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RESEARCH PAPER

Vascular actions of calcimimetics: role of Ca^{2+}-sensing receptors versus Ca^{2+} influx through L-type Ca^{2+} channels

Pratish Thakore and W -S Vanessa Ho

Division of Basic Medical Sciences, St George's University of London, Cranmer Terrace, London, UK

Correspondence
W.-S.V. Ho, Division of Basic Medical Sciences, St George's University of London, Cranmer Terrace, London SW17 0RE, UK.
E-mail: vho@sgul.ac.uk

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BACKGROUND AND PURPOSE

The calcimimetic, (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet), which activates Ca^{2+}-sensing receptors (CaR) in parathyroid glands, is used to treat hyperparathyroidism. Interestingly, CaR in perivascular nerves or endothelial cells is also thought to modulate vascular tone. This study aims to characterize the vascular actions of calcimimetics.

EXPERIMENTAL APPROACH

In rat isolated small mesenteric arteries, the relaxant responses to the calcimimetics, cinacalcet and (R)-2-[[[1-(1-naphthyl) ethyl]amino]methyl]-1H-indole hydrochloride (calindol) were characterized, with particular emphasis on the role of CaR, endothelium, perivascular nerves, K^+ channels and Ca^{2+} channels. Effects of L-ornithine, which activates a Ca^{2+}-sensitive receptor related to CaR (GPRC6A), were also tested.

KEY RESULTS

Cinacalcet induced endothelium-independent relaxation (pEC_{50} 5.58 ± 0.07, E_{max} 97 ± 6%) that was insensitive to sensory nerve desensitization by capsaicin or blockade of large-conductance Ca^{2+}-activated K^+ channels by iberiotoxin. Calindol, another calcimimetic, caused more potent relaxation (pEC_{50} 6.10 ± 0.10, E_{max} 101 ± 6%), which was attenuated by endothelial removal or capsaicin, but not iberiotoxin. The negative modulator of CaR, calhex 231 or changes in $[Ca^{2+}]_o$ had negligible effect on relaxation to both calcimimetics. The calcimimetics relaxed vessels precontracted with high $[K^+]_o$ and inhibited Ca^{2+} influx in endothelium-denuded vessels stimulated by methoxamine, but not ionomycin. They also inhibited contractions to the L-type Ca^{2+} channel activator, BayK8644. L-ornithine induced small relaxation alone and had no effect on the responses to calcimimetics.

CONCLUSION AND IMPLICATIONS

Cinacalcet and calindol are potent arterial relaxants. Under the experimental conditions used, they predominantly act by inhibiting Ca^{2+} influx through L-type Ca^{2+} channels into vascular smooth muscle, whereas Ca^{2+}-sensitive receptors (CaR or GPRC6A) play a minor role.

Abbreviations

BayK8644, (4R)- and (4S)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-trifluoromet hyl)phenyl]-3-pyridinecarboxylic acid methyl ester; calhex 231, 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl]amino]cyclohexyl]benzamide; calindol, (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride; cinacalcet, (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride

Introduction

The cell surface, extracellular Ca^{2+}-sensing receptor (CaR), which is activated by Ca^{2+} ions, is crucial for maintaining a stable systemic $[Ca^{2+}]_o$, primarily through regulation of parathyroid hormone secretion and renal Ca^{2+} excretion (see reviews Brown and MacLeod, 2001). CaR is mainly coupled to $G_{q/11}$ proteins and subsequent activation of phospholipase C, generating intracellular Ca^{2+} signals, but it can also activate G_i proteins and mitogen-activated protein kinases (Brown and MacLeod, 2001). Of note, a number of endogenous (L-α-amino acids) and synthetic substances act as allosteric modulators of CaR (Jensen & Brauner-Osborne, 2007). Calcimimetics are positive modulators of CaR by potentiating the action of extracellular Ca^{2+}. One particular example is (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet), which has been approved for the clinical treatment of uraemic secondary hypercalcaemia parathyroidism and hypercalcaemia in parathyroid cancer (Steddon and Cunningham, 2005). On the other hand, calcilytics, which negatively modulate CaR, might be useful in osteoporosis as parathyroid hormone also stimulates bone growth (Steddon and Cunningham, 2005).

Interestingly, CaR is also present in tissues not involved in systemic Ca^{2+} balance, including cardiovascular tissues (Brown and MacLeod, 2001). Indeed, several studies have shown that cinacalcet or other calcimimetics can lead to acute hypertension as well as chronic hypotension, possibly due to normalization of parathyroid hormone level and direct cardiovascular actions (Ogata *et al.*, 2003; Odenwald *et al.*, 2006; Fryer *et al.*, 2007). Expression of CaR has been demonstrated in the vasculature; in perivascular sensory nerves (Bukoski *et al.*, 1997), the endothelium (Weston *et al.*, 2005; Molostvov *et al.*, 2007) and the vascular smooth muscle (Smajilovic *et al.*, 2006; Molostvov *et al.*, 2007). Although the physiological $[Ca^{2+}]$ in serum is tightly regulated, changes in interstitial $[Ca^{2+}]$ might be sufficient to activate vascular CaR and thus CaR are involved in the regulation of vascular tone and blood pressure. Bukoski and co-workers demonstrated that increases in $[Ca^{2+}]_o$ (from 1 to 5 mM), presumably via CaR, cause mesenteric relaxation that is dependent on perivascular sensory nerves and large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}; Bukoski *et al.*, 1997; Ishioka and Bukoski, 1999). More recently, activation of endothelial CaR, which is closely coupled to intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}), has also been found to elicit vasorelaxation (Weston *et al.*, 2005).

The vasorelaxant actions of Ca^{2+} or CaR would be consistent with the beneficial effect of increased dietary Ca^{2+} intake in some forms of hypertension (McCarty, 2004). However, CaR in vascular smooth muscle cells has also been associated with vasocontraction (Wonneberger *et al.*, 2000) and signalling via mitogen-activated protein kinases (Smajilovic *et al.*, 2006; Molostvov *et al.*, 2007). Therefore, the precise vascular effects of CaR and calcimimetics remain to be defined. In this study, using rat isolated small mesenteric arteries, we have characterized the relaxant responses to cinacalcet and the more recently developed calcimimetic, (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) (Kessler *et al.*, 2004). In particular, the involvement of CaR, the endothelium, perivascular sensory nerves, K^+ channels and Ca^{2+} channels was investigated. Recently, GRPC6A, a novel receptor that is sensitive to Ca^{2+} and closely related to CaR (Wellendorph *et al.*, 2007), has also been identified in the endothelium of mesenteric and coronary arteries (Harno *et al.*, 2008). Thus, the potential contribution of GPRC6A to the calcimimetic responses was also explored. Our results suggest that the two calcimimetics act as potent vasorelaxants mainly through inhibition of Ca^{2+} influx in smooth muscle cells independent of CaR or GPRC6A. Activation of K^+ channels and, in the case of calindol, the endothelium and capsaicin-sensitive nerves also significantly contribute to their mesenteric relaxations.

Methods

Myographic studies

Male Wistar rats (200–350 g; Charles River UK Ltd, Kent, UK) were stunned by a blow to the back of their neck and killed by cervical dislocation. All animal care and use was in accordance with the UK Animal (Scientific Procedures) Act 1986. The third-order branches of the superior mesenteric artery, which provides blood supply to the intestine, were removed and cleaned of adherent tissue. Segments (2 mm in length) were mounted in a Mulvany–Halpern type wire myograph (Model 610 M; Danish Myo Technology, Aarhus, Denmark) and maintained at 37°C in gassed (95% O_2/5% CO_2) Krebs–Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10 as previously described, unless otherwise stated (Ho and Randall, 2007). Arteries were equilibrated and set to a basal tension of 2 to 2.5 mN. The integrity of the endothelium was assessed by precontracting the vessel with 10 μM methoxamine (a α_1-adrenoceptor agonist), followed by relaxation with 10 μM carbachol (a muscarinic acetylcholine receptor agonist); vessels showing relaxations of greater than 90% were designated as endothelium-intact. When endothelium was not required, it was removed by rubbing the intima with a human hair; carbachol-induced relaxation of less than 10% indicated successful removal.

In some experiments, the third-order branches of the superior mesenteric vein were similarly dissected and mounted on a wire myograph. Veins were equilibrated and set to a basal tension of 0.5 mN (Zhang *et al.*, 2007) and precontracted with 60 mM KCl. Nomenclature of molecular targets, including receptors and ion channels, is used in accordance with the Guide to Receptor and Channels, *British Journal of Pharmacology* (Alexander *et al.*, 2008).

Experiments in the presence of extracellular Ca^{2+}

After the test for endothelial integrity, arteries were left for 30 min and then precontracted with 10 μM methoxamine. This was followed by construction of a cumulative concentration–relaxation curve to cinacalcet, calindol or L-ornithine. Preliminary experiments showed that washing could not fully reverse the effects of cinacalcet or calindol;

therefore, only a single concentration–response curve to the calcimimetic was constructed in each preparation. The vehicle of calcimimetics had no significant relaxation (up to 0.6% ethanol vv^{-1}; data not shown) in methoxamine-precontracted arteries. Most experiments were performed in matched vessels; effects of putative modulators or endothelial removal were compared with the control responses obtained in separate vessels of the same rat.

To investigate the relaxation mechanisms of calcimimetics, 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl] amino]cyclohexyl]benzamide (calhex) 231 (a negative modulator of CaR; 3 μM) and iberiotoxin (a selective BK_{Ca} blocker; 50 nM) were added to the myograph bath 30 min before, kept present during, construction of the concentration–response curve. To examine the potential interaction between GRPC6A and calcimimetics, L-ornithine was added to myograph for 5 min before determination of relaxant responses to either cinacalcet or calindol. For functional desensitization of capsaicin-sensitive perivascular nerves, vessels were incubated with 10 μM capsaicin for 1 h, followed by washing out (Zygmunt *et al.*, 1999). In some experiments, vessels were precontracted with high K^+ (60 mM) Krebs–Henseleit solution, which was prepared by equimolar substitution of NaCl for KCl in the standard Krebs–Henseleit buffer described above. Further addition of a small amount of methoxamine (1 μM) was often required to achieve a stable precontracted tone. The tension generated by 60 mM KCl (plus methoxamine top-up: 10.6 ± 1.1 mN) was similar to that induced by 10 μM methoxamine in the test for endothelial integrity (9.0 ± 1.1 mN; 16 vessels).

To further investigate the interaction between the calcimimetics and CaR, some experiments were conducted using Krebs–Henseleit solution containing 0.5 mM, instead of 2 mM, $CaCl_2$ after the endothelial integrity test. Mesenteric arteries were precontracted with 10 μM methoxamine, followed by construction of a cumulative concentration–relaxation curve to cinacalcet, calindol or $CaCl_2$ (1–5 mM). The effects of calhex 231, iberiotoxin or capsaicin on relaxation to the calcimimetics or $CaCl_2$ were determined as described above. Some arteries were pretreated with cinacalcet (1 μM) or calindol (0.3 μM) for 30 min, before construction of the relaxation curve to $CaCl_2$. Preliminary experiments found that the lower $[Ca^{2+}]_o$ significantly reduced methoxamine contractions (0.1–30 μM; data not shown); however, a similar precontracted tone was achieved, where necessary, by using a higher concentration of methoxamine (20 μM) (8.7 ± 0.7 mN as compared with 8.7 ± 0.7 mN under 2 mM $[Ca^{2+}]_o$; 25 vessels). For relaxation studies at 0.5 or 2 mM $[Ca^{2+}]_o$, the mean tension generated in the test for endothelial integrity was similar to that generated after incubation with a potential modulator (control, 8.6 ± 0.9 mN; vs. + calhex 231, 6.9 ± 0.5 mN, 20 vessels; control, 8.6 ± 0.8 mN; vs. + iberiotoxin, 9.6 ± 0.9 mN, 17 vessels; control, 9.3 ± 0.9 mN vs. + capsaicin, 10.1 ± 0.8 mN, 15 vessels; control, 8.4 ± 0.9 mN; vs. + L-ornithine, 9.7 ± 0.8 mN, 8 vessels; control, 10.8 ± 1.9 mN vs. + cinacalcet, 8.8 ± 1.4 mN, 7 vessels; control, 10.0 ± 1.2 mN vs. + calindol, 9.2 ± 1.2 mN, 7 vessels).

In endothelium-denuded mesenteric arteries, effects of calcimimetics on contractions induced by (4R)- and (4S)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-trifluoromet hyl)phenyl]-3-pyridinecarboxylic acid methyl ester (BayK8644) (an activator of the voltage-gated, L-type Ca^{2+} channels; 3 μM, Sanguinetti and Kass, 1984) were also determined. Preliminary experiments found that 3 μM BayK8644 produced consistent, although often transient, contractions in the presence of 15 mM KCl. This is consistent with previous reports that BayK8644 exerts greater effects on L-type Ca^{2+} channels at more depolarized conditions (Sanguinetti and Kass, 1984). Addition of KCl (15 mM) alone caused minimal contractions (0.2 ± 0.1 mN; eight vessels). In separate vessels, cinacalcet (10 μM), calindol (3 μM), verapamil (a L-type Ca^{2+} channel blocker; 10 μM) or their vehicle (0.1% vv^{-1} ethanol) was added to the bath, then 30 min later 15 mM KCl followed by 3 μM BayK8644.

In an additional set of experiments, cumulative concentration–relaxation curves to cinacalcet or calindol were also obtained from small mesenteric veins precontracted with 60 mM KCl.

Ca^{2+}-free experiments

Influx of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels was examined in endothelium-denuded mesenteric arteries depleted of intracellular Ca^{2+} stores according to the methods described previously (Ho and Hiley, 2003). Briefly, extracellular Ca^{2+} was removed by washing vessels with Ca^{2+}-free Krebs–Henseleit solution (composition the same as normal Krebs–Henseleit buffer but with $CaCl_2$ omitted). EGTA (1 mM) was added to the myograph bath, followed by a series of additions of 10 μM methoxamine in order to deplete intracellular Ca^{2+} stores as shown by loss of the contractile response. Vessels were then washed with Ca^{2+}-free Krebs–Henseleit solution, 10 μM methoxamine was added, and a cumulative concentration–response curve to $CaCl_2$ (10 μM–10 mM) was then obtained. Then, the washing process was repeated and a second $CaCl_2$ curve constructed in the presence of vehicle (0.1% vv^{-1} ethanol), cinacalcet or calindol (with 30 min pre-incubation). Contractions were expressed as a percentage of the maximum contraction induced by $CaCl_2$ in the vessel in the presence of 10 μM methoxamine alone. It was noted that the ethanol vehicle significantly potentiated contractions to $CaCl_2$ (data not shown), thus effects of the calcimimetics were determined by comparing the second concentration–response curves with $CaCl_2$ (in the presence of cinacalcet or calindol vs. vehicle).

To further examine the roles of voltage-gated Ca^{2+} channels, ionomycin (a Ca^{2+} ionophore) was used to facilitate Ca^{2+} entry. However, as the effects of ionomycin are irreversible, a modified protocol was used. Endothelium-denuded mesenteric arteries were depleted of intracellular Ca^{2+} stores as described above, followed by incubation with 10 μM ionomycin for 30 min. Contraction was then induced by readdition of 2 mM $CaCl_2$, followed by vehicle (0.1% vv^{-1} ethanol), cinacalcet (10 μM), calindol (3 and 10 μM) or verapamil (10 μM). Preliminary experiments showed that an initial addition of ethanol vehicle (0.1% vv^{-1}) caused an exaggerated contraction on top of the ionomycin-evoked response, but subsequent additions of ethanol vehicle had little effect (data not shown). Thus, in all experiments, ethanol vehicle (0.1% vv^{-1}) was applied,

before determination of responses to a further addition of vehicle, a calcimimetic or verapamil.

Data and statistical analysis

All relaxant responses are expressed as percentage relaxation of the tone induced by methoxamine or KCl. Values are given as mean ± SEM and *n* represents the number of animals used. Concentration–relaxation curves, with variable slopes, were obtained by fitting data using a sigmoidal logistic equation (Prism 4, GraphPad Software, Inc, San Diego, CA, USA). [Y = Bottom + (Top – Bottom)/(1 + $10^{((\text{LogEC50}-X) \times \text{Hillslope})}$), where X is a logarithm of drug concentration and Y is the response that starts from the Bottom and goes to the Top in a sigmoid shape]. The sigmoidal curves were also used to calculate pEC_{50}, negative logarithm of the concentration of relaxant giving 50% of maximum response and E_{max}, maximal response. Statistical comparisons of pEC_{50} and E_{max} were made by Student's *t*-tests or one-way analysis of variance followed by Dunnett's *post hoc* tests, where appropriate (Prism 4, GraphPad Software, Inc, San Diego, CA, USA).

Relaxations and contractions to $CaCl_2$ in the presence of methoxamine were examined by two-way analysis of variance followed by Bonferroni *post hoc* tests, whereas contractions to $CaCl_2$ in the presence of ionomycin, or contractions to BayK8644, were analysed by one-way analysis of variance followed by Dunnett's *post hoc* tests (Prism 4, GraphPad Software, Inc, San Diego, CA, USA). $P < 0.05$ was taken as statistically significant.

Drugs

Methoxamine, carbachol, L-ornithine (Sigma Chemical Co., Poole, UK), iberiotoxin (Tocris Biosciences, Bristol, UK) were dissolved in deionized water. Cinacalcet [(R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride], calindol [(R)-2-[[[1-(1-naphthyl)ethyl]amino] methyl]-1H-indole hydrochloride], S-cinacalcet [(S)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride], S-calindol [(S)-2-[[[1-(1-naphthyl)ethyl] amino]methyl]-1H-indole hydrochloride], calhex 231 (4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl]amino] cyclohexyl]benzamide) (Toronto Research Chemicals Inc., Ontario, Canada), capsaicin and verapamil (Sigma) were dissolved in 100% ethanol. Ionomycin (Sigma) and (+/–)-BayK8644 ((4R)- and (4S)-1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-trifluoromet hyl)phenyl]-3-pyridinecarboxylic acid methyl ester; Merck Chemicals Ltd, Nottingham, UK) were dissolved in 100% dimethyl sulphoxide.

Results

Relaxation to cinacalcet, calindol and L-ornithine in mesenteric arteries

Figure 1 demonstrates that cinacalcet and calindol, a more recently developed calcimimetic, induced concentration-dependent relaxation of rat small mesenteric arteries. The chemical structures of the two compounds are also shown in Figure 1. Relaxation to cinacalcet was endothelium-independent (Table 1; Figure 2A), and slightly less potent (pEC_{50}, $P < 0.01$) than that of calindol (pEC_{50} = 6.10 ± 0.10, E_{max} = 101 ± 6, n = 6 vs. cinacalcet: pEC_{50} = 5.58 ± 0.07, E_{max} = 97 ± 6, n = 5; Figure 2B). Removal of the endothelium caused an apparent rightward displacement (P < 0.01)

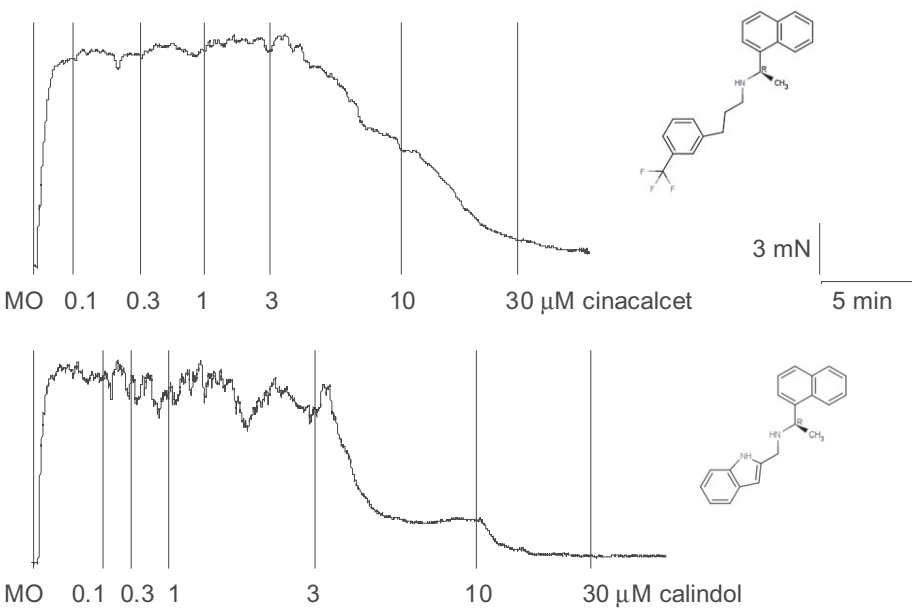

Figure 1

Original tracings showing concentration-dependent relaxation to (A) (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) and (B) (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) in separate mesenteric arteries of the rat. Vertical lines denote addition of drugs. MO, methoxamine (at 10 µM). Chemical structures of the calcimimetics are shown next to their corresponding tracings.

Table 1

Concentration-dependent relaxation to cinacalcet in rat small mesenteric arteries

	pEC_{50}	E_{max} (%)	*n*
+endothelium	5.58 ± 0.07	97 ± 6	5
–endothelium	5.59 ± 0.06	91 ± 5	5
–endothelium			
Methoxamine-precontracted	5.56 ± 0.07	96 ± 6	4
KCl-precontracted	5.24 ± 0.09**	103 ± 11	5
–endothelium	5.60 ± 0.06	97 ± 5	4
+50 nM iberiotoxin	5.49 ± 0.10	95 ± 10	4
+10 μM capsaicin	5.51 ± 0.04	94 ± 5	5
–endothelium	5.50 ± 0.04	95 ± 7	4
+3 μM calhex 231	5.50 ± 0.04	96 ± 4	4
–endothelium (at 0.5 mM $[Ca^{2+}]_o$)	5.55 ± 0.04	97 ± 4	4
+endothelium	5.64 ± 0.08	98 ± 6	4
+1 mM L-ornithine	5.63 ± 0.07	100 ± 5	4

Data are given as mean ± SEM. pEC_{50} and E_{max} were obtained as stated in Methods. *n* indicates number of animals used. All responses were obtained at 2 mM $[Ca^{2+}]_o$, unless otherwise stated. $^{**}P < 0.01$ significantly different from the corresponding controls.
calhex 231, 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl) ethyl]amino]cyclohexyl]benzamide; cinacalcet, (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride.

Table 2

Concentration-dependent relaxation to calindol in rat small mesenteric arteries

	pEC_{50}	E_{max} (%)	*n*
With endothelium	6.10 ± 0.10	101 ± 6	6
Without endothelium	5.77 ± 0.06**	100 ± 5	6
+endothelium			
Methoxamine-precontracted	6.20 ± 0.09	103 ± 4	5
KCl-precontracted	5.66 ± 0.05**	99 ± 5	5
–endothelium			
Methoxamine-precontracted	5.87 ± 0.05	97 ± 4	6
KCl-precontracted	5.48 ± 0.04**	92 ± 4	4
+endothelium	6.04 ± 0.05	96 ± 3	4
+50 nM iberiotoxin	6.08 ± 0.11	100 ± 6	4
+10 μM capsaicin	5.76 ± 0.05*	97 ± 3	4
+endothelium	5.97 ± 0.04	98 ± 3	7
+3 μM calhex 231	5.84 ± 0.07	101 ± 5	5
+endothelium (at 0.5 mM $[Ca^{2+}]_o$)	5.76 ± 0.06#	105 ± 4	5
+3 μM calhex 231	5.81 ± 0.07	102 ± 5	5
+endothelium	6.04 ± 0.06	102 ± 4	4
+1 mM L-ornithine	6.00 ± 0.04	98 ± 3	4

Data are given as mean ± SEM. pEC_{50} and E_{max} were obtained as stated in Methods. *n* indicates number of animals used. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from the corresponding controls. All responses were obtained at 2 mM $[Ca^{2+}]_o$, unless otherwise stated.
$^{\#}P < 0.05$ compared with relaxations at 2 mM $[Ca^{2+}]_o$. calhex 231, 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl] amino]cyclohexyl]benzamide; calindol, (R)-2-[[[1-(1-naphthyl) ethyl]amino]methyl]-1H-indole hydrochloride.

of the concentration–response curve to calindol (Table 2; Figure 2B).

In addition to CaR, a related G-protein coupled receptor, GPRC6A is also expressed in the endothelium of mesenteric arteries (Harno *et al.*, 2008) although its function remains unclear. We found that L-ornithine, an amino acid that activates rat GPRC6A (EC_{50} = 264 μM, Wellendorph *et al.*, 2007) induced small relaxations (% relaxation at 3 mM = 39 ± 6%; *n* = 6; Figure 2C).

Role of K^+ channels and perivascular sensory nerves in relaxation to calcimimetics

In endothelium-denuded vessels, precontracting arteries with 60 mM KCl, instead of 10 μM methoxamine (see *Methods*), reduced ($P < 0.01$) the pEC_{50} for cinacalcet without affecting the maximum response (Table 1; Figure 3A). Similar results were also obtained with calindol, with or without the endothelium (Table 2; Figure 4A,B).

Further experiments found that cinacalcet responses were not significantly attenuated by iberiotoxin, which is a selective BK_{Ca} blocker, or prolonged treatment with capsaicin, which desensitized TRPV1-expressing perivascular nerves (Table 1; Figure 3B). In contrast, the pEC_{50} value of calindol-induced relaxation was significantly ($P < 0.05$) attenuated by capsaicin treatment but not iberiotoxin (Table 2; Figure 4C). Iberiotoxin (50 nM) also had no effect on the small relaxant responses to L-ornithine (*n* = 4; data not shown).

Role of CaR in relaxation to calcimimetics

Interestingly, the negative modulator of CaR, calhex 231 had no significant effect on arterial relaxation induced by cinacalcet (Table 1; Figure 5A). Reducing the $[Ca^{2+}]$ in Krebs–Henseliet solution (from 2 to 0.5 mM) also had no effect on cinacalcet relaxations (Table 1; Figure 5A). Responses to calindol were not significantly affected by calhex 231 (Table 2; Figure 5B). Reducing $[Ca^{2+}]_o$ slightly reduced the pEC_{50} ($P < 0.05$) of calindol-induced relaxation (Table 2), but the resultant relaxation was again not affected by calhex 231 (Table 2; Figure 5B).

In endothelium-intact, methoxamine-precontracted vessels under 0.5 mM $[Ca^{2+}]_o$, incremental additions of $CaCl_2$ (1–5 mM) elicited contractions followed by relaxations (Figure 6A). In contrast to the cases for cinacalcet and calindol, the Ca^{2+}-induced relaxation was inhibited by 3 μM calhex 231 (Figure 6A), 50 nM iberiotoxin (Figure 6B) or 10 μM capsaicin (Figure 6B). It was noted that while

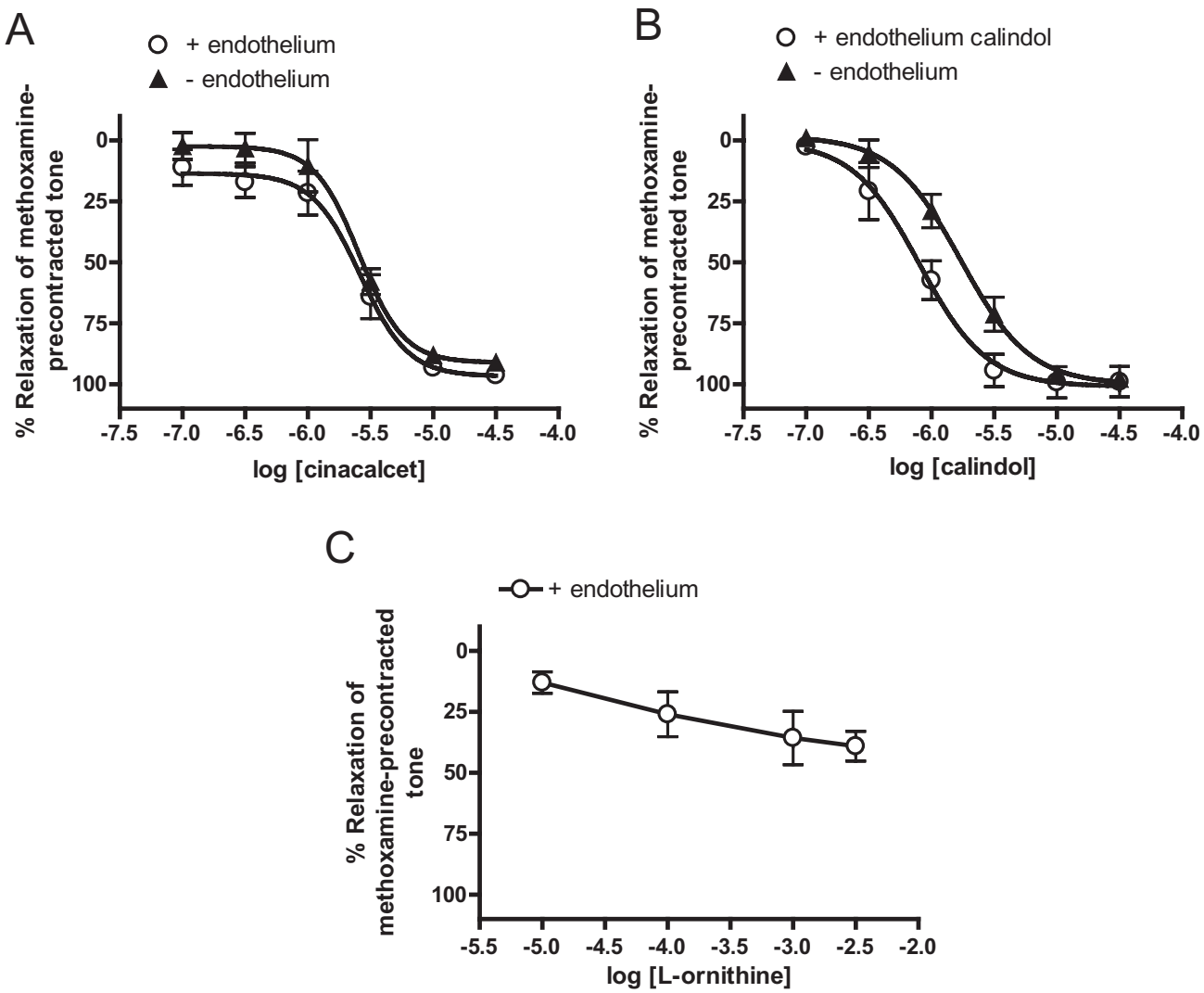

Figure 2

Relaxation to (A) (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) (B) (R)-2-[[[1-(1-naphthyl) ethyl]amino]methyl]-1H-indole hydrochloride (calindol) and (C) L-ornithine in endothelium-intact and -denuded mesenteric arteries. n = 5–6. Values are shown as means and vertical bars represent SEM.

iberiotoxin induced a rightward displacement of the $CaCl_2$ relaxation curve, capsaicin resulted in a reduced E_{max} (Figure 6B). On the other hand, pretreatment of vessels with cinacalcet (1 μM) or calindol (0.3 μM) did not potentiate the responses to $CaCl_2$; in fact, 1 μM cinacalcet significantly reduced responses to $CaCl_2$ at 1 and 2 mM (Figure 6C). Increasing the concentration of calindol to 1 μM also had no effect on $CaCl_2$-induced relaxation (data not shown).

To explore if GPRC6A was involved in arterial relaxations to the calcimimetics, the effect of a brief treatment (for 5 min) with L-ornithine, which can potentiate agonist activity at GPRC6A (Harno *et al.*, 2008), was tested. L-ornithine, at 1 mM, had no effect on cinacalcet (Table 1) or calindol responses (Table 2).

Effects of calcimimetics on $CaCl_2$-induced contractions in the presence of methoxamine

All experiments were performed in endothelium-denuded arteries depleted of intracellular Ca^{2+} and then exposed to methoxamine (10 μM) in the absence of extracellular Ca^{2+}. Under these conditions, addition of $CaCl_2$ (0.01–10 mM) caused concentration-dependent contractions up to 1–3 mM and produced relaxation at higher concentrations. This protocol is frequently used to examine the effect of pharmacological agents on Ca^{2+} entry in vascular smooth muscle (e.g. Ho and Hiley, 2003). Figure 7A shows that cinacalcet, in a concentration-dependent manner, inhibited $CaCl_2$-induced contractions; these contractions were abolished by 10 μM cinacalcet. These effects were mimicked by calindol

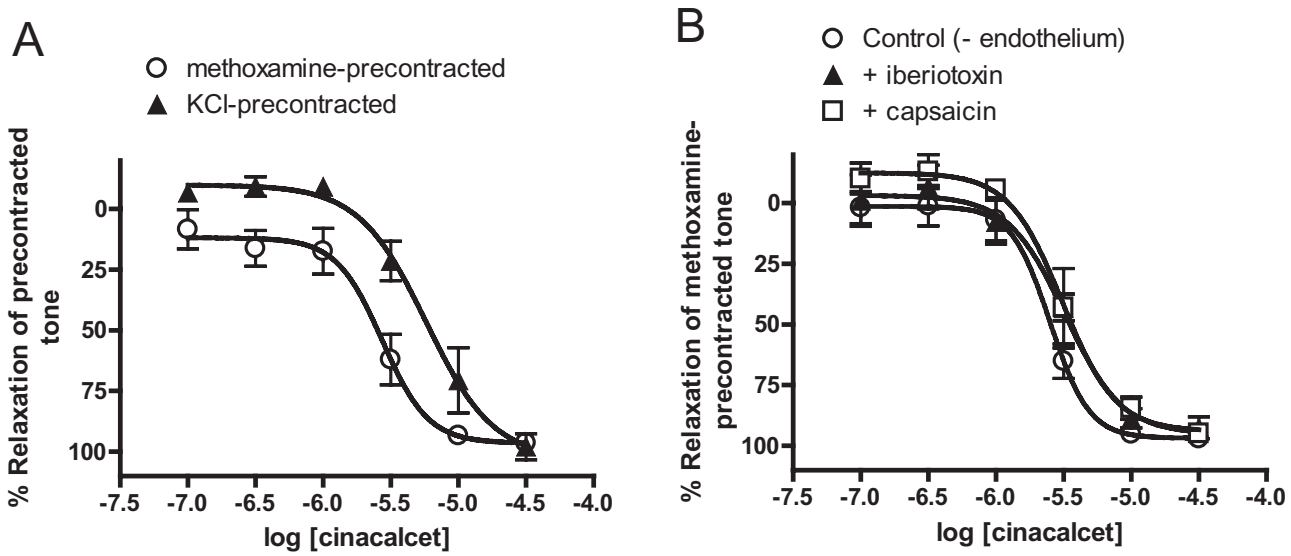

Figure 3

(A) Relaxation to (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) in endothelium-denuded arteries precontracted with 60 mM KCl and 10 μM methoxamine. (B) Effects of iberiotoxin (50 nM) and capsaicin (10 μM) on relaxation to cinacalcet in methoxamine-precontracted, endothelium-denuded arteries. n = 4–5. Values are shown as means and vertical bars represent SEM.

(Figure 7B), which abolished the $CaCl_2$-induced contractions at 3 μM.

Effects of calcimimetics on contractions induced by BayK8644

Activation of voltage-gated, L-type Ca^{2+} channels by BayK8644 (3 μM) induced contractions of endothelium-denuded vessels previously exposed to 15 mM KCl (see *Methods*). These contractions were significantly inhibited by 10 μM cinacalcet, 3 μM calindol or the phenylalkaylamine L-type Ca^{2+} channel blocker, verapamil (at 10 μM; Figure 8A).

Effects of calcimimetics on $CaCl_2$-induced contractions in the presence of ionomycin

As described in Methods, contractions were evoked by ionomycin in arteries that were denuded of endothelium, depleted of intracellular Ca^{2+} stores and initially maintained in the absence of extracellular Ca^{2+}. Intriguingly, under these conditions, addition of cinacalcet (10 μM) and calindol (3 and 10 μM) induced contractions (Figure 8B). In contrast, verapamil (10 μM) had no significant effect on ionomycin-evoked contractions (0.1 ± 0.4 mN; n = 6). It was noted that contractions to the calcimimetics developed more slowly (time taken to attain maximum response ~15 min) than their relaxations seen under methoxamine-precontracted tone (~5 min).

Relaxation to the S-enantiomers of cinacalcet and calindol in mesenteric arteries

S-Cinacalcet, the S-enantiomer of cinacalcet that is inactive at CaR (Brown and MacLeod, 2001), also caused concentration-dependent relaxation (with endothelium, S-cinacalcet, pEC_{50} = 5.66 ± 0.07; E_{max} = 100 ± 5%; n = 6; R-cinacalcet, pEC_{50} = 5.46 ± 0.15; E_{max} = 101 ± 14%; n = 5; Figure 9A). Similar results were also obtained for the S-enantiomer of calindol, except pEC_{50} for S-calindol was significantly (P < 0.05) lower than that for calindol (with endothelium, S-calindol, pEC_{50} = 5.90 ± 0.05; E_{max} = 101 ± 3%; n = 4; R-calindol, pEC_{50} = 5.66 ± 0.15; E_{max} = 100 ± 5%; n = 5; Figure 9B).

Relaxation to cinacalcet and calindol in mesenteric veins

In contrast to results obtained in mesenteric arteries, calindol induced small relaxations in endothelium-denuded, 60 mM KCl-precontracted mesenteric veins (Figure 10). An even smaller effect was detected for cinacalcet (Figure 10, P < 0.05); significant relaxation was only observed at 30 μM.

Discussion and conclusions

Calcimimetics, such as cinacalcet and calindol, are known to activate CaR by potentiating the agonist effects of Ca^{2+} ions (Jensen and Brauner-Osborne, 2007). Functional CaRs are thought to be expressed in the mesenteric arteries (Wang and Bukoski, 1998; Weston *et al.*, 2005) and indeed we found that both cinacalcet and calindol were potent (pEC_{50} – 5.58 and 6.10 respectively) vasorelaxants in rat small mesenteric arteries. CaR has been detected in perivascular sensory nerves (Wang and Bukoski, 1998), endothelial cells (Weston *et al.*,

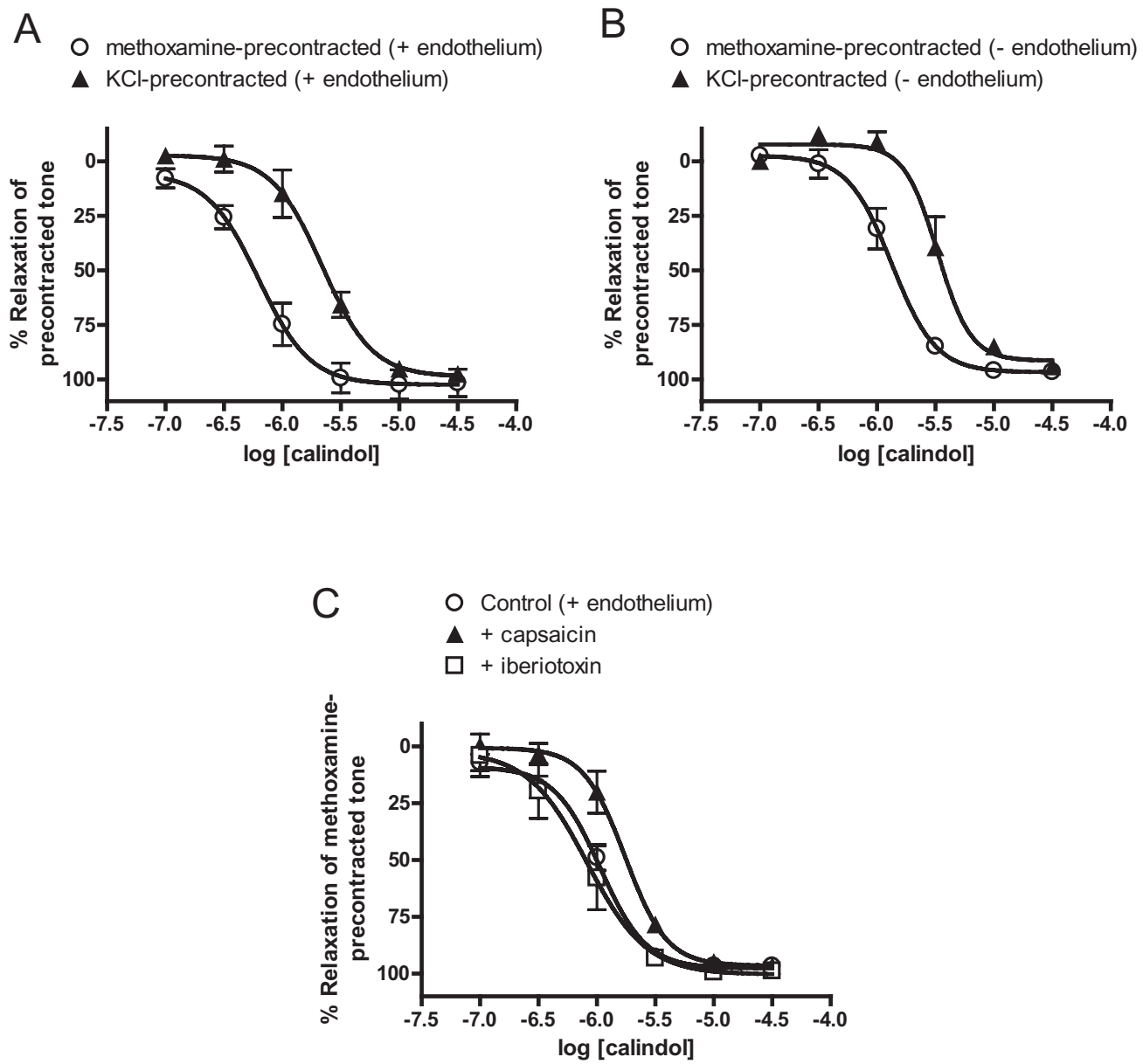

Figure 4

Relaxation to (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) in (A) endothelium-intact and (B) endothelium-denuded arteries precontracted with 60 mM KCl and 10 μM methoxamine. (C) Effects of iberiotoxin (50 nM) and capsaicin (10 μM) on relaxation to calindol in methoxamine-precontracted, endothelium-intact arteries. n = 4–6. Values are shown as means and vertical bars represent SEM.

2005) and, in some cases, smooth muscle cells (Molostvov *et al.*, 2007). In particular, Bukoski and co-workers have proposed that increasing extracellular $[Ca^{2+}]$ within the physiological range (from 1 to 5 mM) relaxes rat mesenteric arteries through excitation of capsaicin-sensitive nerves and subsequent release of endocannabinoids and activation of BK_{Ca} in vascular smooth muscle (Bukoski *et al.*, 1997; Ishioka and Bukoski, 1999; Awumey *et al.*, 2008). The authors also reported that the Ca^{2+}-induced relaxation was independent of a functional endothelium (Bukoski *et al.*, 1997). In contrast, we found that, at 2 mM $[Ca^{2+}]_o$, cinacalcet relaxations were not affected by prolonged treatment with capsaicin, which is an agonist of transient receptor potential vanilloid type 1 (TRPV1) receptors and causes desensitization of TRPV1-expressing sensory nerves. BK_{Ca} blockade with iberiotoxin or endothelial removal also had no effect. While iberiotoxin did not affect calindol responses, the pEC_{50} though not E_{max} of calindol was attenuated in denuded or capsaicin-treated arteries. This is somewhat surprising because the two calcimimetics are thought to have similar potencies and efficacies

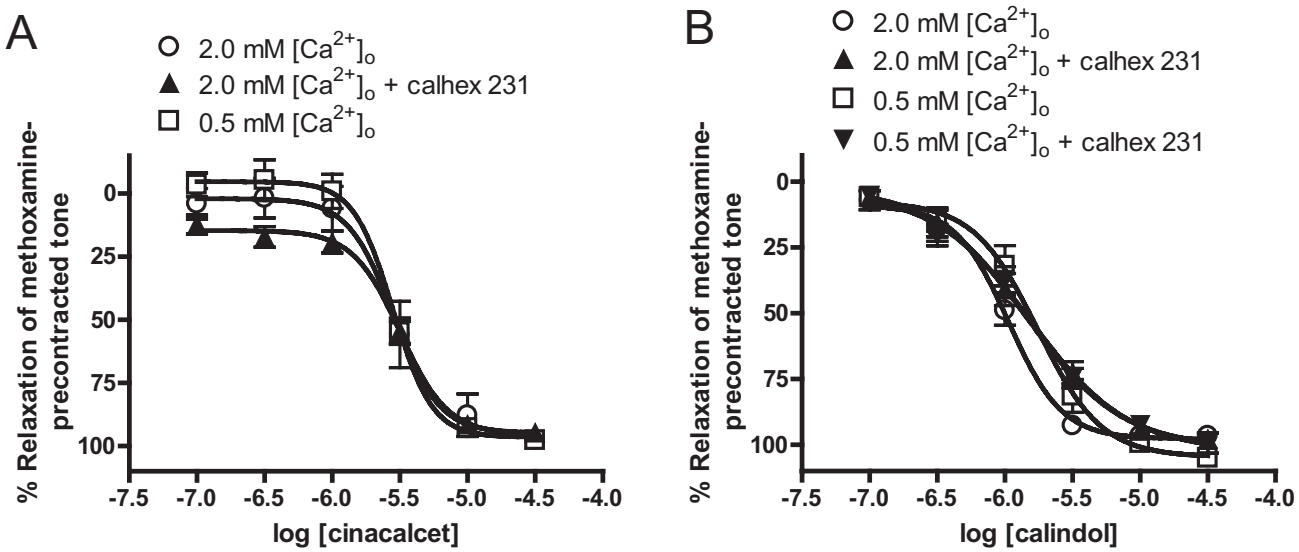

Figure 5

(A) Relaxation to (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) in endothelium-denuded arteries at 2 and 0.5 mM $[Ca^{2+}]_o$, in the absence or presence of 3 μM 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl] amino]cyclohexyl]benzamide (calhex 231). (B) Relaxation to (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) in endothelium-intact arteries at 2 and 0.5 mM $[Ca^{2+}]_o$, in the absence or presence of 3 μM calhex 231. Values are shown as means and vertical bars represent SEM. Note that, on the graph, responses in the presence of calhex 231 at 2 mM $[Ca^{2+}]_o$ overlap almost entirely with those obtained at 0.5 mM $[Ca^{2+}]_o$.

on CaR, as shown in cells stably expressing rat CaR (Kessler *et al.*, 2004).

It should be pointed out that our data with calindol appear at odds with the recent report that relaxation to calindol is only observed after BK_{Ca} blockade (Weston *et al.*, 2008). The authors, using isolated mesenteric arteries mounted in a pressure myograph, found that calindol hyperpolarized vascular smooth muscle cells via endothelial intermediate- conductance Ca^{2+}-activated K^+ channels (IK_{Ca}), an effect which could be prevented by an iberiotoxin-sensitive 'K^+ cloud' in precontracted vessels (Weston *et al.*, 2008; see also Edwards and Weston, 2004). The basis of these discrepancies remains unclear; perhaps under the current experimental conditions, relaxation mechanisms independent of IK_{Ca} activity are exaggerated. We did observe a small endothelium-dependent component of calindol responses. However, because precontraction with 60 mM KCl, which abolished K^+ efflux via K^+ channels, attenuated relaxation to calindol in both endothelium-intact and -denuded arteries, the participation of endothelial K^+ channels remains unclear. High $[K^+]_o$ also attenuated cinacalcet-induced relaxation in denuded vessels, suggesting the involvement of K^+ channels in vascular smooth muscle. Our data argue against BK_{Ca} activation but the precise subtypes of K^+ channels will require further investigation.

Notably, vascular actions of the two calcimimetics were insensitive to calhex 231, a negative modulator of CaR (Jensen and Brauner-Osborne, 2007), suggesting the lack of involvement of CaR. Calhex 231 tended to reduce the steepness the concentration–response curves to calindol (cf. Figure 5B); however, there was no significant change in pEC_{50} values. In this study, calhex 231 was used at a concentration (3 μM) that has previously been shown to abolish CaR-mediated responses in transfected cells (Petrel *et al.*, 2003) and mesenteric arteries (Weston *et al.*, 2008). One theoretical possibility is that, in methoxamine-precontracted arteries, CaR is maximally active and addition of a calcimimetic has no further effects on CaR. However, reducing $[Ca^{2+}]_o$ from 2 to 0.5 mM only slightly shifted the concentration–response curve to calindol and had no effect on cinacalcet responses. Importantly, calhex 231 greatly inhibited relaxation to Ca^{2+} itself, confirming the effectiveness of the calcilytic in our preparation. Moreover, Ca^{2+}-induced relaxation was also attenuated by iberiotoxin and capsaicin. Our results support the proposal that Ca^{2+} activates CaR on perivascular nerves, leading to the release of a diffusible substance, which in turn activates BK_{Ca} in vascular smooth muscle (Bukoski *et al.*, 1997). On the other hand, the presence of calcimimetics (at ≤1 μM) also failed to potentiate Ca^{2+}-induced mesenteric relaxation. We therefore conclude that CaR plays a minor role, if any, in mesenteric relaxation to the calcimimetics. This would suggest that CaR has a minimal contribution to the activation by calcimimetics of K^+ channels, and endothelium-dependent or capsaicin-sensitive pathways.

Given that cinacalcet and calindol exert similar actions on CaR, it is curious to observe that relaxation to calindol, but not cinacalcet, was sensitive to endothelial removal or capsaicin treatment. One possible explanation is that calindol also positively modulates GPRC6A, which has recently been reported in the endothelium of rat mesenteric arteries (Harno *et al.*, 2008). GPRC6A is a novel receptor that is sensitive to Ca^{2+} and closely related to CaR (Wellendorph *et al.*, 2007). In contrast to cinacalcet and calindol, the GPRC6A agonist L-ornithine (up to 3 mM) caused small relaxations.

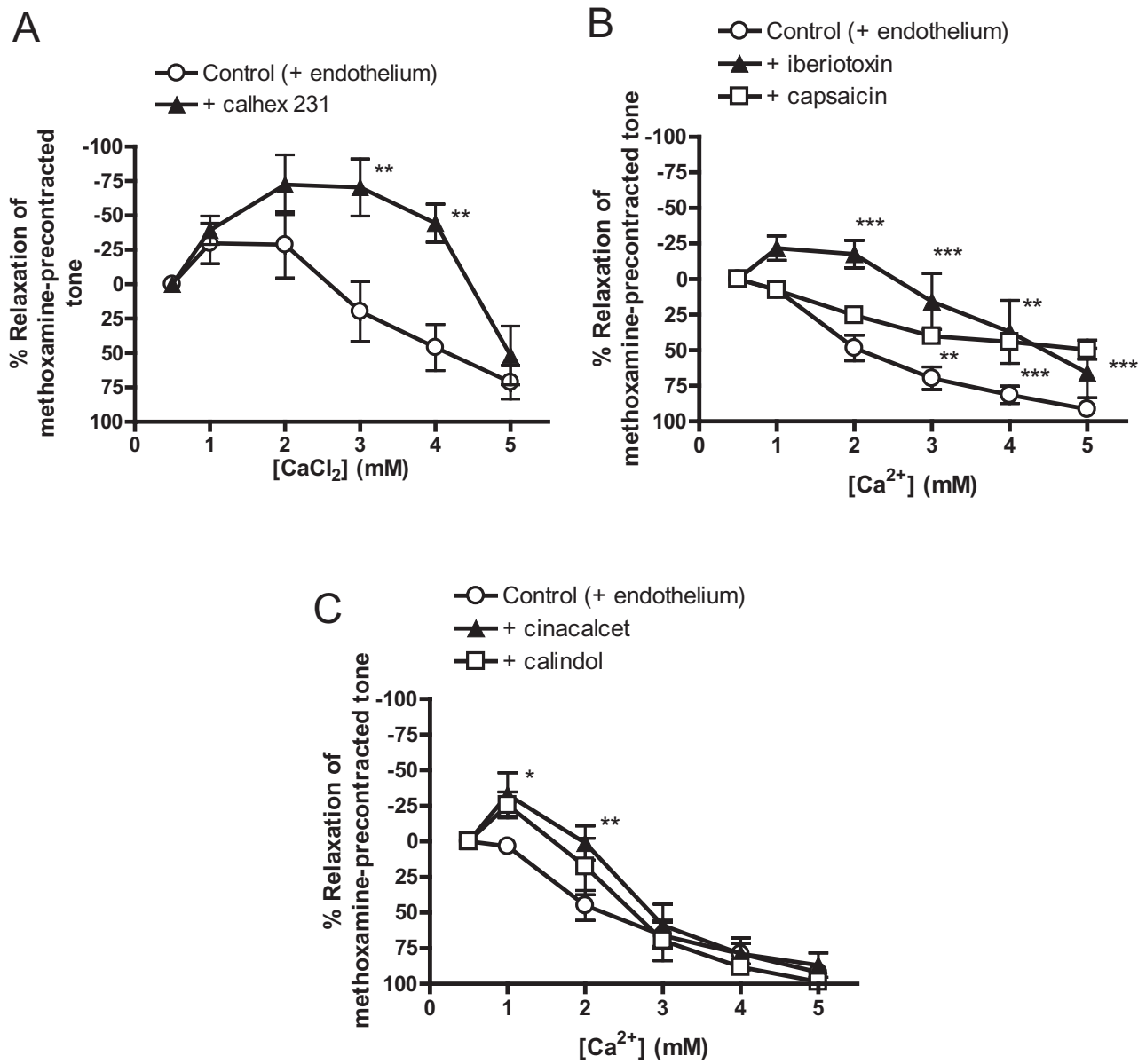

Figure 6

Relaxation to incremental additions of $CaCl_2$ (1–5 mM) in endothelium-intact, methoxamine-precontracted vessels under 0.5 mM $[Ca^{2+}]_o$. (A) Effects of 4-chloro-N-[(1S,2S)-2-[[(1R)-1-(1-naphthalenyl)ethyl]amino]cyclohexyl]benzamide (calhex 231) (3 μM) on $CaCl_2$-induced relaxation. (B) Effects of iberiotoxin (50 nM) or capsaicin (10 μM) on $CaCl_2$-induced relaxation. (C) Effects of (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) (1 μM) or (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) (0.3 μM) on $CaCl_2$-induced relaxation. n = 4–8. *P < 0.05, **P < 0.01, ***P < 0.001 versus control; two-way analysis of variance followed by Bonferroni *post hoc* tests. Values are shown as means and vertical bars represent SEM.

While L-ornithine can potentiate the activity of other agonists or modulators on GPRC6A (Wellendorph *et al.*, 2007; Harno *et al.*, 2008), it had no effect on calcimimetic-induced relaxation in mesenteric arteries. At present, the effect of calindol on GPRC6A is not established (both agonist (Harno *et al.*, 2008) and antagonist actions (Faure *et al.*, 2009) have been reported) and no information is available regarding cinacalcet at GPRC6A. Nonetheless, our data are not consistent with a role of GPRC6A in relaxant responses to the calcimimetics.

The ability of the calcimimetics to fully relax mesenteric arteries precontracted with 60 mM KCl suggests that they might interfere with Ca^{2+} influx via voltage-gated Ca^{2+} channels, or processes downstream, in vascular smooth muscle. Ca^{2+} influx through L-type, voltage-gated Ca^{2+} channels is crucial for the contractile effects of methoxamine, and almost entirely mediates precontraction with depolarizing K^+ solution (Karaki *et al.*, 1997; Ho and Hiley, 2003). Of particular interest, both cinacalcet and calindol bear structural similarities to phenylalkylamines, for example, verapamil (IC_{50} – 1 μM;

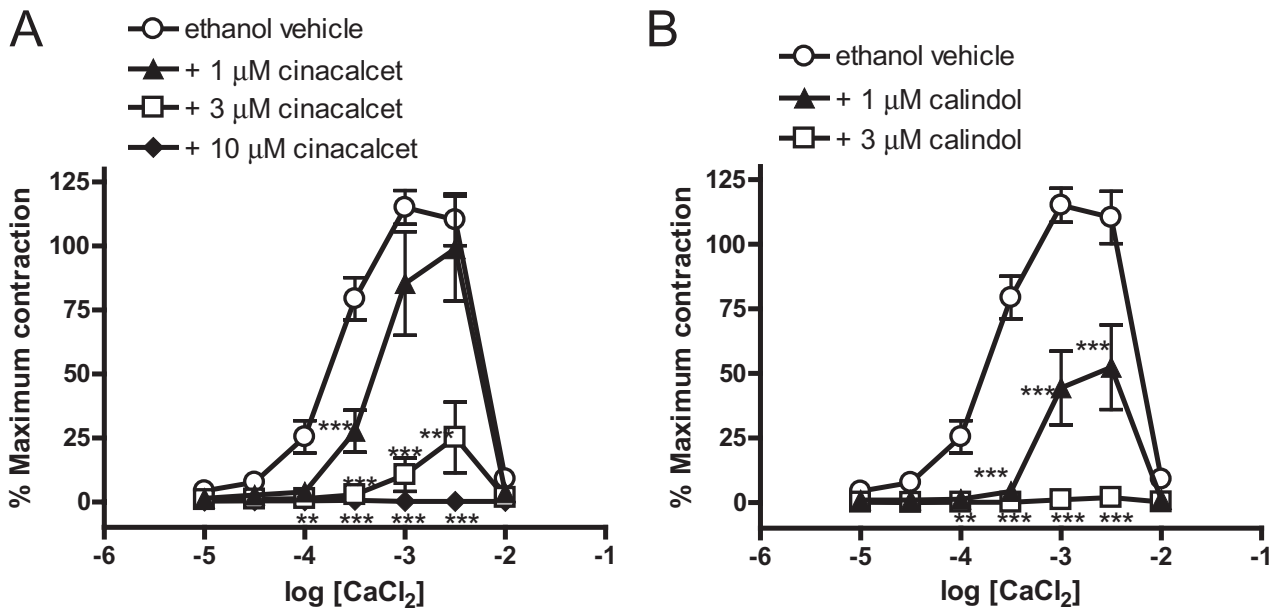

Figure 7

Concentration–response curves for $CaCl_2$-induced contractions of methoxamine-stimulated, endothelium-denuded mesenteric arteries depleted of intracellular Ca^{2+} stores with EGTA as described in Methods. (A) Responses to $CaCl_2$ in the presence of ethanol vehicle (0.1% vv^{-1}) or (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) (at 1, 3, 10 μM). (B) Responses to $CaCl_2$ in the presence of ethanol vehicle (0.1% vv^{-1}) or (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) (at 1 or 3 μM). $n = 4$ for all. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle; two-way analysis of variance, followed by Bonferroni *post hoc* tests. Values are shown as means and vertical bars represent SEM.

Johnson *et al.*, 1996), which are L-type Ca^{2+} channel blockers (Jensen and Brauner-Osborne, 2007). To further test the hypothesis that the calcimimetics block Ca^{2+} influx in mesenteric smooth muscle, we examined their effects on contractions induced by re-additions of Ca^{2+} to endothelium-denuded arteries that had been depleted of intracellular Ca^{2+} stores and stimulated with methoxamine. Using this protocol, Ca^{2+}-induced contraction has been demonstrated to occur mainly through activation of voltage-gated Ca^{2+} channels (White and Hiley, 1998). Both cinacalcet and calindol potently inhibited the contractions to Ca^{2+}, achieving 100% inhibition at 10 μM and 3 μM respectively. Moreover, the calcimimetics inhibited contractions elicited by BayK8644, an activator of L-type Ca^{2+} channels. Stereoisomers of cinacalcet and calindol also induced similar relaxations, consistent with a receptor-independent mechanism of relaxation (Nemeth *et al.*, 2004). Interestingly, we also found that the calcimimetics relax mesenteric arteries to a much greater extent than mesenteric veins. This parallels the preferential relaxant effects of L-type Ca^{2+} channel blockers, e.g. verapamil, on the arterial circulation (Thakali *et al.*, 2010). Taken together, we propose that cinacalcet and calindol induce mesenteric relaxation primarily by inhibiting Ca^{2+} influx, probably via L-type Ca^{2+} channels. It is conceivable that cinacalcet and calindol, which share structural similarities (cf. Figure 1), directly interact with L-type Ca^{2+} channels; however, this remains to be confirmed. It should be emphasized that the calcimimetics might also inhibit other Ca^{2+} influx pathways, for instance receptor-operated or store-operated cation channels, which are involved in agonist-induced contractions. We noted that when Ca^{2+} influx mechanisms were 'by-passed' by contracting mesenteric arteries with ionomycin, the calcimimetics, but not verapamil, elicited contractions. These results indicate that calcimimetics might somehow enhance Ca^{2+}-sensitivity in vascular smooth muscle, an effect that is expected to oppose the predominant vasorelaxant action of the compounds. At present, the involvement of CaR in the contractile effects of calcimimetics remains unclear and is the subject of ongoing investigations.

Our data suggest that CaR-independent, vasorelaxant effects of cinacalcet and calindol should be considered when investigating the direct cardiovascular actions of calcimimetics. In this study, the estimated EC_{50} values (0.6–3 μM) are similar to those obtained from cellular studies of CaR (e.g. Hammerland *et al.*, 1998; Kessler *et al.*, 2004). A recent *in vivo* study has also reported that higher doses of NPS R-568, a structural analogue of cinacalcet, and its enantiomer NPS S-568 (both at 2 $mg \cdot kg^{-1}$) induced very similar hypotension responses in rats (Nakagawa *et al.*, 2009). However, it is unclear if such off-target effects of cinacalcet are clinically relevant as cinacalcet is more effective in suppressing parathyroid hormone levels in humans (EC_{50}, 0.01–0.03 μM; Messa *et al.*, 2008; Serra *et al.*, 2008) and that a lower plasma concentration of cincacalcet has been reported in clinical studies (generally ranges from 0.03 to 0.3 μM; Ohashi *et al.*, 2004; Kumar *et al.*, 2004; Padhi *et al.*, 2007; Serra *et al.*, 2008). Therefore, the relevance of L-type Ca^{2+} channels or other potential off-targets in the therapeutic effects of calcimimetics remains to be determined.

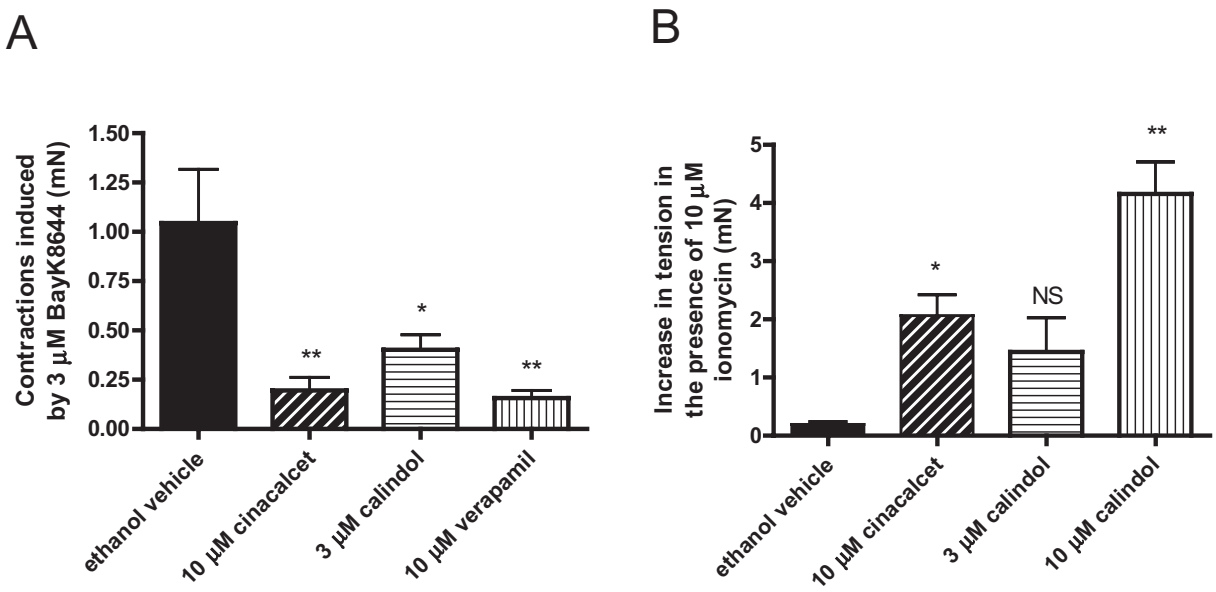

Figure 8

(A) Effects of ethanol vehicle (0.1% vv^{-1}), (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) (10 μM) or (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) (3 μM) on contractions to 3 μM BayK8644 in endothelium-denuded mesenteric arteries as described in Methods. (B) Effects of ethanol vehicle (0.1% vv^{-1}), cinacalcet (10 μM) or calindol (3 μM) on precontracted tone induced by 2 mM $CaCl_2$ in ionomycin-stimulated, endothelium-denuded mesenteric arteries depleted of intracellular Ca^{2+} stores with EGTA, as described in Methods. $n = 4–5$. *$P < 0.05$, **$P < 0.01$ versus vehicle; one-way analysis of variance, followed by Dunnett's *post hoc* tests. Values are shown as means and vertical bars represent SEM. NS, not significant.

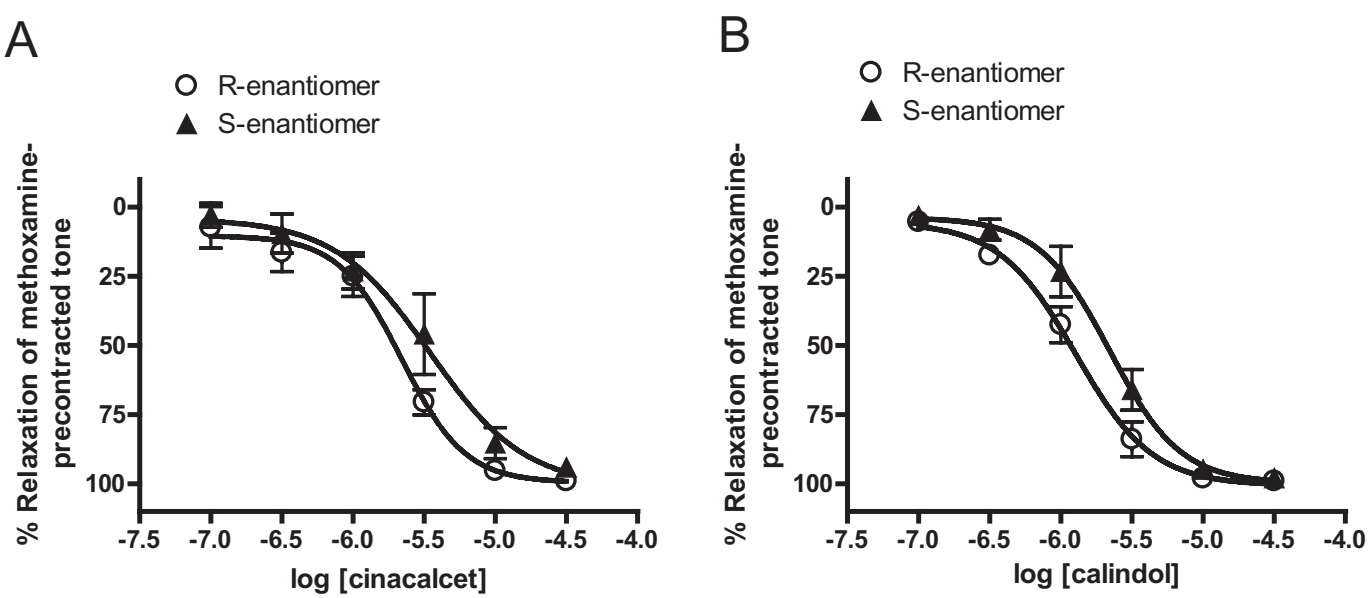

Figure 9

Relaxation to S-and R-enantiomers of (A) (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) and (B) (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) in endothelium-intact mesenteric arteries. $n = 4–6$. Values are shown as means and vertical bars represent SEM.

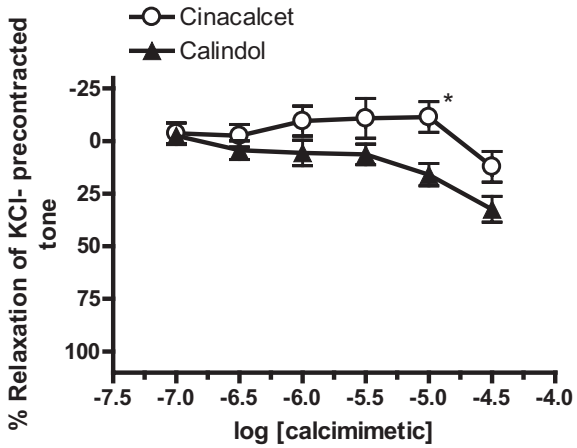

Figure 10

Relaxation to (R)-N-(3-(3-(trifluoromethyl)phenyl)propyl)-1-(1-napthyl)ethylamine hydrochloride (cinacalcet) and (R)-2-[[[1-(1-naphthyl)ethyl]amino]methyl]-1H-indole hydrochloride (calindol) in endothelium-denuded mesenteric veins precontracted with 60 mM KCl. $n = 5$ for all. *$P < 0.05$ versus calindol, two-way analysis of variance, followed by Bonferroni *post hoc* tests. Values are shown as means and vertical bars represent SEM.

To conclude, our data indicate that cinacalcet and calindol act as potent arterial relaxants primarily through inhibiting Ca^{2+} influx into the vascular smooth muscle. Other relaxation mechanisms involve the endothelium, K^+ channels and capsaicin-sensitive, perivascular nerves. However, the established CaR and the novel Ca^{2+}-sensitive receptor, GPRC6A appear to have little role in relaxation induced by the calcimimetics.

Acknowledgements

None.

Conflict of interest

The authors state no conflict of interest.

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