

Principal role of adenylyl cyclase 6 in K^+ channel regulation and vasodilator signalling in vascular smooth muscle cells

Carl P. Nelson¹, Richard D. Rainbow², Jennifer L. Brignell¹, Matthew D. Perry¹, Jonathon M. Willets^{1,3}, Noel W. Davies¹, Nicholas B. Standen¹, and R.A. John Challiss^{1*}

¹Department of Cell Physiology and Pharmacology, University of Leicester, Henry Wellcome Building, Lancaster Road, Leicester LE1 9HN, UK; ²Department of Cardiovascular Sciences, University of Leicester, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK; and ³Department of Cancer Studies and Molecular Medicine, University of Leicester, Robert Kilpatrick Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, UK

Received 19 January 2011; revised 6 May 2011; accepted 12 May 2011; online publish-ahead-of-print 23 May 2011

Time for primary review: 28 days

Aims	Membrane potential is a key determinant of vascular tone and many vasodilators act through the modulation of ion channel currents [e.g. the ATP-sensitive potassium channel (K_{ATP})] involved in setting the membrane potential. Adenylyl cyclase (AC) isoenzymes are potentially important intermediaries in such vasodilator signalling pathways. Vascular smooth muscle cells (VSMCs) express multiple AC isoenzymes, but the reason for such redundancy is unknown. We investigated which of these isoenzymes are involved in vasodilator signalling and regulation of vascular ion channels important in modulating membrane potential.
Methods and results	AC isoenzymes were selectively depleted (by >75%) by transfection of cultured VSMCs with selective short interfering RNA sequences. AC6 was the predominant isoenzyme involved in vasodilator-mediated cAMP accumulation in VSMCs, accounting for ~60% of the total response to β -adrenoceptor (β -AR) stimulation. AC3 played a minor role in β -AR signalling, whereas AC5 made no significant contribution. AC6 was also the principal isoenzyme involved in β -AR-mediated protein kinase A (PKA) signalling (determined using the fluorescent biosensor for PKA activity, AKAR3) and the substantial β -AR/PKA-dependent enhancement of K_{ATP} current. K_{ATP} current was shown to play a vital role in setting the resting membrane potential and in mediating the hyperpolarization observed upon β -AR stimulation.
Conclusion	AC6, but not the closely related AC5, plays a principal role in vasodilator signalling and regulation of the membrane potential in VSMCs. These findings identify AC6 as a vital component in the vasodilatory apparatus central to the control of blood pressure.
Keywords	Adenylyl cyclase • Cyclic AMP • Vascular smooth muscle • ATP-sensitive potassium channel • β -Adrenoceptor

1. Introduction

Vascular tone is determined by the contractile state of the smooth muscle cells within the vessel wall and, as such, represents the balance between vasodilator and vasoconstrictor influences. A variety of endogenous vasodilators can act via G protein-coupled receptors (GPCRs) present in vascular smooth muscle cells

(VSMCs) to mediate vasodilation. Vasodilator-activated GPCRs, such as β -adrenoceptors (β -ARs), primarily signal through G_s proteins to stimulate cyclic AMP (cAMP) synthesis by adenylyl cyclase (AC) isoenzymes. Stimulation of cAMP-dependent protein kinase (PKA) leads to the phosphorylation of a variety of cellular targets, including the activation of a number of K^+ channels present in VSMCs, leading to hyperpolarization and vasodilation.^{1,2}

* Corresponding author. Tel: +44 116 2297146; fax: +44 116 2525045, Email: jc36@le.ac.uk

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2011. For permissions please email: journals.permissions@oup.com.

The online version of this article has been published under an open access model. Users are entitled to use, reproduce, disseminate, or display the open access version of this article for non-commercial purposes provided that the original authorship is properly and fully attributed; the Journal, Learned Society and Oxford University Press are attributed as the original place of publication with correct citation details given; if an article is subsequently reproduced or disseminated not in its entirety but only in part or as a derivative work this must be clearly indicated. For commercial re-use, please contact journals.permissions@oup.com.

VSMC tone is highly dependent on the membrane potential, which is principally determined by the activity of K^+ channels. ATP-sensitive K^+ (K_{ATP}) channels, which consist of hetero-octamers of four pore-forming subunits (encoded by members of the Kir6 family) and four additional sulphonylurea subunits,² are sensitive to the metabolic status of the cell, being closed by the binding of intracellular ATP. The K_{ATP} channel contributes to the resting membrane potential of VSMCs, as its pharmacological blockade increases basal tone in both coronary³ and systemic⁴ circulations. In addition, genetically modified mice lacking a functional vascular K_{ATP} channel display elevated blood pressure, coronary vasospasm, and sudden death,^{5,6} although the vascular basis of this phenotype has been questioned.⁷

K_{ATP} channel activation by a variety of vasodilators has been observed,¹ with several reports implicating K_{ATP} channel activation in β -AR-mediated vasodilation in a range of vascular beds.^{4,8} Mice lacking the β_2 -AR develop hypertension during exercise,⁹ whereas spontaneously hypertensive rats display impaired vascular β -AR responses,¹⁰ and a subset of hypertensive patients exhibit compromised β -AR-mediated vasodilation.¹¹ In addition, single-nucleotide polymorphisms in the β_2 -AR gene are associated with impaired vasodilator responses to β -AR stimulation.¹² Along with other vasodilator signals, β -AR activation has been shown to enhance K_{ATP} channel activity via stimulation of PKA, resulting from AC-mediated cAMP generation.¹³

The AC family comprises nine membrane-bound (ACs 1–9) and one soluble (sAC) isoenzymes, each with distinct expression and regulatory profiles.¹⁴ In common with most other cell types, VSMCs express multiple AC isoenzymes, with strongest evidence for ACs 3, 5, and 6.^{15–17} Reasons for such apparent redundancy are unclear and little is known regarding which of these isoenzymes mediate the functional effects of vasodilator signalling in the vasculature. Previous studies have focused on AC isoenzyme expression rather than function^{16,17} or have suggested potential vasodilator GPCR-AC isoenzyme functional coupling based on caveolar co-localization,^{15,18} or the recombinant over-expression of AC isoenzymes.¹⁹

Here, we have investigated the roles of a number of the most highly expressed AC isoenzymes in β -AR-mediated signalling in VSMCs. Our findings indicate that AC6 is the primary endogenous AC isoenzyme involved in β -AR-mediated cAMP/PKA signalling and activation of the K_{ATP} current, which plays a key role in setting the membrane potential and thereby regulating vascular tone. In contrast, the closely related AC5 plays no discernible part in this signalling cascade in VSMCs, highlighting an important functional distinction between these two closely related isoenzymes.

2. Methods

2.1 VSMC isolation, culture, and transfection

Adult male Wistar rats (150–400 g) were killed by stunning and cervical dislocation. The care of animals was in accordance with the UK Animals (Scientific Procedures) Act 1986. The investigation also conforms to the Guide for Care and Use of Laboratory Animals US (NIH Publication No. 85-23, revised 1996). Dissection and enzymatic digestion was carried out as previously described for aortic smooth muscle cells.²⁰ VSMCs were cultured in Dulbecco's modified Eagle's medium (with glutamax) supplemented with 10% foetal calf serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 2.5 μ g/mL amphotericin B. Cells were transfected with short interfering (si)RNA 48 h prior to experimentation using the Lonza nucleofection device.

2.2 RT-PCR and western blotting

RT-PCR was performed as previously reported²¹ for AC1–8 isoenzymes. PCR amplification was performed using a LightCycler instrument (Roche Applied Science). Cycling conditions were: 95°C (600 s), followed by 45 cycles of 95°C (10 s), 60°C (10 s), and 70°C (1 s). Data were presented as ΔC_T [C_T (negative-control siRNA-transfected cells) – C_T (AC-targeting siRNA-transfected cells)] values. AC3 protein expression was determined by immunoblotting as described previously.¹⁸ Cell lysates were electrophoretically separated and transferred to a nitrocellulose membrane. AC3 expression was assessed using a specific anti-rabbit polyclonal antibody against AC3 (Santa Cruz Biotechnology, CA, USA) and detected by the addition of enhanced chemiluminescence-plus (ECL-plus) reagent (GE Healthcare, UK) and exposure to Hyperfilm (GE Healthcare). Relative protein expression was quantified using the GeneGnome image analysis system (Syngene, Cambridge, UK).

2.3 cAMP determination

Cells were pre-incubated with the non-selective phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX, 300 μ M) for 10 min, followed by stimulation with agonist for 5 min. Samples were extracted on ice in 0.5 M TCA and, following neutralization, were assayed for cAMP by competition assay, as described previously.²²

2.4 Fluorescence resonance energy transfer experiments

VSMCs expressing AKAR3 constructs²³ were imaged using a Zeiss Axiovert 200 microscope (Carl Zeiss, Welwyn Garden City, UK). Cells were excited at 440 nm and fluorescence resonance energy transfer (FRET) ratios measured as a change in the ratio of fluorescence intensities at 470 and 535 nm. Images were acquired and processed using MetaFluor software (Molecular Devices, Downingtown, PA, USA).

2.5 Electrophysiological experiments

Whole-cell K_{ATP} currents from acutely dissociated VSMCs were recorded at –60 mV (with external K^+ raised to 140 mM) as reported previously.¹⁸ Currents from cultured VSMCs (visually identified as transfected by eGFP fluorescence) were recorded at 0 mV using a modified version of the technique reported by Yuan *et al.*²⁴ All experiments were performed at 30–32°C.

2.6 Data analysis and statistics

Data are presented throughout as means $\pm$ SEM from four or more experiments obtained in different preparations. Statistical comparisons used Student's paired or unpaired *t*-test or one- or two-way ANOVA followed by appropriate *post hoc* testing (GraphPad Prism, San Diego, CA, USA).

See also Supplementary material online for further details of experimental procedures.

3. Results

3.1 Activation of the β -AR hyperpolarizes VSMCs via K_{ATP} activation

In acutely dissociated VSMCs, the mean membrane potential recorded in whole-cell current clamp under control conditions was -35.1 ± 1.4 mV ($n = 8$; Figure 1A and B). Stimulation of the endogenous β -AR population with isoprenaline (ISO; 100 nM) caused a substantial hyperpolarization (to -62.5 ± 3.3 mV; $n = 8$; Figure 1A and B), which was reversed on addition of the K_{ATP} channel blocker glibenclamide (10 μ M); indeed, the membrane potential in the presence of ISO and glibenclamide (-22.0 ± 1.5 mV; $n = 8$) was significantly

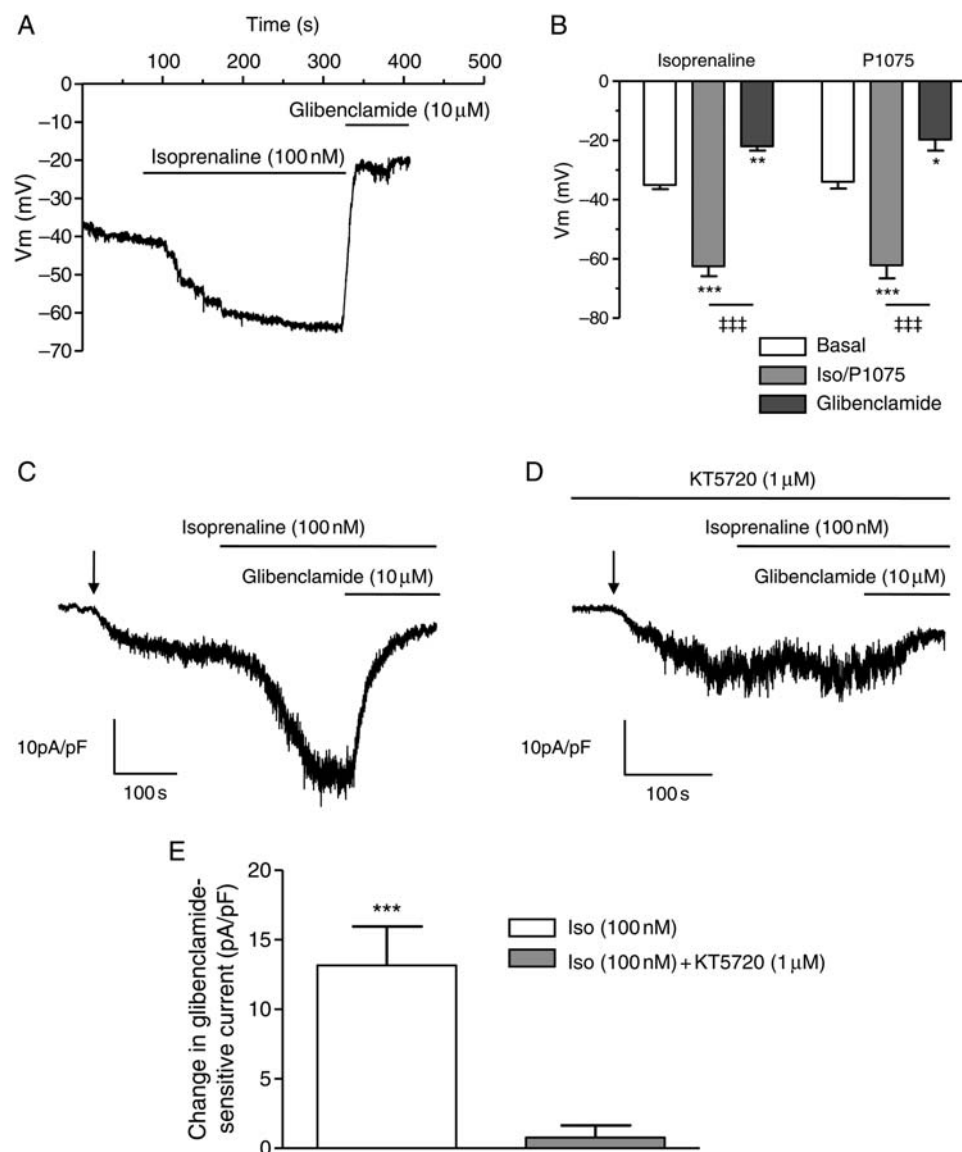


Figure 1 β -AR stimulation hyperpolarizes VSMCs, via activation of K_{ATP} current. (A) ISO (100 nM)-mediated hyperpolarization of acutely dissociated VSMCs and its reversal by the K_{ATP} channel blocker glibenclamide (10 μ M). (B) Mean data for the effects of ISO (100 nM) or the K_{ATP} channel opener P1075 (10 μ M) on membrane potential in the absence and presence of glibenclamide (10 μ M). (C and D) K_{ATP} current recordings in response to ISO (100 nM) in the absence (C) and presence (D) of the PKA inhibitor KT5720 (1 μ M). The arrow indicates the point at which extracellular K^+ was switched to 140 mM. (E) Mean data for ISO (100 nM)-mediated change in glibenclamide-sensitive K_{ATP} current in the absence and presence of KT5720 (1 μ M). Data are expressed as means $\pm$ SEM from $n \geq 6$ cells, from three or more preparations. Statistical significance is indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. basal; ††† $P < 0.001$ for ISO/P1075 $\pm$ glibenclamide.

depolarized relative to control ($P < 0.01$, one-way ANOVA, Bonferroni's *post hoc* test; Figure 1A and B). Similar effects were observed using the specific K_{ATP} channel opener P1075 (10 μ M; Figure 1B). VSMC resting membrane potential can be influenced by K_{ATP} channel activity and β -AR activation causes a marked hyperpolarization by enhancing the K_{ATP} current.

We further investigated this by performing whole-cell recordings of the K_{ATP} current in acutely dissociated VSMCs. On switching to 140 mM extracellular K^+ (indicated by the arrow in Figure 1C and D), spontaneous, glibenclamide-sensitive currents developed, reflecting basal K_{ATP} channel activity. The magnitude of this current was not significantly altered by pre-incubation with the PKA inhibitor

KT5720 (1 μ M) (data not shown). The addition of 100 nM ISO revealed a substantial increase in the glibenclamide-sensitive current (increased by 13.2 ± 2.8 pA/pF; $n = 6$, $P < 0.001$, two-way ANOVA, Bonferroni's *post hoc* test; Figure 1C and E). In the presence of the PKA inhibitor, KT5720 (1 μ M), the addition of ISO had no significant effect (change in current of 0.8 ± 0.9 pA/pF; $n = 6$; Figure 1D and E). Similar data were obtained with another PKA inhibitor, Rp-cAMPS (100 μ M), where the glibenclamide-sensitive current was unaltered by the addition of ISO (change in current of 1.1 ± 0.7 pA/pF; $n = 5$). Endogenous VSMC K_{ATP} current is therefore significantly enhanced by β -AR stimulation in a PKA-dependent manner.

3.2 Endogenous AC expression and siRNA-mediated knockdown in VSMCs

Due to a lack of suitably selective antibodies for many of the AC isoenzymes, we investigated the AC expression profile in VSMCs by

Table 1 AC expression in VSMCs

AC isoenzyme	Acutely dissociated VSMCs	Cultured VSMCs
1	42.5 ± 1.6 (5)	37.1 ± 0.7 (6)
2	37.8 ± 2.7 (5)	29.0 ± 0.1 (6)
3	28.9 ± 1.1 (5)	24.4 ± 0.4 (7)
4	35.1 ± 1.1 (5)	36.1 ± 1.3 (6)
5	28.4 ± 0.6 (5)	29.7 ± 0.6 (7)
6	26.7 ± 0.6 (5)	26.8 ± 0.6 (7)
7	26.5 ± 0.7 (4)	24.2 ± 0.7 (6)
8	40.7 ± 3.0 (5)	28.5 ± 0.8 (6)

RT-PCR for AC isoenzyme expression in acutely dissociated and cultured VSMCs. Data are expressed as mean cycle threshold (C_T) values ± SEM from four or more separate preparations (number indicated in parentheses). Highly expressed (C_T value <30) isoenzymes are highlighted in bold.

RT-PCR. Mean cycle threshold (C_T) values are presented for transcripts corresponding to AC1–8, indicating relatively high transcript levels of AC3 and 5–7 in acutely dissociated VSMCs (Table 1). A broadly similar profile was observed in SMCs acutely isolated from the mesenteric arteries (data not shown), a resistance arterial bed. In VSMCs maintained in culture for between three and six passages, C_T values corresponding to AC3 and 5–7 again indicated that these transcripts are expressed at high levels in cultured VSMCs, although there was some indication that AC2 and 8 transcripts were higher in passaged VSMCs (Table 1).

siRNA sequences designed specifically to target AC3, 5, 6, or a negative-control (scrambled) sequence were transfected into VSMCs, using the Lonza nucleofection system, to obtain high (>90%) levels of transfection efficiency with siRNA (see Supplementary material online, Figure S1). AC isoenzymic expression was assessed 48 h later by RT-PCR (Figure 2). AC3 expression was suppressed in the presence of AC3-targeting siRNA (ΔC_T = −3.2), whereas AC5–7 were unaffected (Figure 2A–D). Similarly, levels of AC5 and 6 were selectively reduced by their respective siRNA sequences (Figure 2A–D). siRNA-mediated suppression of AC3 could be confirmed at the protein level, by immunoblotting with an AC3-specific antibody (Figure 2E). The mean AC3 expression in

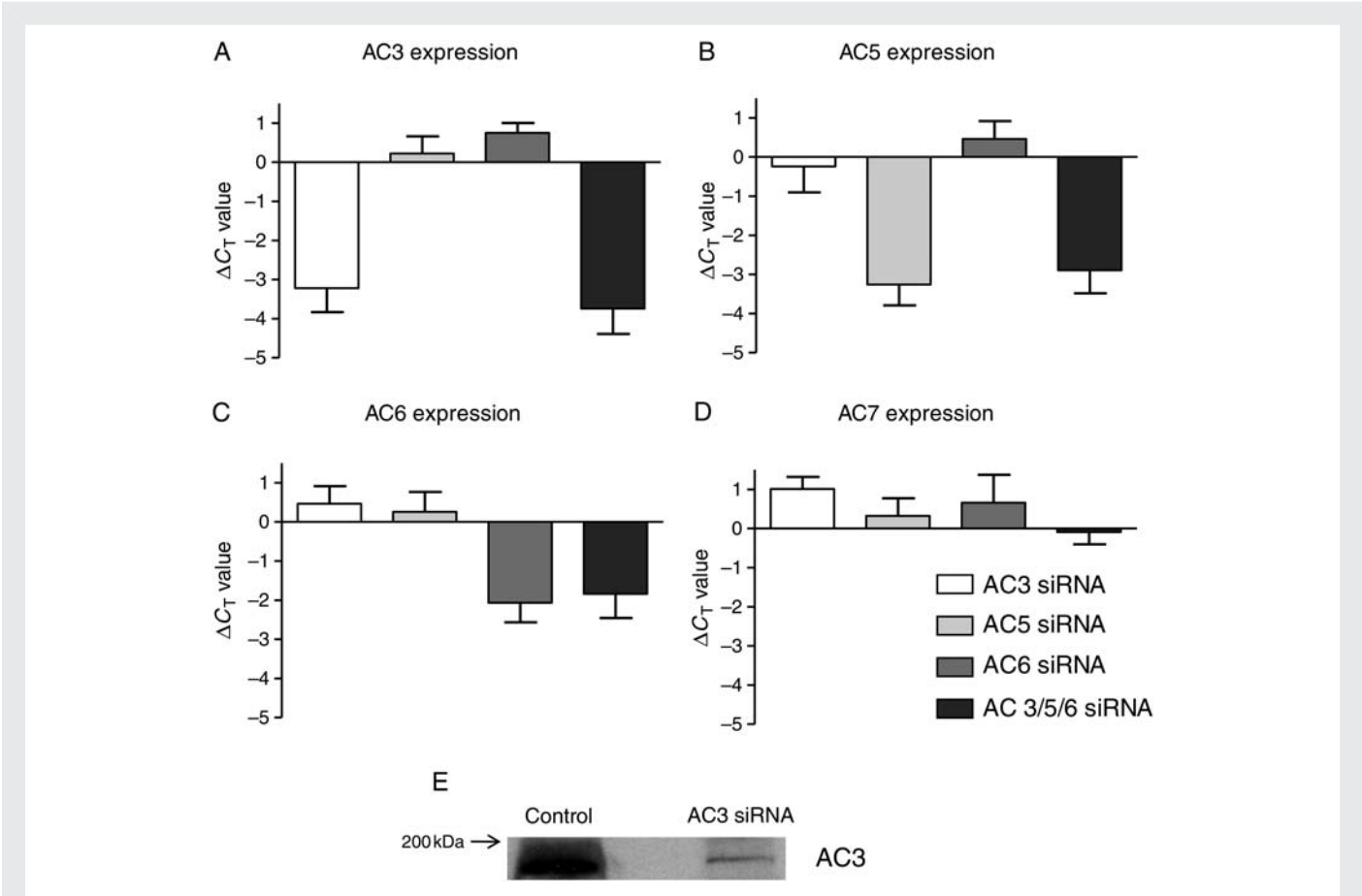


Figure 2 siRNA-mediated knockdown of AC isoenzymes in VSMCs. AC3 (A), AC5 (B), AC6 (C), and AC7 (D) expression was assessed by RT-PCR in cells transfected with siRNA sequences targeting ACs 3, 5, or 6 alone or in combination. Data are expressed as mean ΔC_T [C_T (negative-control siRNA-transfected cells) – C_T (AC-targeting siRNA-transfected cells)] values ± SEM from four or more samples. Negative ΔC_T values indicate a depletion of AC isoenzyme mRNA. (E) Immunoblot demonstrating AC3 protein depletion in VSMCs transfected with AC3-targeting siRNA, representative of four similar experiments.

cells transfected with AC3-targeting siRNA was reduced to $16 \pm 8\%$ of that in negative-control siRNA-transfected VSMCs ($P < 0.05$, Student's unpaired t -test; $n = 4$).

3.3 The role of AC isoenzymes in cAMP signalling in VSMCs

Stimulation of endogenous β -ARs with ISO ($1 \mu\text{M}$) elicited a robust increase in cAMP in VSMCs. Experiments conducted in the presence or absence of the β_1 - (CGP20712A, 100 nM) and β_2 -selective (ICI118551, 100 nM) antagonists demonstrated that the majority of the cAMP response to ISO was mediated by a β_2 -AR population (see Supplementary material online, Figure S2A). In AC3/5/6-depleted VSMCs, levels of cAMP produced by either direct AC activation with forskolin ($10 \mu\text{M}$) or β -AR activation by ISO ($1 \mu\text{M}$) in each case in the presence of the PDE inhibitor IBMX ($300 \mu\text{M}$) were substantially reduced (by 70–75%; Figure 3A). Basal cAMP levels were not significantly decreased (Figure 3A). ISO-mediated cAMP responses were smaller in the absence ($328 \pm 106 \text{ pmol/mg protein}$; $n = 4$) than in the presence ($939 \pm 243 \text{ pmol/mg protein}$; $n = 7$) of IBMX and AC3/5/6 depletion almost completely abolished the response to ISO ($40 \pm 15 \text{ pmol/mg protein}$; $n = 4$) in the absence of IBMX (data not shown). However, as a result of the greater consistency and signal to noise achieved, all subsequent cAMP measurements were performed in the presence of IBMX ($300 \mu\text{M}$). In AC3/5/6-depleted VSMCs, ISO-mediated cAMP accumulation was substantially attenuated across a range of concentrations, with no change in potency (see Supplementary material online, Figure S2B).

In AC6-depleted cells, responses to forskolin were reduced to 41% of control, whereas depletion of AC3, and to a lesser extent AC5, also significantly attenuated cAMP accumulation (Figure 3B). A similar profile was observed for ISO-mediated cAMP responses, with AC6 the principal isoenzyme mediating the response to β -AR stimulation (42% of control response in AC6-depleted cells; Figure 3C). However, although AC3 depletion significantly reduced the response (to 70% of control), AC5 depletion had no statistically significant effect on β -AR-mediated cAMP accumulation (Figure 3C).

Responses to the (G_s -coupled) vasodilators CGRP, PACAP, iloprost, and PGE_2 alone were too small to allow accurate assessment of the roles of individual AC isoenzymes (data not shown). However, in the presence of a very low level of forskolin ($0.1 \mu\text{M}$), which itself caused only a modest cAMP response (from 31 ± 13 in control to $96 \pm 26 \text{ pmol/mg protein}$ in the presence of forskolin; $n = 4$), each agonist stimulated enhanced cAMP responses (see Supplementary material online, Figure S2C), as has been previously demonstrated.²⁵ ISO-stimulated cAMP accumulation was highly sensitive to AC6 depletion in either the absence or presence of forskolin ($0.1 \mu\text{M}$) (see Supplementary material online, Figure S2C). A similar profile was observed for each of the other four vasodilators investigated (see Supplementary material online, Figure S2C). AC6, therefore, seems to be the primary isoenzyme involved in vasodilator-mediated cAMP signalling in vascular smooth muscle.

Although depletion of AC3/5/6 together substantially reduced forskolin- and ISO-mediated cAMP signalling (by 87 and 77%, respectively; Figure 3), it is possible that the residual responses were mediated by alternative AC isoenzymes. In our earlier PCR screen, the AC7 transcript appeared to be present at similar levels to AC3/5/6 (Table 1). We therefore validated an siRNA

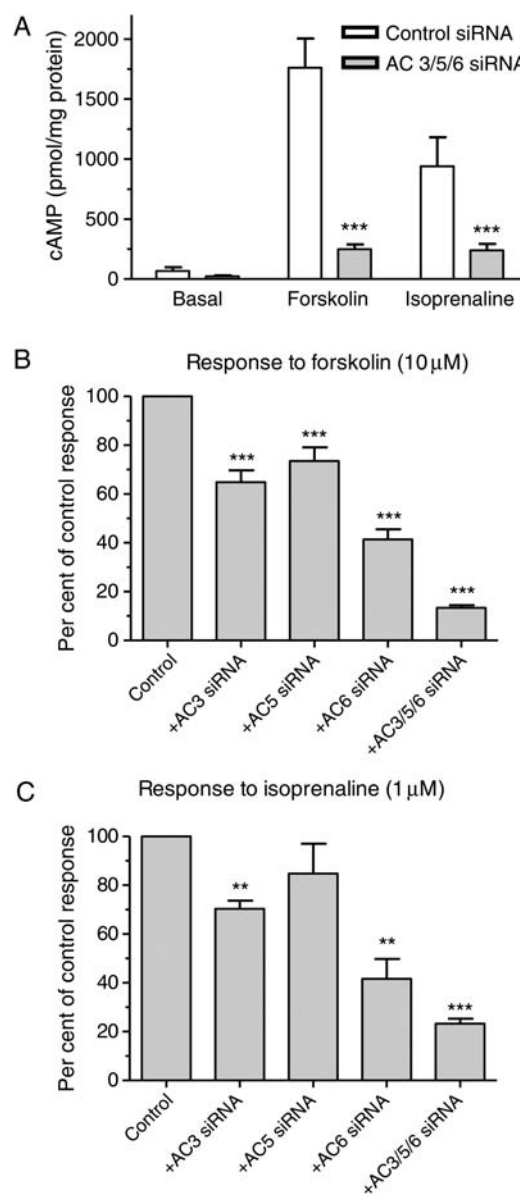


Figure 3 AC6 is the predominant isoenzyme mediating β -AR-stimulated cAMP accumulation in VSMCs. Mean cAMP accumulations in VSMCs transfected with negative-control siRNA or siRNA sequences targeting ACs 3, 5, or 6 (alone or all three in combination) and stimulated with forskolin ($10 \mu\text{M}$) (A and B) or ISO ($1 \mu\text{M}$) (A and C). Data are presented as mean cAMP (pmol/mg protein) (A) or as a percentage of the response in cells transfected with negative-control siRNA (B and C). Data are expressed as means $\pm$ SEM from $n \geq 5$ experiments, from three or more separate cultures. Statistical significance is indicated as ** $P < 0.01$ and *** $P < 0.001$ vs. control.

targeting AC7 (see Supplementary material online, Figure S3A). In VSMCs transfected with this siRNA sequence, forskolin- and ISO-mediated cAMP responses were not reduced and, in fact, were slightly enhanced (see Supplementary material online, Figure S3B). AC7 does not, therefore, appear to play a significant role in β -AR-mediated cAMP signalling in VSMCs.

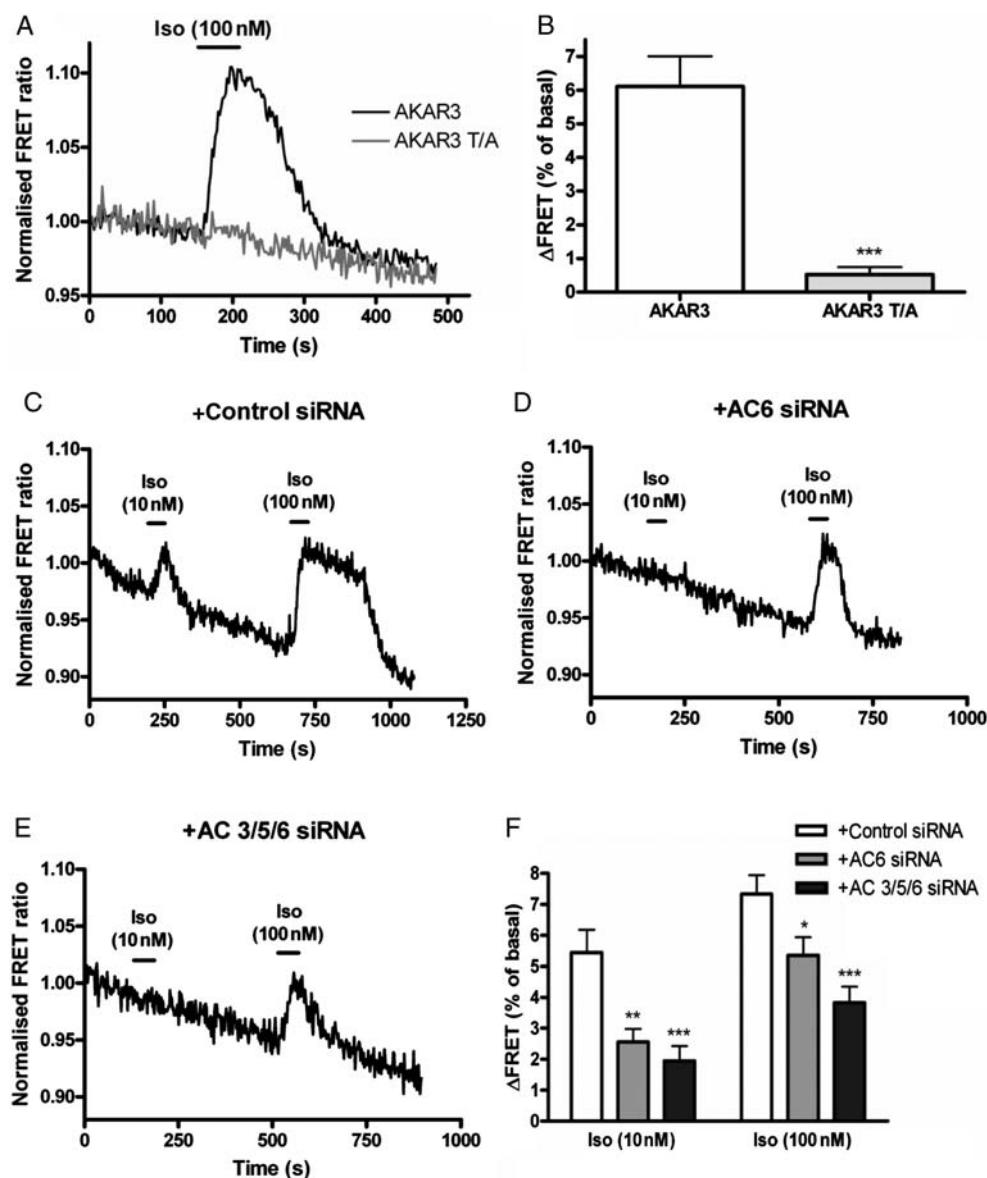


Figure 4 The role of AC isoenzymes in β -AR-mediated PKA activation in VSMCs. Representative traces (A) and mean data (B) for changes in the FRET ratio in response to ISO (100 nM) in VSMCs transfected with AKAR3 [black trace (A) and open bar (B)] or AKAR3 T/A [grey trace (A) and closed bar (B)]. Data are expressed as mean normalized FRET ratio (A) or percentage change in the FRET ratio over baseline (Δ FRET, B) $\pm$ SEM from $n \geq 10$ cells from three separate cultures. (C–E) Changes in the FRET ratio in response to ISO (10 or 100 nM, as indicated) in VSMCs transfected with AKAR3 and either negative-control (C), AC6-targeting (D), or AC3/5/6-targeting (E) siRNA sequences. (F) Mean data for the per cent changes in the FRET ratio above baseline (Δ FRET) in VSMCs transfected with AKAR3 and siRNA sequences. Data are expressed as means $\pm$ SEM from ≥ 38 cells taken from three different cultures. Statistical significance is indicated as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. AKAR3 (B) or control (F) responses.

3.4 AC6 is essential for β -AR-mediated PKA signalling in VSMCs

We utilized a FRET-based biosensor for PKA activity (AKAR3²³) to examine the role of individual AC isoenzymes in ISO-mediated PKA signalling. Brief (60 s) stimulation of AKAR3-transfected VSMCs with ISO (100 nM) elicited a rapid and reversible increase in the FRET ratio (Figure 4A and B). In contrast, in cells expressing a negative-control version of AKAR3 (AKAR3 T/A), application of ISO (100 nM) failed to alter significantly the FRET ratio (Figure 4A and

B). In AC6 (Figure 4D and F) or AC3/5/6 (Figure 4E and F) depleted VSMCs, AKAR3 responses to either 10 or 100 nM ISO were significantly attenuated, relative to control siRNA-transfected cells [Figure 4C and F; $P < 0.01$ (AC6, 10 nM ISO); $P < 0.05$ (AC6, 100 nM ISO); $P < 0.001$ (AC3/5/6, 10 or 100 nM ISO); one-way ANOVA, Bonferroni's *post hoc* test]. Depletion of AC6 or AC3/5/6 had similar effects on the AKAR3 responses to ISO, suggesting that in agreement with our data for cAMP signalling, AC6 is the key cAMP-synthesizing isoenzyme for β -AR-mediated PKA activation.

3.5 AC6 is the principal isoenzyme mediating β -AR- K_{ATP} channel modulation in VSMCs

To examine the roles of AC isoenzymes in β -AR-mediated K_{ATP} channel modulation, we characterized the K_{ATP} current in cultured VSMCs, measuring P1075-, ISO-, and metabolic inhibition-induced current and membrane potential changes (see Supplementary material online, Figure S4). We also confirmed that ISO had similar PKA-dependent effects on K_{ATP} current and membrane potential in cultured VSMCs as in acutely dissociated cells (see Supplementary material online, Figure S5). In untransfected (data not shown) or negative-control siRNA-transfected (Figure 5A and F) VSMCs, ISO (100 nM) caused a robust increase in current (from $0.47 \pm$

0.07 pA/pF in control to 5.99 ± 0.45 pA/pF in the presence of ISO; $n = 16$), which was glibenclamide-sensitive (0.06 ± 0.05 pA/pF remaining in the presence of the K_{ATP} blocker). Depletion of AC3 reduced the response to ISO by 34% ($P < 0.01$, two-way ANOVA and Bonferroni's *post hoc* test; Figure 5B and F), whereas knockdown of AC5 was without significant effect (Figure 5C and F). AC6 depletion had the largest effect, with ISO-mediated enhancement of K_{ATP} channel activity reduced by 74% relative to control-transfected cells ($P < 0.001$; two-way ANOVA and Bonferroni's *post hoc* test; Figure 5D and F). Depletion of AC3/5/6 reduced the ISO response by $\sim 95\%$ ($P < 0.001$; two-way ANOVA and Bonferroni's *post hoc* test; Figure 5E and F). In contrast, basal K_{ATP} current and that initiated by the K_{ATP} channel opener P1075 were unaffected by the knockdown of these AC isoenzymes, singly or in combination (Figure 5). β -AR-mediated modulation of K_{ATP} channel activity in VSMCs is therefore highly dependent on AC6 (and to a lesser extent AC3) activity, whereas AC5 plays no significant role.

4. Discussion

We have shown in VSMCs that AC6 plays a critical role in β -AR-mediated signalling. The activation of this signalling pathway elicits a substantial hyperpolarization (by ≥ 27 mV) of VSMCs through the sequential activation of AC6, PKA, and K_{ATP} current. Since the relationship between membrane potential and contractile tone in VSMCs is an extremely steep one, such a change in membrane potential would be expected to elicit a significant change in arterial diameter. This pathway is therefore a critical determinant of both blood pressure and blood flow to organs.

K_{ATP} channels have been previously demonstrated to contribute to the regulation of vascular tone. Genetic disruption of the pore-forming Kir6.1 subunit in mouse causes elevated blood pressure, a Prinzmetal angina-like phenotype and a sensitivity to vasoconstrictors.⁵ In addition, β -AR signalling initiates VSMC hyperpolarization via AC/PKA activation and the opening of K_{ATP} channels.^{26,27} We have confirmed that in VSMCs, ISO-mediated stimulation of the β -AR (predominantly β_2 -AR) population elicited a robust, PKA-dependent increase in K_{ATP} current, which developed with a 20–30 s delay consistent with the onset of PKA activation in our system (Figure 4). These observations, together with the fact that glibenclamide fully reversed ISO-mediated hyperpolarization, indicate that K_{ATP} channels are a physiologically important target of the G_{α_s} /AC/cAMP/PKA signalling pathway, both at rest and during vasodilation.

In common with earlier studies,^{15–17} we found evidence, at the mRNA level, for expression of multiple AC isoenzymes in VSMCs. Taking these findings as a whole, it is clear that mRNAs for AC3, 5, and 6 consistently appear to be most highly expressed, and for this reason, we focused on these isoenzymes in the majority of our subsequent investigation. Indeed, depletion of AC3, 5, and 6 together was highly effective in attenuating the cellular cAMP response to ISO (by $>75\%$ in the presence and $\sim 90\%$ in the absence of IBMX) and several other vasodilators. We therefore believe that it is unlikely that any additional AC isoenzyme has a significant functional role in vasodilator-mediated cAMP generation in VSMCs.

We provide evidence for a modest, but significant role for AC3 in both forskolin- and ISO-mediated signalling in VSMCs. AC3 is reported to be the principal AC isoenzyme activated by PGE_2 in VSMCs,²⁸ with PGE_2 -mediated cAMP responses being attenuated by

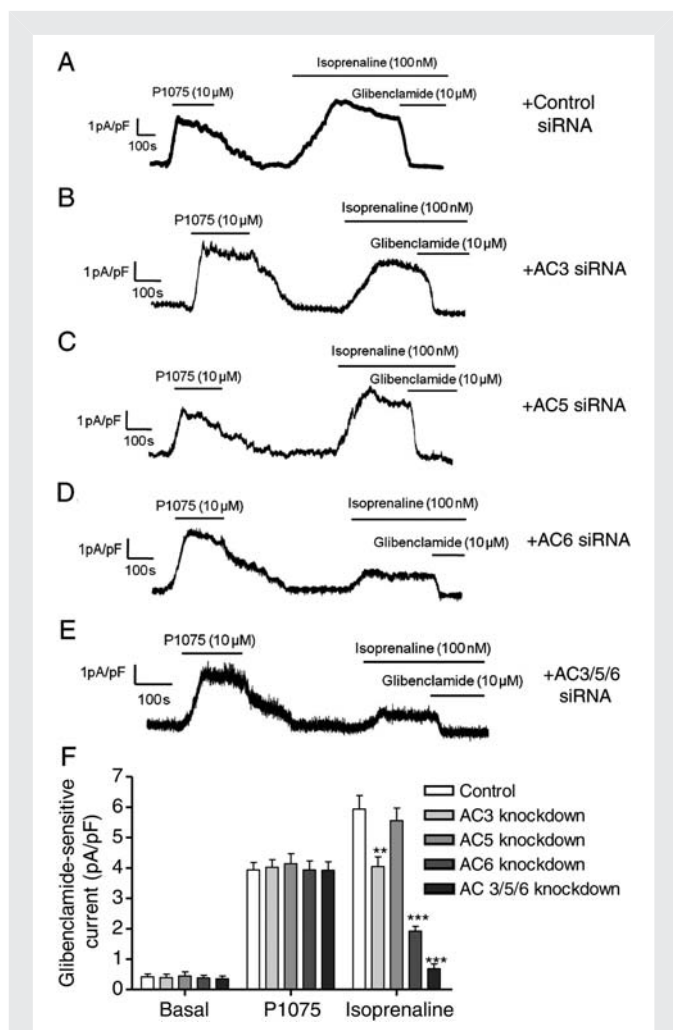


Figure 5 AC6 is the predominant isoenzyme mediating the β -AR-stimulated K_{ATP} current in VSMCs. K_{ATP} current recordings in VSMCs transfected with negative control (A), AC3 (B), AC5 (C), AC6 (D), or AC3/5/6 (E)-targeting siRNA sequences. Cells were held at 0 mV and P1075 (10 μ M), ISO (100 nM), and glibenclamide (10 μ M) were added as indicated. (F) Mean glibenclamide-sensitive current values (normalized to cell capacitance) in VSMCs transfected with negative-control or AC-targeting siRNA sequences. Data are expressed as means $\pm$ SEM for $n \geq 8$ cells, from three separate cultures. Statistical significance is indicated as ** $P < 0.01$ and *** $P < 0.001$ vs. control.

~50% in mice heterozygous for the disrupted AC3 gene. Our findings (and those by Ostrom *et al.*¹⁵) indicate a more minor role for AC3 in PGE₂-mediated cAMP signalling (see Supplementary material online, Figure S2C), perhaps suggesting species-specific differences in the roles of AC isoenzymes. However, AC3 has also been shown to play a significant role in Ca²⁺-stimulated rat VSMC cAMP signalling,¹⁵ providing further evidence for its functional expression in this tissue.

Perhaps, the most surprising finding of our study was the striking difference in the relative importance of AC5 and AC6 isoenzymes in VSMCs. AC5 and AC6 display a high level of homology in amino acid sequence (65%) and share many regulatory properties,¹⁴ so the dominant role of AC6 and the apparent absence of a significant role for AC5 in β -AR-mediated cAMP signalling in aortic cells are intriguing results. This selectivity does not appear to be peculiar to β -AR signalling, as a panel of alternative vasodilators displayed a similar AC6 dependence. In support of a central role for AC6 in vasodilator signalling, Ostrom *et al.*¹⁵ found that modest (approximately two-fold) over-expression of AC6 in VSMCs enhanced forskolin-, ISO-, and PGE₂-mediated cAMP signalling. A further study in VSMCs found that AC6 over-expression enhanced ISO-mediated morphological change (which has been related to vasodilator reactivity), as well as forskolin-stimulated vasodilator-stimulated phosphoprotein phosphorylation (an index of PKA activity).¹⁹ These findings are consistent with a predominant role for AC6 in vasodilator/PKA signalling, but are limited by their reliance on over-expression. siRNA-mediated depletion of both AC5 and AC6 in VSMCs has been shown to attenuate uridine diphosphate-mediated inhibition of ISO-stimulated cAMP signals.²⁹ This was considered as correlative evidence that β -AR may couple preferentially to endogenous AC5/6.²⁹ However, puzzlingly the ISO response was not reduced *per se* in the AC5/6-depleted cells. These studies hint at the involvement of AC5/6, whereas our findings demonstrate the principal role for AC6 in vasodilator signalling in VSMCs.

Although our study has focused on defining the relative importance of AC isoenzymes, it must be noted that PDE isoenzymes also exhibit considerable diversity and compartmentalization. The functional compartmentalization of distinct PDE isoenzymes (particularly PDE3 and PDE4) has previously been reported to influence the differential activation of PKA and PKG following β -AR activation in the rat aorta.³⁰ It is therefore possible that different PDEs, perhaps co-located with specific AC isoenzymes, also play a critical role in regulating local or global cAMP levels.

The absence of a significant role for AC5 in vasodilator signalling in the vasculature could potentially be of clinical relevance. AC5 inhibition, or genetic disruption, is well established to be cardioprotective in mice exposed to chronic pressure overload³¹ and to enhance longevity,³² leading to the suggestion that its inhibition might provide an effective treatment for congestive heart failure (CHF).³³ An obvious problem with such a strategy would be that AC5 is not expressed exclusively in the heart, with off-target effects in the vasculature being cited as a potential problem.³³ However, in light of our findings (albeit presently limited to aortic smooth muscle cells), it is possible that any such side effects would be minimal, provided that any potential agent was highly selective for AC5 over AC6.

In contrast, AC6 over-expression in cardiac tissue improves function and may be beneficial in the failing heart,^{34,35} leading to the notion that intracoronary AC6 gene transfer might be a rational approach to CHF therapy.³⁶ If AC6 is a key determinant of vasorelaxation, might its over-expression/activation in the vasculature also

be beneficial? Enhancing vasodilation in CHF would reduce afterload and myocardial oxygen consumption (while perhaps increasing coronary blood supply), which might be anticipated to improve cardiac function. A single-nucleotide polymorphism in the β_2 -AR (Ile164), which results in reduced AC coupling, is associated with an increased relative risk of death³⁷ as well as a decreased exercise capacity and vasodilatory response to exercise³⁸ in CHF patients. Although results contradictory to these have been reported,¹² these observations suggest that augmented vasodilation might indeed be protective in CHF. If AC6 expression/activity is the limiting factor in vasodilator signalling, as is suggested by previous studies,^{15,39} the possible beneficial effects of enhancing AC6 activity in vascular smooth muscle are worthy of further investigation.

The principal importance of AC6 in vascular smooth muscle physiology is highlighted by our demonstration of the dependence of β -AR-mediated PKA signalling and PKA-dependent K_{ATP} modulation on the expression of this isoenzyme. K_{ATP} channel activity, which we and others have established as a key determinant of the membrane potential, is substantially increased by β -AR activation, predominantly via AC6-mediated cAMP generation and PKA activation. This identifies AC6 as a vital regulator of vascular tone, with implications for cardiovascular function in both health and disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We gratefully acknowledge Jin Zhang (Johns Hopkins University, USA) for the generous provision of the AKAR3 constructs and thank Prof. Dermot Cooper (University of Cambridge, UK) for his critical reading of the manuscript.

Conflict of interest: none declared.

Funding

This work was supported by the British Heart Foundation (grant no. RG06/008/22062). Funding to pay Open Access charges was provided by the British Heart Foundation.

References

- Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* 1995;**268**:C799–C822.
- Quayle JM, Nelson MT, Standen NB. ATP-sensitive and inwardly rectifying potassium channels in smooth muscle. *Physiol Rev* 1997;**77**:1165–1232.
- Duncker DJ, Van Zon NS, Altman JD, Pavsek TJ, Bache RJ. Role of K_{ATP} channels in coronary vasodilation during exercise. *Circulation* 1993;**88**:1245–1253.
- Jackson WF. Arteriolar tone is determined by activity of ATP-sensitive potassium channels. *Am J Physiol* 1993;**265**:H1797–H1803.
- Miki T, Suzuki M, Shibasaki T, Uemura H, Sato T, Yamaguchi K *et al.* Mouse model of Prinzmetal angina by disruption of the inward rectifier Kir6.1. *Nat Med* 2002;**8**:466–472.
- Chutkow WA, Pu J, Wheeler MT, Wada T, Makielski JC, Burant CF *et al.* Episodic coronary artery vasospasm and hypertension develop in the absence of Sur2 KATP channels. *J Clin Invest* 2002;**110**:203–208.
- Kakkar R, Ye B, Stoller DA, Smalley M, Shi NQ, Galles K *et al.* Spontaneous coronary vasospasm in K_{ATP} mutant mice arises from a smooth muscle-extrinsic process. *Circ Res* 2006;**98**:682–689.
- Randall MD, McCulloch AL. The involvement of ATP-sensitive potassium channels in β -adrenoceptor-mediated vasorelaxation in the rat isolated mesenteric arterial bed. *Br J Pharmacol* 1995;**115**:607–612.
- Chruscinski AJ, Rohrer DK, Schauble E, Desai KH, Bernstein D, Kobilka BK. Targeted disruption of the β_2 -adrenergic receptor gene. *J Biol Chem* 1999;**274**:16694–16700.

10. Asano M, Masuzawa K, Matsuda T. Evidence for reduced β -adrenoceptor coupling to adenylate cyclase in femoral arteries from spontaneously hypertensive rats. *Br J Pharmacol* 1988;**94**:73–86.
11. Stein CM, Nelson R, Deegan R, He H, Wood M, Wood AJ. Forearm β -adrenergic receptor-mediated vasodilation is impaired, without alteration of forearm norepinephrine spillover, in borderline hypertension. *J Clin Invest* 1995;**96**:579–585.
12. Leineweber K, Heusch G. β_1 - and β_2 -Adrenoceptor polymorphisms and cardiovascular diseases. *Br J Pharmacol* 2009;**158**:61–69.
13. Wellman GC, Quayle JM, Standen NB. ATP-sensitive K^+ channel activation by calcitonin gene-related peptide and protein kinase A in pig coronary arterial smooth muscle. *J Physiol* 1998;**507**:117–129.
14. Willoughby D, Cooper DM. Organization and Ca^{2+} regulation of adenylyl cyclases in cAMP microdomains. *Physiol Rev* 2007;**87**:965–1010.
15. Ostrom RS, Liu X, Head BP, Gregorian C, Seasholtz TM, Insel PA. Localization of adenylyl cyclase isoforms and G protein-coupled receptors in vascular smooth muscle cells: expression in caveolin-rich and noncaveolin domains. *Mol Pharmacol* 2002;**62**:983–992.
16. Webb JG, Yates PW, Yang Q, Mukhin YV, Lanier SM. Adenylyl cyclase isoforms and signal integration in models of vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 1998;**276**:H1545–H1552.
17. Matsumoto T, Wakabayashi K, Kobayashi T, Kamata K. Diabetes-related changes in cAMP-dependent protein kinase activity and decrease in relaxation response in rat mesenteric artery. *Am J Physiol Heart Circ Physiol* 2004;**287**:H1064–H1071.
18. Sampson LJ, Hayabuchi Y, Standen NB, Dart C. Caveolae localize protein kinase A signalling to arterial ATP-sensitive potassium channels. *Circ Res* 2004;**95**:1012–1018.
19. Gros R, Ding Q, Chorzyczewski J, Pickering JG, Limbird LE, Feldman RD. Adenylyl cyclase isoform-selective regulation of vascular smooth muscle proliferation and cytoskeletal reorganization. *Circ Res* 2006;**99**:845–852.
20. Davies LM, Purves GI, Barrett-Jolley R, Dart C. Interaction with caveolin-1 modulates vascular ATP-sensitive potassium (K_{ATP}) channel activity. *J Physiol* 2010;**588**:3255–3266.
21. Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RAJ et al. G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoreceptor signalling. *Cardiovasc Res* 2011;**89**:193–203.
22. Brown BL, Albano JD, Ekins RP, Sgherzi AM. A simple and sensitive saturation assay method for the measurement of adenosine 3':5'-cyclic monophosphate. *Biochem J* 1971;**121**:561–562.
23. Allen MD, Zhang J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun* 2006;**348**:716–721.
24. Yuan XJ, Goldman WF, Tod ML, Rubin LJ, Blaustein MP. Ionic currents in rat pulmonary and mesenteric arterial myocytes in primary culture and subculture. *Am J Physiol* 1993;**264**:L107–L115.
25. Darfler FJ, Mahan LC, Koachman AM, Insel PA. Stimulation of forskolin of intact S49 lymphoma cells involves the nucleotide regulatory protein of adenylate cyclase. *J Biol Chem* 1982;**257**:11901–11907.
26. Nakashima M, Vanhoutte PM. Isoproterenol causes hyperpolarization through opening of ATP-sensitive potassium channels in vascular smooth muscle of the canine saphenous vein. *J Pharmacol Exp Ther* 1995;**272**:379–384.
27. Goto K, Fujii K, Abe I, Fujishima M. Sympathetic control of arterial membrane potential by ATP-sensitive K^+ -channels. *Hypertension* 2000;**35**:379–384.
28. Wong ST, Baker LP, Trinh K, Hetman M, Suzuki LA, Storm DR et al. Adenylyl cyclase-3 mediates prostaglandin E_2 -induced growth inhibition in arterial smooth muscle cells. *J Biol Chem* 2001;**276**:34206–34212.
29. von Hayn K, Werthmann RC, Nikolaev VO, Hommers LG, Lohse MJ, Bunemann M. G_q -mediated Ca^{2+} signals inhibit adenylyl cyclases 5/6 in vascular smooth muscle cells. *Am J Physiol Cell Physiol* 2010;**298**:C324–C332.
30. Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. *Pharmacol Ther* 2006;**109**:366–398.
31. Okumura S, Takagi G, Kawabe J, Yang G, Lee MC, Hong C et al. Disruption of type 5 adenylyl cyclase gene preserves cardiac function against pressure overload. *Proc Natl Acad Sci USA* 2003;**100**:9986–9990.
32. Vatner SF, Yan L, Ishikawa Y, Vatner DE, Sadoshima J. Adenylyl cyclase type 5 disruption prolongs longevity and protects the heart against stress. *Circ J* 2009;**73**:195–200.
33. Pierre S, Eschenhagen T, Geisslinger G, Scholich K. Capturing adenylyl cyclases as potential drug targets. *Nat Rev Drug Discov* 2009;**8**:321–335.
34. Gao MH, Bayat H, Roth DM, Yao Zhou J, Drumm J, Burhan J et al. Controlled expression of cardiac-directed adenylylcyclase type VI provides increased contractile function. *Cardiovasc Res* 2002;**56**:197–204.
35. Lai NC, Tang T, Gao MH, Saito M, Takahashi T, Roth DM et al. Activation of cardiac adenylyl cyclase expression increases function of the failing ischemic heart in mice. *J Am Coll Cardiol* 2008;**51**:1490–1497.
36. Phan HM, Gao MH, Lai NC, Tang T, Hammond HK. New signalling pathways associated with increased cardiac adenylyl cyclase 6 expression: implications for possible congestive heart failure therapy. *Trends Cardiovasc Med* 2007;**17**:215–221.
37. Liggett SB, Wagoner LE, Craft LL, Hornung RW, Hoit BD, McIntosh TC et al. The Ile164 β_2 -adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. *J Clin Invest* 1998;**102**:1534–1539.
38. Wagoner LE, Craft LL, Singh B, Suresh DP, Zengel PW, McGuire N et al. Polymorphisms of the β_2 -adrenergic receptor determine exercise capacity in patients with heart failure. *Circ Res* 2000;**86**:834–840.
39. Gros R, Van Uum S, Hutchinson-Jaffe A, Ding Q, Pickering JG, Hegele RA et al. Increased enzyme activity and β -adrenergic mediated vasodilation in subjects expressing a single-nucleotide variant of human adenylyl cyclase 6. *Arterioscler Thromb Vasc Biol* 2007;**27**:2657–2663.