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The calcilytics Calhex-231 and NPS 2143 and the calcimimetic Calindol reduce vascular reactivity via inhibition of voltage-gated Ca^{2+} channels

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ABSTRACT

The present study investigates the effect of commonly used negative and positive allosteric modulators of the calcium-sensing receptor (CaSR) on vascular reactivity. In wire myography studies, increasing $[Ca^{2+}]_o$ from 1 mM to 6 mM induced concentration-dependent relaxations of methoxamine-induced pre-contracted rabbit mesenteric arteries, with 6 mM $[Ca^{2+}]_o$ producing almost complete relaxation. $[Ca^{2+}]_o$-induced relaxations were attenuated in the presence of the calcilytics Calhex-231 and NPS 2143, and abolished by the removal of the endothelium. In addition to their calcilytic effects, Calhex-231 and NPS 2143 also produced concentration-dependent inhibitions of methoxamine- or KCl-induced precontracted tone, which were unaffected by removal of the endothelium and unopposed in the presence of the calcimimetic Calindol. In vessels with depleted Ca^{2+} stores, contractions mediated by Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCCs) were inhibited by Calhex231. In freshly isolated single rabbit mesenteric artery smooth muscle cells, Calhex-231 and NPS 2143 inhibited whole-cell VGCC currents. Application of Calindol also inhibited methoxamine- and KCl-induced pre-contracted tone, and inhibited whole-cell VGCC currents. In conclusion, in addition to their CaSR-mediated actions in the vasculature, Calhex-231, NPS 2143 and Calindol reduce vascular contractility via direct inhibition of VGCCs.

1. Introduction

The extracellular calcium-sensing receptor (CaSR) has a well-characterised role in regulating plasma Ca^{2+} homeostasis through regulating the secretion of parathyroid hormone (PTH) from the parathyroid gland. (Brown and MacLeod, 2001; Hofer and Brown, 2003; Ward and Riccardi, 2012). The CaSR is a therapeutic target for diseases linked to calcium homeostasis, and a series of allosteric modulators of the CaSR are available (Hebert, 2006; Jensen and Bräuner-Osborne, 2007; Mancilla and De Luca, 1998; Nemeth et al., 2001; Petrel et al., 2003; Steddon and Cunningham, 2005). Positive CaSR modulators such as Cinacalcet and Calindol, termed calcimimetics, potentiate the action of extracellular Ca^{2+} at the receptor to suppress PTH release (Hebert, 2006; Jensen and Bräuner-Osborne, 2007; Steddon and Cunningham, 2005). As such, Cinacalcet (Mimpara®), the only allosteric modulator of Gprotein coupled receptors currently approved for clinical use, is used to treat uraemic secondary hypercalcaemia, and hyperparathyroidism, associated with parathyroid malignancy (Hebert, 2006; Jensen and Bräuner-Osborne, 2007; Steddon and Cunningham, 2005). Conversely, negative CaSR modulators such as NPS 2143 and Calhex-231, termed calcilytics, decrease stimulation of CaSRs to increase PTH release (Mancilla and De Luca, 1998; Nemeth et al., 2001; Petrel et al., 2003; Steddon and Cunningham, 2005). Calcilytics have been proposed to treat patients with gain-of-function CaSR-mutations, and osteoporosis as increases in plasma PTH levels may have anabolic effects on trabecular and compact bone (Fitzpatrick et al., 2011; Han and Wan, 2012).

Functional CaSR expression has also been demonstrated in tissues not associated with regulating calcium homeostasis, including the vasculature. Stimulation of endothelial CaSRs by increasing the external Ca^{2+} concentration ($[Ca^{2+}]_o$) and with calcimimetics induces nitric oxide (NO) production which leads to vasorelaxations through stimulation of BK_{Ca} channels in vascular smooth muscle cells (VSMCs) (Awumey et al., 2013; Greenberg et al., 2016; Loot et al., 2013; Smajilovic et al., 2007; Ziegelstein et al., 2006). Endothelial CaSR stimulation also activates IK_{Ca} channels to induce endothelium-derived hyperpolarisations (EDH) and vasorelaxations (Awumey et al., 2013; Dora et al., 2008; Greenberg et al., 2016; Weston et al., 2005, 2008). In addition, stimulation of CaSRs expressed on perivascular neurons (Bukoski et al., 1997, 2002; Ishioka and Bukoski, 1999; Mupanomunda et al., 1998; Wang and Bukoski, 1998) and VSMCs (Li et al., 2011; Molostvov et al., 2008, 2007; Schepelmann et al., 2016;

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Smajilovic et al., 2006; Wonneberger et al., 2000) have also been linked to changes in vascular reactivity.

These findings indicate that calcimimetics and calcilytics may represent potential therapeutic targets to control vascular contractility. However, the mechanism of action of these agents on vascular tone is unclear, with both CaSR-dependent and -independent effects described (Bonomini et al., 2012; Loot et al., 2013; Rybczynska et al., 2006a,b, 2010; Smajilovic et al., 2007; Thakore and Ho, 2011). Calcimimetics have been shown to directly induce NO production in endothelial cells (ECs) and directly inhibit voltage-gated Ca^{2+} channels (VGCCs) in VSMCs (Bonomini et al., 2012; Thakore and Ho, 2011). Calcilytics have also been proposed to attenuate phenylephrine- and KCl-evoked contractions in mouse aorta and induce an acute hypertensive effect in vivo studies that may involve a CaSR-dependent or independent action on the vasculature (Loot et al., 2013; Rybczynska et al., 2006a,b, 2010).

Our limited understanding of the effects of CaSR modulators in the vasculature is an important omission if the CaSR is to become a potential therapeutic target to control vascular contractility. In addition, understanding how these agents affect the vasculature may predict potential adverse effects of CaSR-related drugs used to regulate PTH secretion and Ca^{2+} homeostasis. The present study addresses these issues by examining how the commonly used CaSR calcilytics, Calhex-231 and NPS 2143 affect vascular contractility at concentrations which have previously been shown to inhibit CaSR responses in a variety of tissues. The effects of the calcimimetic compound calindol on vascular reactivity are also examined. Our results reveal that in addition to regulating CaSR-mediated changes in vascular tone, these drugs also inhibit vascular reactivity via CaSR-independent mechanisms which predominantly arises from direct blockade of VGCCs. Importantly, both the CaSR-dependent and independent actions are likely to occur at similar concentrations.

2. Materials and methods

2.1. Cell and vessel segment preparation

Male New Zealand White rabbits (2.5–3 kg) were killed by intravenous injection of sodium pentobarbitone (120 mg/kg) in accordance with Schedule I of the UK Animals Scientific Procedures Act, 1986. Second-order branches of rabbit superior mesenteric artery were dissected and cleaned of adherent tissue in physiological salt solution (PSS) containing (mM): NaCl 126, KCl 6, Glucose 10, HEPES 11, $MgCl_2$ 1.2, and $CaCl_2$ 1.5, with pH adjusted to 7.2 with 10 M NaOH. Following dissection, vessels were either cut into 2 mm segments for wire myography studies or enzymatically dispersed to obtain freshly isolated single VSMCs. To isolate VSMCs, the vessels were cut open longitudinally and the endothelium was gently removed from the vessel wall with a cotton bud, and vessels were then washed in PSS containing 50 μm $[Ca^{2+}]_o$ for 5 min at 37 °C and placed in fresh 50 μm $[Ca^{2+}]_o$ PSS containing collagenase (1 mg/ml) and protease (0.2 mg/ml) for 15 min at 37 °C. Following this, vessels were triturated in fresh PSS and the cell-containing solution was collected and centrifuged for 1 min at 1000 rpm. The supernatant was removed and the cells re-suspended in fresh PSS containing 0.75 mM $[Ca^{2+}]_o$, plated onto coverslips, and left at 4 °C for 1 h before use.

2.2. Isometric tension recordings

The effects of increasing concentrations of $[Ca^{2+}]_o$, Calhex-231, NPS 2143 and Calindol on vascular tone were investigated using wire myography. Vessel segments of 2 mm in length were mounted in a wire myograph (Model 610 M; Danish Myo Technology, Aarhus, Denmark) and equilibrated for 30 min at 37 °C in 5 ml of 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_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1, D-glucose 10. The mean resting diameter of the vessel segments was 402 ± 6 μm (n=32 animals, 122 vessel segments, ± S.E.M). Once mounted, vessel segments were then normalized to 90% of the internal circumference predicted to occur under a transmural pressure of 100 mmHg (Mulvany and Halpern, 1977). Mean resting vessel tension following normalisation was 5.6 ± 0.1 mN (n=32 animals, 122 vessel segments, ± S.E.M). Vessels were left for 10 min and were then challenged with 60 mM KCl for 5 min before being washed out with fresh Krebs solution. Endothelium integrity was then assessed by stably pre-contracting vessels with the α_1 adrenoceptor agonist 10 μm methoxamine (the concentration which gives 80% of the maximal methoxamine-induced response) followed by the addition of 10 μm Carbachol (CCh). Vessels in which CCh-induced relaxations were > 90% of pre-contracted tone were designated as having a functional endothelium. When necessary, endothelium was removed by rubbing the intima with a human hair, and CCh-induced relaxations of < 10% of pre-contracted arteries indicated successful removal. Vessel segments were incubated for 30 min in fresh Krebs solution and then pre-contracted with 10 μm methoxamine or 60 mM KCl as required. This was followed by cumulative additions of $CaCl_2$, Calhex-231 (4-Chloro-*N*-[(1 *S*,2 *S*)−2-[[(1 *R*)−1-(1naphthalenyl)ethyl]amino]cyclohexyl]-benzamide Hydrochloride), NPS 2143 (2-Chloro-6[(2 *R*)−3-[[1,1-dimethyl-2-(2-naphthalenyl)ethyl]amino-2-hydroxypropoxy]benzonitrile Hydrochloride), Calindol (N-[(1 R)−1-(1-Naphthalenyl)ethyl]−1H-indole-2-methanamine Hydrochloride; (R)−2-[[[1-(1-Naphthyl)ethyl] amino]methyl]−1H-indole Hydrochloride) or their respective vehicles. When required, inhibitors were added to the vessel segments 30 min before the construction of the concentration-response curves and were present throughout the experiments after this initial exposure period.

Experiments carried out in the presence of an inhibitor following an initial 30 min exposure period are described throughout as performed 'in the presence of' the relevant inhibitor tested.

In a separate set of experiments, the inhibitory effects of Calhex-231 on vascular contractility mediated by Ca^{2+} influx through activation of VGCCs was examined in endothelium removed vessels following depletion of intracellular Ca^{2+} stores. Vessels were equilibrated in Krebs solution containing 0 mM $[Ca^{2+}]_o$, and the cell-permeable Ca^{2+} chelator BAPTA-AM (50 μm) was added to the bath followed by a series of 10 μm methoxamine additions until no contractile response was observed. Vessels were then washed in fresh Ca^{2+}-free Krebs solution together with 3 μm or 10 μm Calhex-231, or its vehicle dimethyl sulfoxide (DMSO) and left for 30 mins. This was followed by the addition of 10 μm methoxamine, and after 3 mins, 2 mM $[Ca^{2+}]_o$ was added to the bath.

For each experiment described above, controls were performed using vessel segments isolated from the same animal. All relaxant responses are expressed as percentage relaxation of pre-contracted tone induced by either 10 μm methoxamine or 60 mM KCl. In the Ca^{2+} influx experiment, contractions are expressed as a percentage of the maximum contraction induced by 2 mM $[Ca^{2+}]_o$ following stimulation with 10 μm methoxamine, in the absence of any drug or vehicle (DMSO). Control vessels also had their calcium stores depleted and were lacking a functional endothelium. Data points on all graphs and bars on all bar charts are mean values and error bars represent S.E.M. For each experiment *n*=number of animals, with at least 3–4 vessel segments used from each animal.

Responses were analysed by 2-Way ANOVA followed by Bonferroni *post hoc* tests or by Student's *t*-test where appropriate. Where required, drug concentrations inhibiting 50% of the contraction (IC_{50}) as well as the maximal inhibition (E_{max}) were calculated from an inspection of the dose-response curves and compared using Student's *t*-test. In all analyses, $P < 0.05$ was taken as statistically significant. Bonferroni comparisons are shown above the graph data points whereby: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. controls. Statistical analyses and graphs were made using Graphpad Prism 6 software (GraphPad Software, Inc, San Diego, CA, USA).