, increases myosin light chain (MLC) phosphorylation, leading to further contraction [7, 8]. The underlying mechanism of contractile regulation involving cyclic nucleotides and, the ROK and PKC pathways needs further investigation to clarify the inhibition of Ca^{2+} sensitisation.

In detrusor smooth muscle, it remains still under controversial which signalling pathway is responsible for the relaxation during bladder filling phase. Some reports indicate that cAMP but not cGMP seems to be indeed a mediator of relaxation effect in rat [9], guinea-pig [10] rabbit [11] and human [12] detrusor. Alternatively, intensive studies in rat detrusor have demonstrated the direct and

indirect effect of cAMP using β-adreoceptor agonist, isoprenealine, forskolin, and adenylate cyslase inhibitor although its relaxation effect was stronger in passive tension than in the tension induced by KCl or by muscarinic receptor agonists [9, 13, 14, 15]. Also, in human detrusor, similar results were obtained using isoprenealine [16] and forskolin [17, 18] indicating that cAMP operates in non-contracted detrusor (filling phase) but less in contracted detrusor (voiding phase). Further, the relaxation by phosphodiesterase (PDE) inhibitors has been extensively investingated to prove their therapeutic potency, as PDEs can hydrolyse cyclic nucleotides much faster than they are synthesised by the respective cyclises. Hence, the relaxation effect of the PDE inhibitors selective to cAMP indicates that cAMP is a mediator in rat [19], guinea-pig [10] and human [17, 18, 20] detrusor. All of these above studies support some role of cAMP in detrusor, however none of them used cAMP by itself to their studies and few of them addressed the underlying mechanisms of cAMP-induced relaxation. Therefore, the aim of present study is to reveal the underlying mechanism of intracellular cAMP signaling pathway, especially, downstream of PKA. Hence, clarifying the precise intracellular mechanisms of detrusor relaxation by cAMP would support the future clinical use of PDE IV inhibitors for urinary dysfunction.

We previously reported the unique enhancement of Ca^{2+} sensitisation by multipotent activation of Rho kinase (ROK) pathway, regardless of the inhibition of PKC pathway in DSM of a guinea pig bladder outlet obstruction (BOO) model [21]. How cAMP modulates pathophysiological conditions caused by BOO is of additional interest. Recently, exchange protein directly activated by cAMP (Epac) was discovered and identified as a novel cAMP sensor that provides alternative pathway of PKA activation in certain smooth muscle organs [22, 23]. In the airway, smooth muscle Epac may

have therapeutic potential in the treatment of obstructive airway disease [24]. It is likely that this concept is applicable to treatment of obstructive lower urinary dysfunctions.

The present study was thus designed to examine the role of cAMP through a Ca^{2+}-independent mechanism (inhibition of Ca^{2+}-sensitisation) in human DSM contraction activated by muscarinic agonist, carbachol (CCh), using an α-toxin permeabilized preparation. An α-toxin permeabilized preparation that preserves receptor-effector communications allows for equilibration of the cytoplasm with inorganic and small molecules (< 500 Da in molecular weight) indicating complete ignorance of ion channel effect such as β-receptor stimulated Ca^{2+}-activated K^+ channel (BK_{Ca}) channel regardless of coupled protein kinase A (PKA) activation [25, 26, 27]. Using this advantage of α-toxin permeabilization, downstream of β-receptor stimulation; i. e. cross-talk between cAMP and ROK or PKC was investigated in detail. In addition, the expression and role of the PKA-independent cAMP sensor, Epac, in human DSM was evaluated.

MATERIALS AND METHODS

Tissue Specimens

The protocol of this study was approved by the Ethics Committees of the Graduate School of Medical Sciences, Kyushu University and Harasanshin Hospital. Smooth muscle tissue was obtained from the urinary bladders of humans (28 males and 6 females; mean age, 66.1 ± 7.6years) on whom radical cystectomies were performed at Kyushu University Hospital and Harasanshin Hospital due to bladder cancer, urethral cancer or prostate cancer. Written informed consents were obtained from all patients.

The specimens were harvested from a tumour-free area by excising the smooth muscle tissue from the urinary bladder. The specimens were then immediately put into an ice-cold physiological salt solution. Whether tissues were obtained from Kyushu University Hospital or Hara Sanshin Hospital, the experimental protocol started wihin 1 hours and finished within 2 days. [16]

Smooth Muscle Preparation and α-Toxin permeabilization

The permeabilized DSM strips were prepared as described previously [8]. Briefly, DSM strips without mucosa or connective tissues (200 - 300 μm in diameter and 3 - 4 mm in length) were mounted horizontally between two tungsten wires. One end of each strip was connected to a force transducer (UL2 Minebea Co. Ltd., Osaka, Japan) in 100-μL relaxing solution for 1 - 2 min and permeabilized with 5,000 U/mL *Staphylococcus aureus* α-toxin for 1 h [18]. After permeabilization, a resting tension of 9.8 mN was applied for 1 h. All permeabilized strips were treated with the Ca^{2+} ionophore, A 23187 (1 μM), for 30 min and then applied cyclopiazonic acid (CPA; 10 μM) to be completely independent from Ca^{2+} store [28, 29]. All experiments were performed at room temperature (RT; 25°C). A large “N” and a small “n” indicate the number of patients and DSM strips, respectively.

Experimental Design

Initial studies were conducted to characterise the effect of cAMP on the tension force induced by the only increase in $[Ca^{2+}]_i$ up to100 μM after permeabilization. Then, the effect of cAMP on the tension force developed by carbachol (CCh)-induced Ca^{2+}-sensitisation in the presence of 100 μM

GTP plus 10 μM CCh at fixed 1 μM $[Ca^{2+}]_i$. To further examine the effects of cAMP on CCh-induced Ca^{2+}-sensitisation, cAMP was applied after application of the muscarinic receptor 2 (M_2)-selective inhibitor, 1 μM AF-DX116, the M_3 selective inhibitor, 4-diphenylacetoxy-N-me1 μM 4-DAMP [21], the ROK-selective inhibitor, Y-27632 [21, 29, 30], or PKC-selective inhibitor, GF-109302X [21, 29, 31], in the presence of 100 μM GTP plus 10 μM CCh at fixed 1 μM $[Ca^{2+}]_i$. To confirm the effect of cAMP on Ca^{2+}-sensitisation in human DSM, cAMP was applied after the PKC activator, PDBu, or the ROK activator, SPC, induced stable contraction at fixed 1 μM $[Ca^{2+}]_i$. To specifically examine the effect of cAMP on MLCK activation, calyculin A (MLCP inhibitor) was applied prior to the cAMP application at fixed 1 μM $[Ca^{2+}]_i$.

To activate PKA and Epac, the selective PKA activator, 6-Bnz-cAMP (100 μM) [32], and selective Epac activator, 8-pCPT-2'-O-Me-cAMP (100 μM) [33], were applied under the following conditions: 1) in the presence of 1 μM $[Ca^{2+}]_i$, 2) CCh-induced Ca^{2+}-sensitisation (100 μM GTP plus 10 μM CCh at fixed 1 μM $[Ca^{2+}]_i$), or 3) after Y-27632 or GF-109302X application in the presence of 100 μM GTP plus 10 μM CCh at fixed 1 μM $[Ca^{2+}]_i$.

Immunoblotting

Samples were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer system (1 h, 15 V). After blocking for 1 h in 5% skim milk, the membrane was probed with a primary antibody. The membrane was washed three times and incubated with horseradish peroxidase (HRP)-conjugated

anti-mouse immunoglobulin (Ig)G (Bio-Rad Hercules, CA, USA) for 1 h. Immunoreactive proteins on the membrane were visualised following treatment with a detection reagent (LumiGLO®, Cell Signaling Technology Danvers, MA, USA). The monoclonal anti-Epac1 (ab21236) and anti-Epac2 (ab21238) antibodies were purchased from Abcam (Cambridge, UK). Monoclonal anti-Ras-related C3 botulinum toxin substrate 1 (Rac1; 05-389) and anti-Ras-related protein 1 (Rap1) antibodies (07-916) were purchased from Millipore (Darmstadt, Germany).

Data Analysis and Statistical Procedures

Data were obtained using a computerised data acquisition system (MacLab, Analog Digital Instruments, Sydney, New South Wales, Australia and Apple Corp., Sunnyvale, California, USA) and are presented as means ± standard deviation (SD). The activation curves of the following equation were fitted to the experimental data in Fig. 1.:

$$Tension(\%) = \frac{Tension_{(\max)}}{\left[1+\left(\frac{EC_{50}}{[Ca^{2+}]_i}\right)^{nH}\right]} \times 100 .$$

The tension force is expressed in a relative manner; the tension force obtained at 3 μM $[Ca^{2+}]_i$ was normalised as 100%. The EC_{50} is the concentration of Ca^{2+} that activated the half-maximal contraction response (relative value), and *n*H is the Hill coefficient. Statistical analyses were performed using independent Student's *t*-tests in SPSS software version 19. $P < 0.05$ was considered to indicate statistical significance.

Drugs, Chemical Reagents and Other Materials

Carbamoylcholine chloride (CCh), α-toxin, GTP, cyclopiazonic acid (CPA), adenosine 3',5'-cyclic monophosphate (cAMP), 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate (dibutyryl-cAMP), forskolin (FSK), rolipram, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), sphingosylphosphorylcholine (SPC) and phorbol 12,13-dibutyrate (PDBu), were obtained from Sigma (St. Louis, MO, USA). (R)-(+)-trans-N(4-pyridil)-4-(1-aminoethyl)–cyclohexanecarboximide dihydrochloride, monohydrate (Y-27632), bisindolylmaleimide I (GF-109203X) and 5-(methylamino)- 2- [[(2R, 3R, 6S, 8S, 9R, 11R)- 3, 9, 11- trimethyl- 8- [(1S)- 1- methyl- 2- oxo- 2-(1H- pyrrol- 2- yl)ethyl]- 1, 7- dioxaspiro[5.5]undec- 2- yl]methyl- 4- benzoxazolecarboxylic acid（A-23187） were from Calbiochem (La Jolla, CA, USA). Calyculin A was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-(Decyloxy)benzoic acid 4-[(2R)-2-(ethynyloxy)propoxy]phenyl ester（W-7) and 11-[[2-[(Diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one（AF-DX116) were from Tocris Bioscience (Bristol, UK). N^6-benzoyladenosine-3',5'-cyclic monophosphate (6-Bnz-cAMP) and 8-(4-chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate (8-pCPT-2'-*O*-Me-cAMP) were from Biolog (Bremen, Germany). 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) was from Alexis Corporation (Lausen, Switzerland). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one、2-Morpholino-8-phenylchromone、2-Morpholino-8-phenyl-4H-1-benzopyran-4-one (LY-294002) was from Cayman Chemical Company (Ann Arbor, Michigan, USA). The functions of drugs are listed on table 1.

Physiological salt solution (PSS) contained 123 mM NaCl, 15.5 mM $NaHCO_3$, 11.5 mM D-glucose, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 1.2 mM KH_2PO_4 gassed with 5% CO_2 and 95% O_2 (pH 7.4). Relaxing solution was composed of 100 mM potassium methanesulfonate, 20 mM Tris-maleate, 10 mM ethylene glycol-bis (β-aminoethylether)-N',N',N',N' tetra acetic acid (EGTA), 10 mM creatinine phosphate, 3.38 mM $MgCl_2$, 2.2 mM Na_2 adenosine triphosphate (pH 6.8). Activating solutions containing the indicated concentration of free Ca^{2+} were created by adding an exact amount of $CaCl_2$ to the relaxing solution using the Ca^{2+}-EGTA binding constant of 10^6/M.

RESULTS

Cyclic AMP Induced Relaxation of α-Toxin Permeabilized Human DSM upon Tension Development

The relaxation effect of cAMP on $[Ca^{2+}]_i$-dependence was examined using α-toxin permeabilized DSM. Ca^{2+} concentration was varied between 0.1 and 100 μM in the presence or absence of cAMP (100 μM). Tension force was increased by $[Ca^{2+}]_I$ in a concentration dependent manner. 100 μM cAMP significantly attenuated the force development induced by increased $[Ca^{2+}]_i$ at concentrations exceeding 1 μM (N = 3, n = 6; Fig. 1).

The effect of cAMP-related reagents on α-toxin permeabilized DSM

After recognition of the stable contraction induced by 1 μM $[Ca^{2+}]_i$ cAMP obviously induced relaxation by 37.6 ± 9.5% (N = 5, n = 20) (Fig. 2A). Similarly, the adenylyl cyclasse activator, 10 μM forskolin (FSK) and the phosphodiesterase IV inhibitor, 10 μM rolipram induced relaxation by 14.7 ±

9.6 % (N = 3, n = 6) and by 25.2 ± 8.6 % (N = 3, n = 6), respectively (Fig. 2B, C & D). These results indicate that human DSM relaxation is mediated by cAMP.

Effect of cAMP (100 μM) on CCh-induced Ca^{2+} Sensitisation

The effect of cAMP (100 μM) was further examined on CCh-induced Ca^{2+} sensitisation at fixed 1 μM $[Ca^{2+}]_i$. Since muscarinic receptors are coupled with G-proteins, 100 μM GTP was added prior to the application of 10 μM CCh. Although stimulation of muscarinic receptors by CCh in the presence of GTP further increased the tension at fixed 1 μM $[Ca^{2+}]_i$ (Ca^{2+} sensitisation), the relaxation effect of cAMP was decreased from 37.6 ± 9.5 % to 13.2 ± 5.6 % (Figs. 2A & D, 3A & C). Preliminaly, both the selective M_2 inhibitor, AF-DX116 (1 μM), and the selective M_3 inhibitor, 4-DAMP (1 μM) attenuated the tension force induced by GTP plus CCh at fixed 1 μM $[Ca^{2+}]_i$ by 91.0 ± 11.1% and 77.6±7.7%, respectively. In these conditions, the effect of cAMP was recovered by AF-DX116 and 4-DAMP from 13.2 ± 5.6 % to 24.3 ± 7.2 % and 37.5 ± 20.7 %, respectively. Both AF-DX116 and 4-DAMP significantly recovered its relaxation, however the relaxation effect by cAMP was stronger in the presence of 4-DAMP (N = 3, n = 10–11; Fig. 3).

Effects of Y-27632 or GF-109203X on cAMP-induced Relaxation

Ca^{2+} sensitisation is mainly caused by activation of the ROK and PKC pathways. To evaluate whether cAMP targets either of these, we investigated the effect of cAMP in the presence of selective inhibitors of ROK or PKC. While Ca^{2+} sensitisation was induced by 10 μM CCh in the presence of 100 μM GTP at 1 μM $[Ca^{2+}]_i$, pre-application of 10 μM Y-27632 (ROK-selective inhibitor) reduced

the force development only marginally (16.2 ± 7.2 %; N = 3, n = 8). Additional application of cAMP (100 μM) caused a small reduction in force development (37.4 ± 17.2 %, N = 3, n = 8; Fig. 4Ab & B). Conversely, pre-application of 10 μM GF-109203X (PKC-selective inhibitor) markedly reduced the Ca^{2+} sensitisation by 62.4 ± 20.5 % (N = 3, n = 6). Additional application of cAMP (100 μM) caused a significant decrease in the residual force (94.0 ± 54.5 %; Fig. 4Ba & B). The relaxation effect of cAMP was significantly greater in the presence of GF-109203X than in the presence of Y-27632 (N = 3, n = 6~8, $P < 0.05$; Fig. 4Ab & Bb), even though GF-109203X predominantly attenuated the force development of Ca^{2+} sensitisation, as opposed to Y-27632.

Effect of cAMP on Tension Force Induced by Ca^{2+} Sensitisation-related Reagents and MLCP Inhibitors

To confirm the association of the underlying cAMP relaxation mechanism with the ROK pathway or PKC pathway, we used SPC (a ROK-selective activator) or PDBu (a PKC-selective activator) instead of CCh to induce Ca^{2+} sensitisation. SPC (1 μM) and PDBu (1 μM) induced Ca^{2+} sensitisation at fixed 1 μM $[Ca^{2+}]_I$, to 130.8 ± 25.1 % (N = 5, n = 21) and 192.6 ± 141.8 % (N = 4, n = 16), respectively. The relaxation effect of cAMP on SPC-induced contraction was similar to that observed in CCh-induced Ca^{2+} sensitisation in the presence of the PKC-selective inhibitor, GF109203X (Fig. 5A & D). In contrast, Ca^{2+} sensitisation induced by PDBu was not changed following application of 100 μM cAMP (N = 4, n = 16; Fig. 5B & D). While a potent inhibitor of MLCP, calyculin A (1 μM), also induced further contraction at fixed 1 μM $[Ca^{2+}]_i$, 100 μM cAMP failed to induce relaxation on tension development in human DSM (N = 2, n = 8; Fig. 5C & D).

Effects of Contractile-related Drugs on cAMP-induced Relaxation in Human DSM

To further investigate the cAMP relaxation mechanism, we employed the protein kinase A (PKA) inhibitor, H-89, calmodulin inhibitor, W-7, and phosphoinositide 3 kinase (PI3K) inhibitor, LY-294002. H-89 (10 μM) significantly decreased the cAMP-induced relaxation in force development at fixed 1 μM $[Ca^{2+}]_i$ (N = 4, n = 8; Fig. 6A & D). W-7 (100 μM) decreased the tension force induced by fixed 1 μM $[Ca^{2+}]_i$. However, the relaxation effect of cAMP was significantly increased by 401.4 ± 137.3 % in the presence of 100 μM W-7 (N = 4, n = 8; Fig. 6B & D). LY-294002 (10 μM) had no effect on cAMP-induced relaxation at fixed 1 μM $[Ca^{2+}]_i$ (N = 3, n = 6; Fig. 6C & D).

Effect of 6-Bnz-cAMP and 8-pCPT-2'-O-Me-cAMP on Tension Force Induced by Intracellular Calcium and CCh with GTP

To investigate the precise role of PKA and Epac, which are activated by cAMP and are downstream of the cAMP signalling cascade that leads to smooth muscle relaxation, we used the PKA-selective activator, 6-Bnz-cAMP (100 μM), and Epac-selective activator, 8-pCPT-2'-*O*-Me-cAMP (100 μM). 6-Bnz-cAMP induced 44.9 ± 16.1 % relaxation of the contractile response to 1 μM $[Ca^{2+}]_i$ (N = 5; n = 18). There was no significant difference in comparison with that relaxation induced by cAMP. 8-pCPT-2'-*O*-Me-cAMP slightly inhibited the 1 μM $[Ca^{2+}]_i$-induced contraction by 7.4 ± 5.6 % (N = 4; n = 15, Fig. 7A & C). The relaxation effects following 6-Bnz-cAMP and 8-pCPT-2'-*O*-Me-cAMP treatment in CCh-induced Ca^{2+}-sensitisation (100 μM GTP plus 10 μM CCh at fixed 1 μM $[Ca^{2+}]_i$) were inhibited by 15.7 ± 5.0 % (N = 4, n = 17) and by 7.9 ± 3.8 % (N = 5, n = 19), respectively. Although these relaxation by both 6-Bnz-cAMP and 8-pCPT-2'-*O*-Me-cAMP in CCh-induced

Ca^{2+}-sensitisation were attenuated in comparison with those observed in 1 μM $[Ca^{2+}]_i$, the predominant effect of 6-Bnz-cAMP over 8-pCPT-2'-*O*-Me-cAMP was unchanged (Fig. 7B & C).

Expression of Epac and its Related Proteins (Rac1, Rap1) in Human DSM

The expression of proteins involved the Epac pathway was examined. Epac 1 was expressed but Epac 2 was not. Epac-related proteins, Rac1 and Rap1, were also expressed in human DSM (Fig. 8).

DISCUSSION

The present study is the first to address the cAMP relaxation mechanism in the contraction ascribed to Ca^{2+} sensitization of human DSM. The relaxation effect of cAMP was observed in the tension development induced by the only increase of intracellular Ca^{2+} concentration. This relaxation was attenuated by CCh-induced Ca^{2+} sensitization due to the tolerant effect of PKC pathway to cAMP regardless ROK pathway was inhibited by cAMP using α-toxin permeabilized human DSMs. Furthermore, we identified the expression of Epac1, Rac1 and Rap1, which are cAMP-regulated proteins that induce smooth muscle relaxation independent of PKA in human DSM. The Epac signalling pathway in human DSM seems to be functionally minor compared with other smooth muscles (e.g. bronchial smooth muscle).

Investigations of cyclic nucleotide-dependent smooth muscle relaxation have focused on vascular smooth muscle [34]. Cyclic nucleotides (cAMP and cGMP) are the main second messengers that induce muscle relaxation under physiological conditions. Multiple effects of cyclic nucleotides have been reported. However, these effects are mediated through two major pathways as mentioned in introduction, shortly: (i) the decreased Ca^{2+}-dependent pathway due to the reduction of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) [1, 2, 3, 4]. (ii) The decrease of the Ca^{2+}-independent pathway; i.e. inhibition of Ca^{2+}-sensitisation [5, 6]. Although these two pathways have great effect on each other complicatedly, our present study using α-toxin permeabilization clearly demonstrated the novel effect of cAMP on Ca^{2+}-sensitisation in human DSM as discussed in the following section.

In smooth muscle, Ca^{2+} sensitivity can be modulated by MLCK or MLCP [35]. MLCP inhibition, in turn, leads to increased MLCK phosphorylation that enhances force development at constant $[Ca^{2+}]_i$, which is referred to as Ca^{2+}-sensitisation (Ca^{2+}-independent pathway), through two major pathways: the ROK and PKC pathways. The present study addressed the effect of cAMP on the ROK and/or PKC pathways. Activation of the ROK pathway but not the PKC pathway was inhibited by cAMP, when the Ca^{2+}-sensitisation was activated by CCh and GTP, as evidenced by our results following the use of a ROK-selective inhibitor (Y-27632) and activator (SPC) as well as a PKC-selective inhibitor (GF-109203X) and activator (PDBu) (Figs. 4 & 5).

Tension Force Induced by Constant-fixed Intracellular Calcium was Reduced by cAMP

Cyclic-AMP induced relaxation after α-toxin permeabilization, even in the tension force induced by only increase of $[Ca^{2+}]_i$ (Figs. 1, 2A, 6). This relaxation seemed to be similar to that observed in thoracic aortic smooth muscle, where it was assumed that cAMP abolished KCl (40 mM)-induced

contraction by inhibiting Ca^{2+}-mediated Rho activation dependent on PI3K class II α-isoform of (PI3K-C2α) [36]. In our study, the PI3K inhibitor, LY-294002, had no effect on Ca^{2+}-induced contraction of human DSMs (Fig. 6C & D). However, the relaxation effect of cAMP was enhanced by Y-27632 but markedly by GF-109203X in the tension force induced by only increase in $[Ca^{2+}]_i$. (data not shown), It is likely that just an increase in $[Ca^{2+}]_i$ activates not only ROK pathway but PKC pathway in human DSM (via a still unknown mechanism). On the other hand, PI3K reportedly mediates proliferation via M_3 receptors in human DSM, presumably related to bladder hypertrophy [37]. It is also of our another interest.

While the calmodulin selective inhibitor, W-7 [38] as well as the PKC-selective inhibitor, GF-109203X [21,29,31], , dominantly inhibited the force development evoked by only increase in $[Ca^{2+}]_i$, these agents increased the relaxation effects of cAMP , indicating that PKC and the Ca^{2+}-calmodulin complex counteract the relaxation effect of cAMP (Figs. 6B). It was proposed that increased cAMP levels reduce myofilament Ca^{2+} sensitivity by phosphorylating MLCK and thereby decreasing its affinity for the Ca^{2+}-calmodulin complex [38, 39]. This is the reason that W-7 inhibited the phosphorylation of MLCK, presumably supporting cAMP-induced MLCP activation.

Inhibition of CCh-induced Ca^{2+}-sensitisation by cAMP

Using α-toxin permeabilized human DSM, muscarinic stimulation (10 μM CCh and 100 μM GTP) induced further contraction in the presence of 1 μM $[Ca^{2+}]_i$ (i.e. Ca^{2+} sensitisation). However, the cAMP-induced relaxation effect was decreased by CCh-induced Ca^{2+}-sensitisation from 38% to 13%

(Figs.2A, D & 3). Physiologically, bladder contraction is regulated mainly by M_3 receptors. The M_3-selective inhibitor, 4-DAMP (1 μM), increased the relaxation effect by cAMP more than did the M_2-selective inhibitor, AF-DX116 (1 μM; Fig. 3). This is because CCh-induced PKC pathway activation was predominantly inhibited by 4-DAMP. On the other hand, the recovery by M_2 receptor inhibitor, AF-DX116 to the impaired relaxation effect by cAMP in Ca^{2+} sensitisation was weaker but significantly clear indicating that M_2 receptor counteracts cMAP signalling pathway in line with previous reports [13, 14, 15, 40]. The impaired relaxation effect by cAMP in Ca^{2+} sensitisation (10 μM CCh and 100 μM GTP) was recovered slightly by Y-27632 but markedly by GF-109203X (Fig. 4). These results demonstrated that cAMP inhibits Ca^{2+} sensitisation induced by the ROK pathway but not by the PKC pathway. This suggestion is further supported by the finding that cAMP inhibited tension development induced by SPC (ROK-selective agonist) but not by PDBu (PKC-selective agonist; Fig. 5), which was also observed in rat caudal arteries [39]. Taken together, these findings indicate that cAMP inhibits Ca^{2+} sensitisation induced by the ROK pathway; however, Ca^{2+} sensitisation induced by the PKC pathway is tolerant of cAMP.

In a previous study, we found that the PKC pathway was impaired in a BOO model, such as benign prostate hyperplasia (BPH) that induces overactive bladder (OAB) [29]. However, overwhelming enhancement of the ROK pathway induced a pathophysiological increase in Ca^{2+} sensitisation despite an impaired PKC pathway in guinea pig DSM [29]. Therefore, cAMP likely plays a major role in inducing bladder relaxation in the BOO model. Clinical use of a β_3 agonist or PDE IV inhibitor to enhance the effect of cAMP to treat OAB is encouraged by our present findings [18, 41]. Interestingly, cAMP signalling pathway was involved in even PDE V inhibitor sildenafil-induced

relaxation in human detrusor [20]. The comprehensive studies between cAMP and PDE inhibitors would be more required.

The role of Epac in human DSM

The present study also demonstrated for the first time the expression of Epac1 and Epac1-related proteins, Rap1 and Rac1, in human DSM. Epac, which mediates PKA-independent signal transduction, was discovered a decade ago [42, 43]. Two variants of Epac (Epac1 and Epac2) are ubiquitously expressed; Epac1 is abundant in the brain, heart, kidney, pancreas, spleen ovary, thyroid and spinal cord, whereas Epac2 is less expressed than Epac 1. However the expression of Epac2 is clarified in the specific region of the brain, adrenal glands, liver and pancreatic islets of Langerhans [44]. Recently, the expression and role of Epac was also clarified in airway, gastric, and vascular smooth muscle tissues of various species [43]. Activation of Epac by cAMP shifts the balance of Ras homolog gene family, member A (RhoA) and Rap1 (or Rac1) towards Rap1 (or Rac1). However, the functional role of Epac and its underlying mechanism in smooth muscle are just beginning to be defined. In comparison with other smooth muscle tissue, such as airway smooth muscle, it is unlikely that they play a significant physiological role in relaxation of human DSM. However it is still unknown how Epacs and their related proteins regulate Ca^{2+}-dependent/independent pathways and, are modulated by aging or obstruction, such as BOO. Further experiment would be required.

Conclusions

Here, we demonstrated the effect of cAMP on Ca^{2+} sensitisation and described the expression and role of Epac in human DSM. While cAMP inhibited Ca^{2+} sensitisation activated by the ROK pathway, the PKC pathway was tolerant of cAMP. A novel cAMP-activated protein, Epac1, and its related proteins, Rap1 and Rac1, were expressed in human DSM however they play a minor role. It is still unclear how they are affected by lower urinary tract dysfunction such as BOO (Fig. 9).

Conflicts of Interest

Dr. Kajioka reports grants from Astellas Pharma Inc., during the conduct of the study; grants from Astellas Pharma Inc., outside the submitted work; .

All other authors have nothing to disclose.

REFERENCES

1) Raeymaekers L, Eggermont JA, Wuytack F, Casteels R. Effects of cyclic nucleotide dependent protein kinases on the endoplasmic reticulum Ca^{2+} pump of bovine pulmonary artery. *Cell Calcium* 1990; 11(4): 261-268.

2) Mundiña-Weilenmann C, Vittone L, Rinaldi G, Said M, de Cingolani GC, Mattiazzi A. Endoplasmic reticulum contribution to the relaxant effect of cGMP- and cAMP-elevating agents in feline aorta. *Am J Physiol Heart Circ Physiol* 2000; 278(6): H1856-65

3) Robertson BE, Schubert R, Hescheler J, Nelson MT. cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am J Physiol* 1993; 265(1 Pt 1): C299-303.

4) Xin W, Li N, Cheng Q, Petkov GV. BK channel-mediated relaxation of urinary bladder smooth muscle: a novel paradigm for phosphodiesterase type 4 regulation of bladder function. *J Pharmacol Exp Ther* 2014; 349(1): 56-65.

5) Conti MA, Adelstein RS. The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5' cAMP-dependent protein kinase. *J Biol Chem* 1981; 256(7): 3178–3181.

6) Rembold CM. Regulation of contraction and relaxation in arterial smooth muscle. *Hypertension* 1992; 20(2): 129–137.

7) Himpens B, Matthijs G, Somlyo AV, Butler TM, Somlyo AP. Cytoplasmic free calcium, myosin light chain phosphorylation, and force in phasic and tonic smooth muscle. *J Gen Physiol* 1988; 92(6): 713-729.

8) Kitazawa T, Masuo M, Somlyo AP. G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proc Natl Acad Sci U S A* 1991; 88(20): 9307-9310.

9) Frazier EP, Mathy MJ, Peters SL, Michel MC. Does cyclic AMP mediate rat urinary bladder relaxation by isoproterenol? *J Pharmacol Exp Ther* 2005; 313(1): 260-267.

10) Longhurst PA, Briscoe JA, Rosenberg DJ, Leggett RE. The role of cyclic nucleotides in guinea-pig bladder contractility. *Br J Pharmacol* 1997; 121(8): 1665-1672.

11) Morita T, Tsujii T, Dokita S. Regional difference in functional roles of cAMP and cGMP in lower urinary tract smooth muscle contractility. *Urol Int* 1992; 49(4): 191-195.

12) Morita T, Ando M, Kihara K, Oshima H. Species differences in cAMP production and contractile response induced by beta-adrenoceptor subtypes in urinary bladder smooth muscle. *Neurourol Urodyn* 1993; 12(2): 185-190

13) Michel MC, Sand C. Effect of pre-contraction on β-adrenoceptor-mediated relaxation of rat urinary bladder. *World J Urol* 2009; 27(6): 711-715

14) Uchida H, Shishido K, Nomiya M, Yamaguchi O. Involvement of cyclic AMP-dependent and -independent mechanisms in the relaxation of rat detrusor muscle via beta-adrenoceptors. *Eur J Pharmacol* 2005; 518(2-3): 195-202.

15) Witte LP, de Haas N, Mammen M, et al. Muscarinic receptor subtypes and signalling involved in the attenuation of isoprenaline-induced rat urinary bladder relaxation. *Naunyn Schmiedebergs Arch Pharmacol* 2011; 384(6):555-563.

16) Schneider T, Fetscher C, Michel MC. Human Urinary Bladder Strip Relaxation by the β-Adrenoceptor Agonist Isoprenaline: Methodological Considerations and Effects of Gender and Age. *Front Pharmacol* 2011; 25(2): 11.

17) Truss MC, Uckert S, Stief CG, Kuczyk M, Jonas U. Cyclic nucleotide phosphodiesterase (PDE) isoenzymes in the human detrusor smooth muscle. II. Identification and characterization. *Urol Res* 1996; 24(3): 123-128.

18) Oger S, Behr-Roussel D, Gorny D, et al. Relaxation of phasic contractile activity of human detrusor strips by cyclic nucleotide phosphodiesterase type 4 inhibition. *Eur Urol* 2007; 51(3): 772-780.

19) Kaiho Y, Nishiguchi J, Kwon DD, et al. The effects of a type 4 phosphodiesterase inhibitor and the muscarinic cholinergic antagonist tolterodine tartrate on detrusor overactivity in female rats with bladder outlet obstruction. *BJU Int* 2008; 101(5): 615-620.

20) Oger S, Behr-Roussel D, Gorny D, et al. Signalling pathways involved in sildenafil-induced relaxation of human bladder dome smooth muscle. *Br J Pharmacol* 2010; 160(5): 1135-1143

21) Shahab N, Kajioka S, Seki N, Naito S. Functional role of muscarinic receptor subtypes in calcium sensitization and their contribution to rho-kinase and protein kinase C pathways in contraction of human detrusor smooth muscle. *Urology* 2012; 79(5): 1184.e7-13.

22) Zieba BJ, Artamonov MV, Jin L, et al. The cAMP-responsive Rap1 guanine nucleotide exchange factor, Epac, induces smooth muscle relaxation by down-regulation of RhoA activity. *J Biol Chem* 2011; 286(19): 16681-16692.

23) Rajagopal S, Kumar DP, Mahavadi S, et al. Activation of G protein-coupled bile acid receptor, TGR5, induces smooth muscle relaxation via both Epac- and PKA-mediated inhibition of RhoA/Rho kinase pathway. *Am J Physiol Gastrointest Liver Physiol* 2013; 304(5): G527-535.

24) Roscioni SS, Maarsingh H, Elzinga CR, et al. Epac as a novel effector of airway smooth muscle relaxation. *J Cell Mol Med* 2011; 15(7): 1551-1563.

25) Nishimura J, Kolber M, van Breemen C. Norepinephrine and GTP-gamma-S increase myofilament Ca^{2+} sensitivity in alpha-toxin permeabilized arterial smooth muscle. *Biochem Biophys Res Commun* 1988; 157(2): 677-683.

26) Takahashi R, Nishimura J, Hirano K, Seki N, Naito S, Kanaide H. Ca^{2+} sensitization in contraction of human bladder smooth muscle. *J Urol* 2004; 172(2): 748-752.

27) Shahab N, Kajioka S, Takahashi R, et al. Novel effect of 2-aminoethoxydiphenylborate through inhibition of calcium sensitization induced by Rho kinase activation in human detrusor smooth muscle. *Eur J Pharmacol* 2013; 708(1-3): 14-20.

28) Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV, Somlyo AP. Receptor-coupled, permeabilized smooth muscle. Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca^{2+}. *J Biol Chem* 1989; 264(10): 5339-5342.

29) Shahab N, Kajioka S, Takahashi-Yanaga F, et al. Obstruction enhances rho-kinase pathway and diminishes protein kinase C pathway in carbachol-induced calcium sensitization in contraction of α-toxin permeabilized guinea pig detrusor smooth muscle. *Neurourol Urodyn* 2012; 31(4): 593-599.

30) Uehata M, Ishizaki T, Satoh H, et al. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 1997; 389(6654): 990-994.

31) Toullec D1, Pianetti P, Coste H, et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. *J Biol Chem* 1991; 266(24): 15771-15781.

32) Christensen AE, Selheim F, de Rooii J, et al. AMP Analog Mapping of Epac1 and cAMP Kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J Biol Chem* 2003; 278(37): 35394-35402.

33) Rehmann H, Schwede F, Doskeland SO, et al. Ligand-mediated Activation of the cAMP-responsive Guanine Nucleotide Exchange Factor Epac. *J Biol Chem* 2003; 278(40): 38548-38556.

34) Morgado M, Cairrão E, Santos-Silva AJ, Verde I. Cyclic nucleotide-dependent relaxation pathways in vascular smooth muscle. *Cell Mol Life Sci* 2012; 69(2): 247-266.

35) Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994; 372(6503): 231-236.

36) Azam MA, Yoshioka K, Ohkura S, et al. Ca^{2+}-independent, inhibitory effects of cyclic adenosine 5'-monophosphate on Ca^{2+} regulation of phosphoinositide 3-kinase C2alpha, Rho, and myosin phosphatase in vascular smooth muscle. *J Pharmacol Exp Ther* 2007; 320(2): 907-916.

37) Arrighi N, Bodei S, Zani D, et al. Different muscarinic receptor subtypes modulate proliferation of primary human detrusor smooth muscle cells via Akt/PI3K and map kinases. *Pharmacol Res* 2013; 74: 1-6.

38) Asano M, Suzuki Y, Hidaka H. Effects of various calmodulin antagonists on contraction of rabbit aortic strips. *J Pharmacol Exp Ther* 1982; 220(1): 191-196.

39) Grassie ME, Sutherland C, Ulke-Lemée A, et al. Cross-talk between Rho-associated kinase and cyclic nucleotide-dependent kinase signaling pathways in the regulation of smooth muscle myosin light chain phosphatase. *J Biol Chem* 2012; 287(43): 36356-36369.

40) Ehlert FJ1, Ahn S, Pak KJ, et al. Neuronally released acetylcholine acts on the M_2 muscarinic receptor to oppose the relaxant effect of isoproterenol on cholinergic contractions in mouse urinary bladder. *Pharmacol Exp Ther* 2007; 322(2): 631-637.

41) Yamaguchi O, Marui E, Kakizaki H, et al. Phase III, randomised, double-blind, placebo-controlled study of the β_3-adrenoceptor agonist mirabegron, 50 mg once daily, in Japanese patients with overactive bladder. *BJU Int* 2014; 113(6): 951-960.

42) de Rooij J, Zwartkruis FJ, Verheijen MH, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 1998; 396(6710): 474-477.

43) Kawasaki H, Springett GM, Mochizuki N, et al. A family of cAMP-binding proteins that directly activate Rap1. *Science* 1998; 282(5397): 2275-2279.

44) Holz GG, Kang G, Harbeck M, Roe MW, Chepurny OG. Cell physiology of cAMP sensor Epac. *J Physiol* 2006; 577(Pt 1): 5-15.

ACKNOWLEDGEMENTS

This work was supported by a Grant-Aid for Special Purposes (23592371 and 26462445) from the Japan Society for the Promotion of Science. We thank Prof. Alexaj Verkhratsky, University of Manchester, for proofreading the manuscript and useful discussion. Noriko Hakota and Eriko Gunshima assisted with experiments. Dr. Hirofumi Koga, Dr. Hiroyuki Nomura and Mrs. Ikumi Takeuchi cooperated to obtain tissue from patients at Harasanshin Hospital. The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: **http://www.textcheck.com/certificate/X06oxy**

FIGURE LEGENDS

Figure 1

Effects of cAMP on contraction induced by cumulative $[Ca^{2+}]_i$ addition in α-toxin permeabilized human detrusor smooth muscle.

Representative traces obtained from α-toxin permeabilized human detrusor smooth muscle following the cumulative addition of Ca^{2+} in the absence (A) and presence (B) of 100 μM cAMP are shown. The mean concentration-response curves for tension force to $[Ca^{2+}]_i$ without (open circle) and with (filled circle) 100 μM cAMP are shown in C. The tension force is expressed in a relative manner; the tension force obtained at 3 μM $[Ca^{2+}]_i$ is normalised as 100%. Error bars indicate means ± SD. Asterisks indicate a significant difference ($P < 0.05$; N = 3, n = 6).

Figure 2

Effects of cyclic adenosine monophosphate (cAMP), forskolin (FSK) and rolipram on Ca^{2+}-induced contraction in α-toxin permeabilized human detrusor smooth muscle (DSM).

Representative traces demonstrate the effects of 100 μM cAMP (A), 10 μM FSK (B), and 10 μM rolipram (C) on 1 μM $[Ca^{2+}]_i$-induced contraction in intact human detrusor smooth muscle. Graph summarises the relaxation effect of cAMP, FSK and rolipram. The maximum tension force at 1 μM $[Ca^{2+}]_i$ was normalised as 100%. Columns represent means ± SD (N = 3 - 5, n = 6 - 20) (D).

Figure 3

Effects of cAMP on Ca^{2+} sensitisation induced by guanosine-5'-triphosphate (GTP) plus carbachol (CCh) in the absence and presence of AF-DX116 or 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP) in α-toxin permeabilized human detrusor smooth muscle.

Representative traces show the effects of 100 μM cAMP addition on Ca^{2+} sensitisation induced by GTP plus CCh in the absence or presence of 1 μM AF-DX (A) and 1 μM 4-DAMP (B) in α-toxin permeabilized human detrusor smooth muscle. Graph summarises the relaxation effect of cAMP with (N = 3, n = 11) or without AF-DX (N = 3, n = 11) and 4-DAMP (N = 3, n = 10). Columns represent means ± S.E. Only *P* values indicative of significance are presented.

Figure 4

Effects of cAMP on Ca^{2+} sensitisation induced by GTP plus carbachol (CCh) in the absence and presence of Y-27632 or GF109203X in α-toxin permeabilized human detrusor smooth muscle.

Representative traces show the effects of 100 μM cAMP on Ca^{2+} sensitisation induced by 100 μM GTP and 10 μM CCh in the absence or presence of 10 μM Y-27632 (Aa) or GF-109203X (Ba) in α-toxin permeabilized human detrusor smooth muscle. Each graph shows the relationship between the relaxation effect of 100 μM cAMP and the developed tension force induced by Ca^{2+} sensitisation (100 μM GTP and 10 μM CCh) obtained from each skinned fibre. The developed tension force induced by GTP and CCh, and the cAMP-induced relaxation values are expressed in a relative manner; Ca^{2+} (1 μM)-induced tension force is normalised as 1.0. The relative values before (open circle) and after

(filled circle) application of 10 μM Y-27632 (Ab) or 10 μM GF-109203X (Bb) obtained from the same strip were plotted and connected with lines since Ca^{2+} sensitisation-enhancement by GTP plus CCh varies among individual detrusor strips (21). Inner graphs demonstrate the relative value average before and after application of Y-27632 or GF-109203X. Asterisks indicate significant differences within the same strip, as compared with its own control. (Y-27632, N = 3, n = 8, $P = 0.0011$; GF-109203X, N = 3, n = 6, $P = 0.015$).

Figure 5

Effects of cAMP on tension force induced by sphingosylphosphorylcholine (SPC), phorbol 12,13 dibutyrate (PDBu) or calyculin A in α-toxin permeabilized human detrusor smooth muscle.

Representative traces show the effects of 100 μM cAMP on tension force activated by 1 μM SPC (A), 1 μM PDBu (B) or 1 μM calyculin A (C) at 1 μM $[Ca^{2+}]_i$ in α-toxin permeabilized human detrusor smooth muscle. (D) The graph summarises normalised cAMP-induced relaxation following augmentation by 1 μM SPC (N = 5, n = 21), 1 μM PDBu (N = 4, n = 16) or 1 μM calyculin A (N = 2, n = 8). Columns represent means ± SE.

Figure 6

Effects of cAMP on Ca^{2+}-induced contraction in the presence of a protein kinase A (PKA) inhibitor, calmodulin antagonist, or PI3K inhibitor in α-toxin permeabilized human detrusor smooth muscle.

Representative traces show the effects of 100 μM cAMP on 1 μM Ca^{2+}-induced contraction in the presence or absence of 10 μM H-89 (A), 100 μM W-7 (B) or 10 μM LY-294002 (C) in α-toxin permeabilized human detrusor smooth muscle. Graph shows the relative cAMP-induced relaxation value following 10 μM H-89 (slashed column; N=4, n=8), 100 μM W-7 (dotted column; N = 4, n = 8) or 10 μM LY-294002 (open column; N = 3, n = 6). Relaxation without any reagents (control; filled column) is normalised as 100%. Columns represent means ± SE. Only *P* values indicative of significance are presented.

Figure 7

Effects of cAMP analogues on Ca^{2+}-induced contraction in α-toxin permeabilized human detrusor smooth muscle.

Representative traces show the effects of 100 μM cAMP (Aa), 100 μM 6Bnz-cAMP (Ab) and 100 μM 8pCPT-2'-O-Me-cAMP (Ac) on 1 μM Ca^{2+}-induced contraction, and the effects of 100 μM 6Bnz-cAMP (Ba) and 100 μM 8pCPT-2'-O-Me-cAMP (Bb) on Ca^{2+} sensitisation (100 μM GTP plus 10 μM CCh at fixed 1 μM $[Ca^{2+}]_i$) in α-toxin permeabilized human detrusor smooth muscle. Columns show the relaxation effects of cAMP (filled column), 6Bnz-cAMP (slashed column) and 8pCPT-2'-O-Me-cAMP (open column) in C. Columns represent means ± SE. The relaxation effect of 6Bnz-cAMP was insignificant compared with the relaxation effect of cAMP ($P = 0.32$ on 1 μM Ca^{2+}-induced contraction; N = 5, n = 18, $P = 0.075$ on Ca^{2+} sensitisation; N = 4, n = 11). In contrast, the relaxation effect of 8pCPT-2'-O-Me-cAMP was significantly different compared with the relaxation effect of cAMP ($P < 0.0001$ on 1 μM Ca^{2+}-induced contraction; N = 4, n = 17, $P = 0.00017$ on Ca^{2+} sensitisation; N = 5, n = 19).

Figure 8

Western blot examination of exchange protein activated by cAMP (Epac)1, Epac2, Ras-related protein 1 (Rap1) and Ras-related C3 botulinum toxin substrate 1 (Rac1) in human detrusor smooth muscle. Various mouse organs (brain, pancreas, kidney and detrusor smooth muscle) were used as positive and negative controls. Arrows indicate the expected molecular weights (MW) of target proteins.

Figure 9

Schematic diagram illustrating plausible mechanisms underlying the relationship between cAMP and a Ca^{2+} sensitisation-related kinase.; FSK, forskolin

Table 1

chemical reagents	principle action
A-23187	Ca^{2+} ionophore
AF-DX116	selective muscarinic receptor 2 (M_2) inhibitor
6-Bnz-cAMP	selective protein kinase A (PKA) activator
calyculin A	myosin light chain phosphatase (MLCP) inhibitor
carbachol (CCh)	selective muscarinic receptor agonist
cyclopiazonic acid (CPA)	inhibitor of endo-(or sarco-)plasmic reticulum Ca^{2+}-ATPase
4-DAMP	selective muscarinic receptor 3 (M_3) inhibitor
dibutyryl-cAMP	membrane-permeable cAMP
forskolin	adenylyl cyclase activator
GF-109203X	selective protein kinase C (PKC) inhibitor
H-89	selective protein kinase A (PKA) inhibitor
LY-294002	selective phosphoinositide 3 kinase (PI3K) inhibitor
8-pCPT-2'-*O*-Me-cAMP	selective Epac activator
phorbol 12, 13-dibutyrate (PDBu)	selective protein kinase C (PKC) activator
rolipram	selective phosphodiesterase (PDE) IV inhibitor
sphingosylphosphorylcholine (SPC)	selective rho kniase (ROK) activator
W-7	selective calmodulin inhibitor
Y-27632	selective rho kniase (ROK) inhibitor

Fig. 1

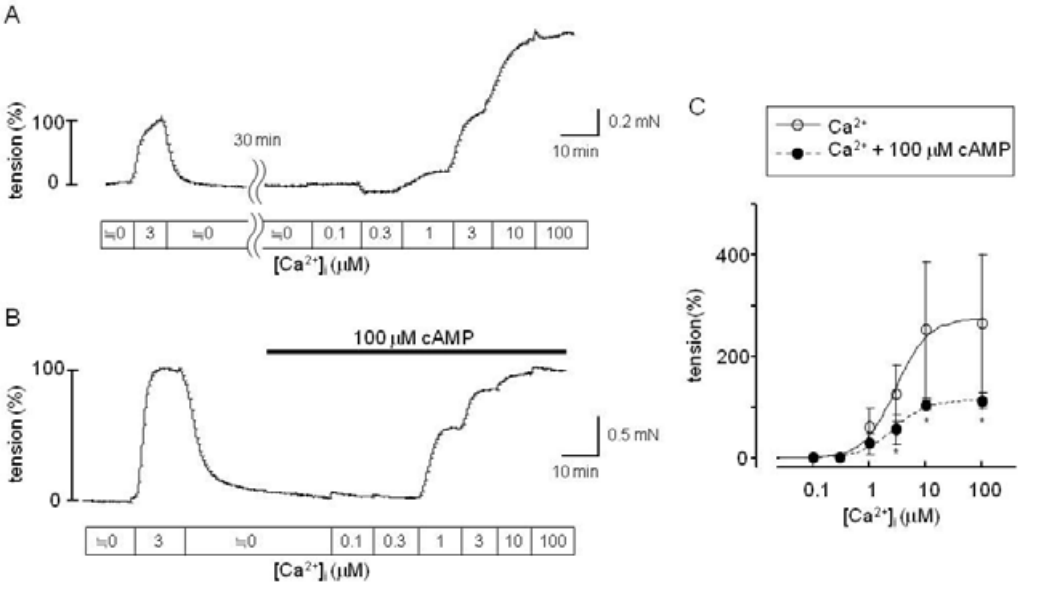


Fig. 2

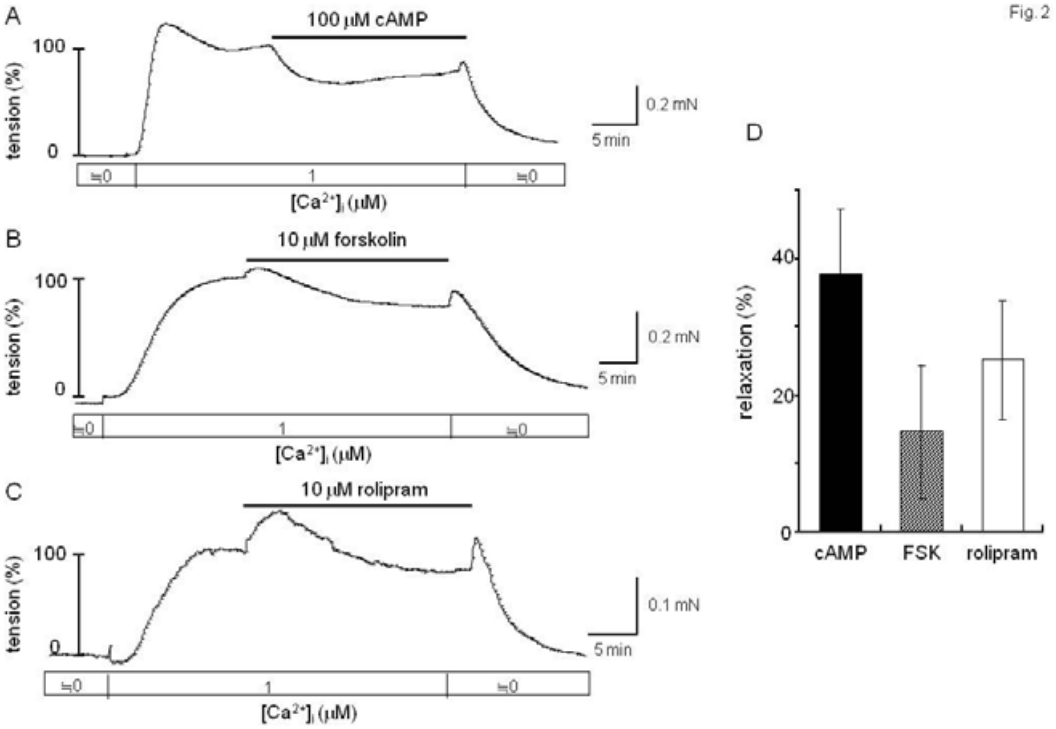


Fig. 3

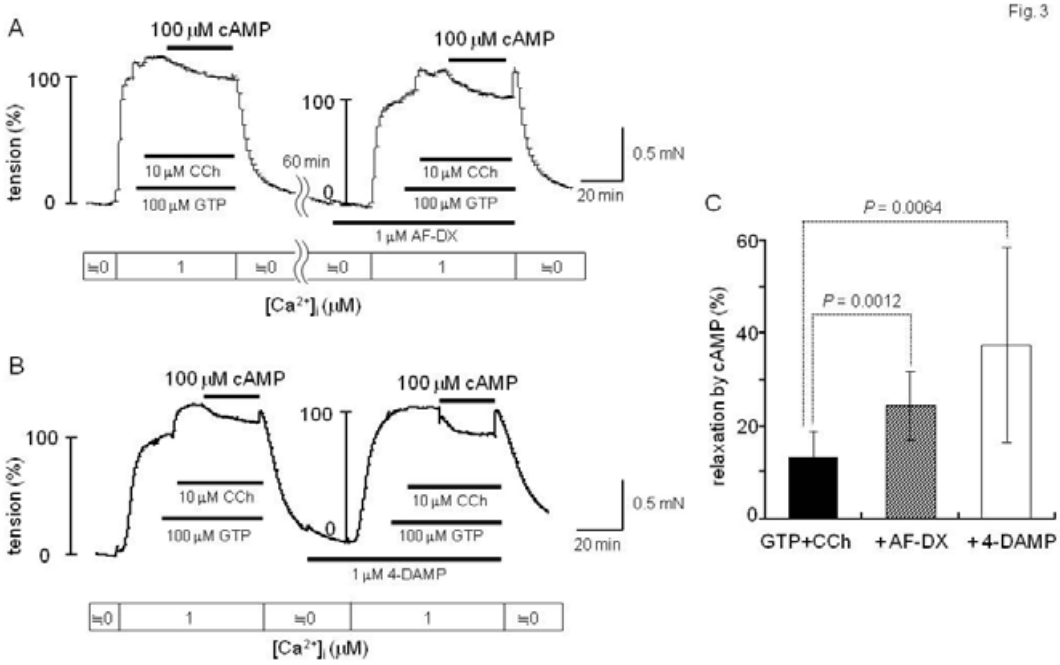


Fig. 4

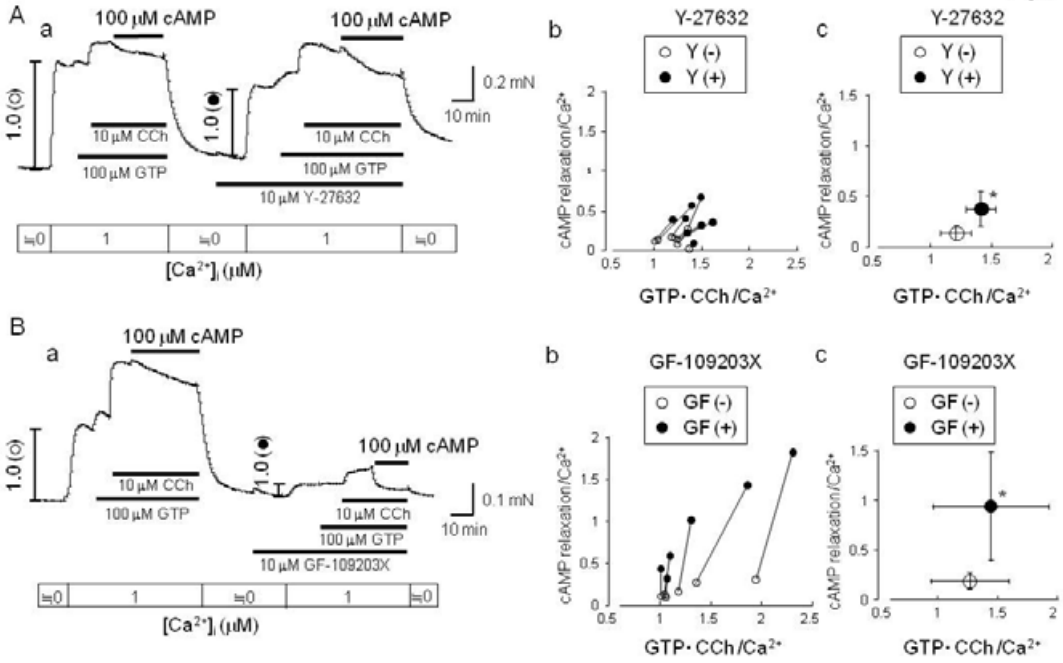


Fig. 5

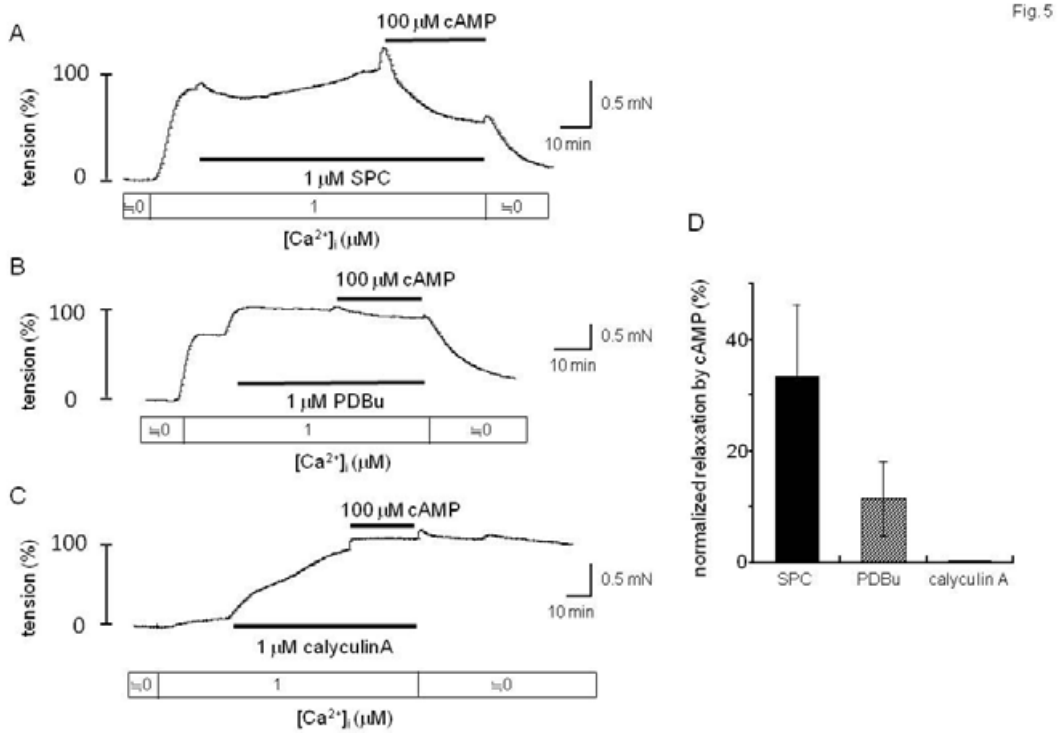


Fig. 6

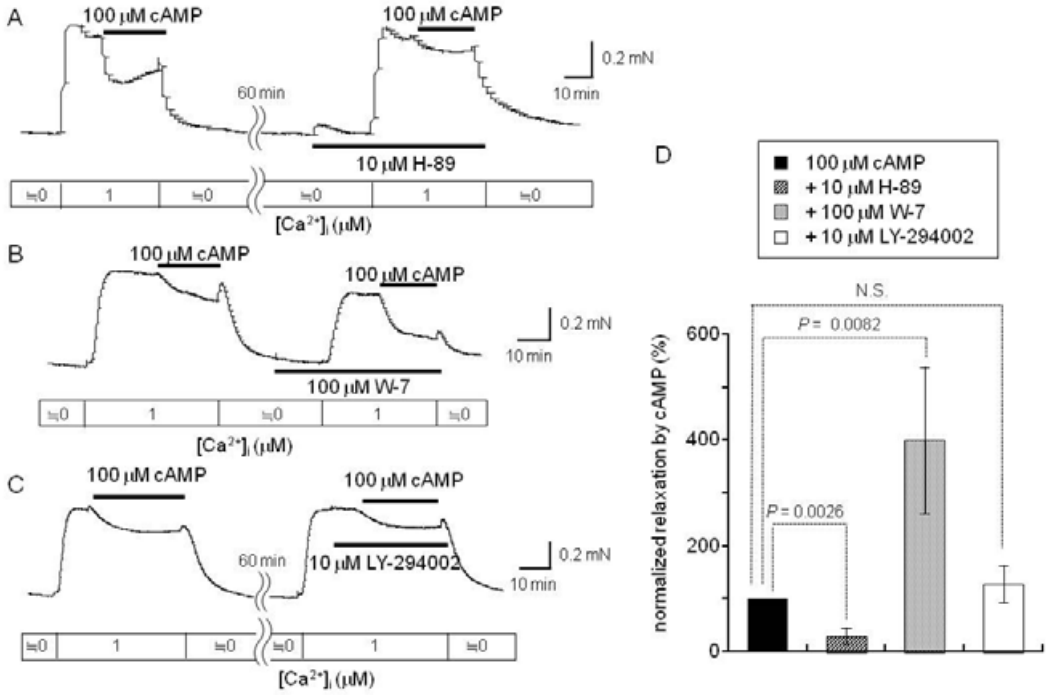


Fig. 7

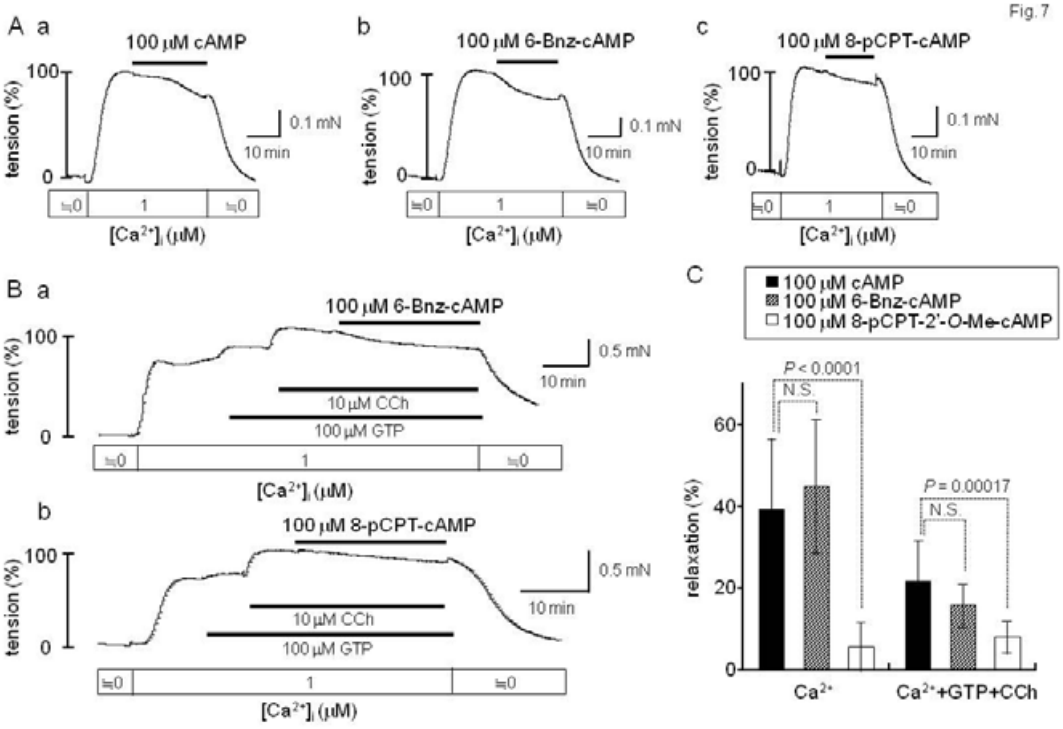
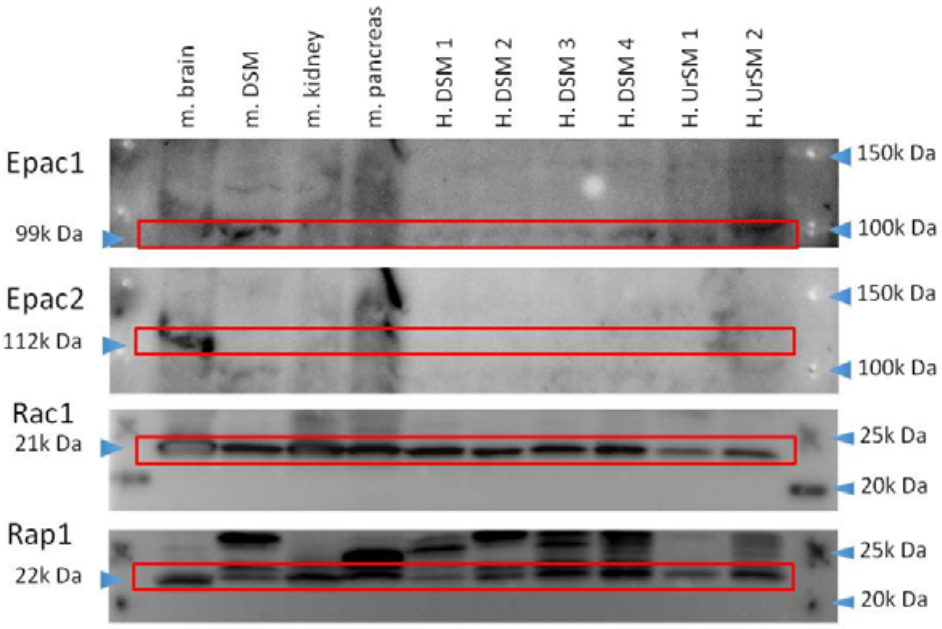


Fig.8



*m.: mouse, *H. Human,
*DSM: detrusor smooth muscle,
*UrSM: urethra smooth muscle

Fig.9