

RESEARCH PAPER

Inhibition of nitric oxide-activated guanylyl cyclase by calmodulin antagonists

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Background and purpose: Nitric oxide (NO) controls numerous physiological processes by activation of its receptor, guanylyl cyclase (sGC), leading to the accumulation of 3'-5' cyclic guanosine monophosphate (cGMP). Ca²⁺-calmodulin (CaM) regulates both NO synthesis by NO synthase and cGMP hydrolysis by phosphodiesterase-1. We report that, unexpectedly, the CaM antagonists, calmidazolium, phenoxybenzamine and trifluoperazine, also inhibited cGMP accumulation in cerebellar cells evoked by an exogenous NO donor, with IC₅₀ values of 11, 80 and 180 µM respectively. Here we sought to elucidate the underlying mechanism(s).

Experimental approach: We used cerebellar cell suspensions to determine the influence of CaM antagonists on all steps of the NO-cGMP pathway. Homogenized tissue and purified enzyme were used to test effects of calmidazolium on sGC activity.

Key results: Inhibition of cGMP accumulation in the cells did not depend on changes in intracellular Ca²⁺ concentration. Degradation of cGMP and inactivation of NO were both inhibited by the CaM antagonists, ruling out increased loss of cGMP or NO as explanations. Instead, calmidazolium directly inhibited purified sGC (IC₅₀ = 10 µM). The inhibition was not in competition with NO, nor did it arise from displacement of the haem moiety from sGC. Calmidazolium decreased enzyme V_{max} and K_m, indicating that it acts in an uncompetitive manner.

Conclusions and implications: The disruption of every stage of NO signal transduction by common CaM antagonists, unrelated to CaM antagonism, cautions against their utility as pharmacological tools. More positively, the compounds exemplify a novel class of sGC inhibitors that, with improved selectivity, may be therapeutically valuable.

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Keywords: guanylyl cyclase; nitric oxide; calmodulin; cGMP; calcium; cerebellum

Abbreviations: CaM, calmodulin; iNOS, inducible NOS; NOS, nitric oxide synthase; PDE, phosphodiesterase; PLA₂, phospholipase A₂; PPIX, protoporphyrin IX; sGC, soluble guanylyl cyclase

Introduction

Accumulation of the second messenger, 3'-5' cyclic guanosine monophosphate (cGMP), following activation of the nitric oxide receptor, soluble guanylyl cyclase (sGC), controls a wide range of physiological processes (Moncada *et al.*, 1991; Garthwaite, 2008). Dysfunction of this signalling pathway is implied in several clinical conditions, including hypertension, erectile dysfunction and septic shock (Bredt, 1999; Vallance and Leiper, 2002). The pharmacology of this pathway is well developed (Rees *et al.*, 1990; Morley and Keefer, 1993; Ko *et al.*, 1994; Schrammel *et al.*, 1996; Stasch *et al.*, 2001) and continues to be of pharmaceutical benefit and interest (Moncada, 1999; Napoli and Ignarro, 2003; Evgenov *et al.*, 2006).

Ever since the NO-cGMP pathway was first identified, its modulation by Ca²⁺ has been recognized (Palmer *et al.*, 1988; Bredt and Snyder, 1990; Mayer *et al.*, 1990). All isoforms of NO synthases (NOS) bind and are activated by calmodulin (CaM). In the case of the inducible isoform (iNOS, inducible nitric oxide synthase), CaM is bound even at resting Ca²⁺ concentrations, and the enzyme is therefore constitutively active after expression (Cho *et al.*, 1992). In contrast, eNOS and nNOS bind CaM only after elevation of cytosolic Ca²⁺ concentration, and are thereby activated in a phasic manner, following, for example, opening of Ca²⁺-permeable NMDA receptors (Bredt and Snyder, 1990; Mayer *et al.*, 1990; Stuehr, 1999). Similarly, Ca²⁺-CaM activates an isoform of phosphodiesterase (PDE1), the enzyme that hydrolyses both cGMP and 3'-5' cyclic adenosine monophosphate (cAMP). This dual effect can lead to suppression of cGMP accumulation in those cells that are stimulated to release NO (Mayer *et al.*, 1992).

A large number of compounds have been developed that disrupt CaM function. These 'calmodulin antagonists' are

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generally molecules with hydrophobic and positively charged groups, which occupy the hydrophobic pockets on CaM, preventing the interaction of these pockets with amphiphilic regions of target proteins, such as NOS and PDE1 (LaPorte *et al.*, 1980; O'Neil and DeGrado, 1990). However, CaM regulates the activity of dozens of proteins (O'Neil and DeGrado, 1990; Jurado *et al.*, 1999), and so antagonizing its binding to target proteins can have complex effects on a given signalling pathway. For example, CaM antagonists have been shown to relieve the CaM-dependent inhibition of phospholipase A₂ (PLA₂), opening store-operated or non-store-operated Ca²⁺ entry channels in several cell types (Peppiatt *et al.*, 2004; Smani *et al.*, 2004). This may be significant for NO signalling, as Ca²⁺ can directly inhibit the NO receptor (sGC) by binding to high and low affinity sites (Parkinson *et al.*, 1999; Kazerounian *et al.*, 2002), although the sensitivity of sGC to Ca²⁺ lies in the micromolar range (IC₅₀ ~ 2.6 µM), and evidence of direct inhibition of sGC (rather than activation of PDE1) in a physiological context is scarce. Conversely, cGMP has been shown to modulate Ca²⁺ signalling, mainly through the phosphorylation by cGMP-dependent protein kinase of several of the channels, pumps and buffers that comprise the Ca²⁺ signalling 'toolkit' (Clementi and Meldolesi, 1997; Berridge *et al.*, 2003).

Given the potential for crosstalk, we investigated the effect of commonly used CaM antagonists – namely calmidazolium, trifluoperazine and phenoxybenzamine – on the NO-cGMP and Ca²⁺ signalling pathways in rat cerebellar cell suspensions. We found that in addition to the predicted effects due to CaM antagonism, these compounds also had pharmacological activity independent of Ca²⁺-CaM. Specifically, CaM antagonists inhibited NO inactivation in cell suspensions and inhibited purified sGC in an uncompetitive manner. Consequently, CaM antagonists could disrupt every step of the NO signalling pathway.

Methods

Preparation of cerebellar cells

All animal care and experimental procedures complied with UK Home Office and local ethics committee regulations. Cerebellar cell suspensions from 8 day old Wistar rats were prepared as previously described (Bellamy and Garthwaite, 2001a). Accumulation of cGMP was measured by inactivating an aliquot of the cell suspension in boiling buffer (containing 50 mM Tris and 4 mM EDTA) after a fixed period of NO exposure at 37°C. Total cGMP content was measured by standard radioimmunoassay or the 'HitHunter' enzyme fragment complementation chemiluminescence assay (GE Healthcare, Little Chalfont, UK), according to manufacturer instructions.

Ca²⁺ imaging

For Ca²⁺ imaging experiments, cell suspensions were prepared as above, and allowed to settle onto poly-l-lysine coated coverslips for 30 min. The coverslips were washed with imaging buffer containing (mM): NaCl 135, KCl 3, HEPES 10, glucose 15, MgSO₄ 2, CaCl₂ 2, and then incubated at room temperature with 3 µM Fluo-4 AM for 30 min. After washing in

imaging buffer, the cells were left for a further 30 min to allow de-esterification of the indicator. Astrocytes were identified by their characteristic morphology – they adhere to the substrate more tightly than the abundant neurons and develop processes that gave them a 'hairy' appearance (Bellamy *et al.*, 2000). Astrocytes readily took up Fluo-4 AM, as previously observed (Singaravelu *et al.*, 2006). Cells were imaged in a static bath on the stage of an Olympus IX70 inverted microscope (objective 60×, NA 1.25; Olympus, Southend-on-Sea, UK), and images were acquired with a Hamamatsu ORCA-ER camera (exposure time, 100 ms; frame rate, 2 Hz; Hamamatsu, Welwyn Garden City, UK). Drugs were added by solution exchange in imaging buffer at the final concentration indicated.

Assay of NO and sGC activity

NO concentration in buffer and cell suspensions (1 mL) was measured in a sealed, stirred chamber at 37°C with an electrochemical probe (ISO-NO; World Precision Instruments, Stevenage, UK).

Purified α1β1 guanylyl cyclase (sGC) from bovine lung was purchased from Alexis Biochemicals (Nottingham, UK). The stock solution was diluted to a concentration of 5 µg·mL⁻¹ in ice-cold buffer containing (mM): Tris 10, dithiothreitol (DTT) 1, and 0.05% bovine serum albumin (BSA), pH 7.4. Activity was measured after further dilution of the enzyme into reaction buffer to give final concentrations of (mM): Tris 50, MgCl₂ 3, DTT 0.01, guanosine triphosphate (GTP) 1, 0.05% BSA, and 50 ng·mL⁻¹ of sGC at pH 7.4 and 37°C before addition of NO donor.

'Clamped' NO concentrations were achieved with the method of Griffiths *et al.* (2003). Briefly, sGC was incubated in a buffer containing (mM): Tris 50, MgCl₂ 3, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) 0.1, DTT 0.01, uric acid 0.3, GTP 1, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [Na⁺ salt] (carboxy-PTIO) 0.2 and 1000 U·mL⁻¹ superoxide dismutase at pH 7.4 and 37°C. After pre-incubation with calmidazolium or dimethyl sulphoxide (DMSO), (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]-diazene-1-ium-1, 2-diolate (SPER/NO) was added at serial dilutions from 0.3 µM to 1 mM. After 2 min, an aliquot of reaction mixture was inactivated and assayed for cGMP content, as for cell suspensions. Free NO concentrations were calculated based on the values for kinetic parameters cited in Griffiths *et al.* (2003), and the EC₅₀ for NO was ~1 nM, closely similar to the previous report.

For experiments to test for haem dissociation, purified sGC (20 µL at 5 µg·mL⁻¹) was incubated with Tween-20 (0.5%), calmidazolium Cl (30 µM) or DMSO (10%) for 15 min at 37°C. Thereafter, the samples were diluted 1:10 in buffer containing (mM): Tris 10, DTT 1, and 0.05% BSA, pH 7.4, and centrifuged at 12 500×g for 10 min at 4°C, through 30 kDa size-exclusion filters (Microcon YM-30; Millipore UK Ltd., Watford, UK). The samples were eluted from the filters in 20 µL of buffer by a further 5 min centrifugation, and then assayed for activity in the usual way.

Cerebellar homogenate was prepared from the cerebella of 16–20 day old Wistar rats using an Ultra-Turrax blade

homogenizer (IKA, Staufen, Germany) in a buffer containing (mM): Tris 10, DTT 1, pH 7.4. Homogenate was diluted 1:4 into reaction buffer containing (mM): Tris 50, DTT 1, MgCl₂ 3, GTP 1, 3-isobutyl-1-methyl xanthine (IBMX) 1, phosphocreatine 5 and 150 µg·mL⁻¹ creatine kinase, pH 7.4 at 37°C. Synthesis of cGMP was triggered by addition of 2-(N,N-diethylamino)-diazolate-2-oxide [diethylammonium salt] (DEA/NO; 100 µM) for 10 min. Aliquots were inactivated and assayed for cGMP content as for cell suspensions.

Data analysis

Unless otherwise indicated, data are the means of three experiments ± SEM. Concentration–response curves were fitted with a logistic equation: $y = c + [(a - c)/(1 + (x/x_0)^p)]$ to allow estimation of potency ($x_0 = EC_{50}$ or IC_{50}) using Origin 7.5 (Microcal, Northampton, MA, USA).

Materials

Superoxide dismutase, IBMX, phosphocreatine, creatine kinase, GTP, calmidazolium chloride, phenoxybenzamine, trifluoperazine and N-(6-aminoethyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) were obtained from Sigma Aldrich (Poole, UK), Fluo 4-AM was obtained from Invitrogen (Paisley, UK), and 2-(N,N-diethylamino)-diazolate-2-oxide [diethylammonium salt] (DEA/NO), (Z)-1-[N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino]-diazene-1-ium-1,2-diolate (SPER/NO), and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [Na⁺ salt] (carboxy-PTIO) were obtained from Alexis (Nottingham, UK). All other reagents were from VWR (Lutterworth, UK).

The NO donor DEA/NO was made up as a stock solution in ice-cold 10 mM NaOH, and diluted 1:100 into the cell suspension at $t = 0$, to give a final concentration of 1 µM. CaM antagonists were prepared as stock solutions in DMSO and diluted 1:100 to give final concentrations as indicated.

Drug target nomenclature conforms to the Guide to Receptors and Channels, 3rd edition (Alexander *et al.*, 2008).

Results

Inhibition of cGMP accumulation by CaM antagonists

Addition of the nitric oxide donor, DEA/NO, to cerebellar cell suspensions caused an increase in intracellular cGMP concentration, which reached a plateau after ~2 min (Figure 1A). This accumulation is largely confined to astrocytes (Southam *et al.*, 1992; Bellamy and Garthwaite, 2001b). Preincubation of the cell suspension with the CaM antagonist, calmidazolium chloride (30 µM) for 10 min significantly inhibited both the amplitude of the NO-evoked cGMP response and the rate of onset of cGMP accumulation (Figure 1A). The concentration–response relationship for DEA/NO in the presence and absence of calmidazolium indicates that the antagonist decreases both the maximal cGMP concentration reached and the potency of the NO donor ($EC_{50} \sim 80$ nM in the absence and 170 nM in the presence of antagonist; Figure 1B).

To explore whether this effect on cGMP accumulation was limited to calmidazolium, other CaM antagonists with different chemical structures were tested, namely trifluoperazine and phenoxybenzamine (Zimmer and Hofmann, 1987; Cimino and Weiss, 1988). Both of these compounds inhibited the accumulation of cGMP in cerebellar cells (Figure 1C), but the lag in cGMP accumulation caused by phenoxybenzamine was less pronounced than for calmidazolium (Figure 1A), and apparently absent for trifluoperazine. The comparative potencies of the CaM antagonists were tested by measuring cGMP accumulation after 2 min incubation with 1 µM DEA/NO (Figure 1D). The potencies were calmidazolium ($IC_{50} \sim 11$ µM) > phenoxybenzamine ($IC_{50} \sim 80$ µM) > trifluoperazine ($IC_{50} \sim 180$ µM).

Effect of Ca²⁺ on cGMP accumulation and inhibition by calmidazolium

The inhibition of cGMP accumulation in cells by CaM antagonists could result from several mechanisms, but in the first instance, we investigated whether the known actions of calmidazolium on Ca²⁺-CaM signalling accounted for the inhibitory effect.

The interactions between Ca²⁺ and cGMP signalling pathways are complex (Clementi and Meldolesi, 1997). In addition to activating NO synthase and PDE1 via CaM, Ca²⁺ has been shown to directly inhibit purified sGC at concentrations in the micromolar range in an uncompetitive manner (Parkinson *et al.*, 1999; Kazerounian *et al.*, 2002). Calmidazolium has been shown to trigger Ca²⁺ entry in several cell types, including cerebellar astrocytes (Singaravelu *et al.*, 2006), by relief of the CaM-dependent inhibition of PLA₂. In principle, therefore, calmidazolium could inhibit sGC activation through elevation of cytosolic Ca²⁺ concentration. To test this possibility, we loaded cerebellar cell suspensions with the Ca²⁺ indicator Fluo 4-AM and measured the change in fluorescence intensity after exposing the cells to known Ca²⁺-mobilizing agonists (Verkhatsky *et al.*, 1998) and calmidazolium, and tested the effect of these interventions on DEA/NO-evoked cGMP accumulation.

The excitatory neurotransmitter glutamate caused a large increase in Ca²⁺ concentration, as measured by Fluo-4 fluorescence, which, in many cells, became dysregulated after an initial peak (Figure 2A,B). This probably reflects the high concentration of glutamate used (1 mM), which can be toxic to astrocytes during prolonged exposure (Matute *et al.*, 2006). Despite generating large Ca²⁺ signals, however, pre-incubation of cells with 1 mM glutamate for 30 s had no significant effect on the amplitude or time course of subsequent NO-evoked cGMP accumulation (Figure 2D). Agonists for metabotropic glutamate receptors (*trans*-ACPD, 100 µM) and AMPA/kainate receptors (kainate, 100 µM) also failed to inhibit cGMP formation (data not shown). Ca²⁺-dependent inhibition of sGC could be achieved, however, with the Ca²⁺ ionophore A23187 (10 µM), which reduced cGMP accumulation by approximately two-thirds (Figure 2E). Collectively, these results suggest that neurotransmitter receptors linked to Ca²⁺ elevation in astrocytes do not cause detectable inhibition of sGC – permeabilization of the cell membrane is required to reach sufficiently high concentrations of Ca²⁺ within the vicinity of cellular sGC.

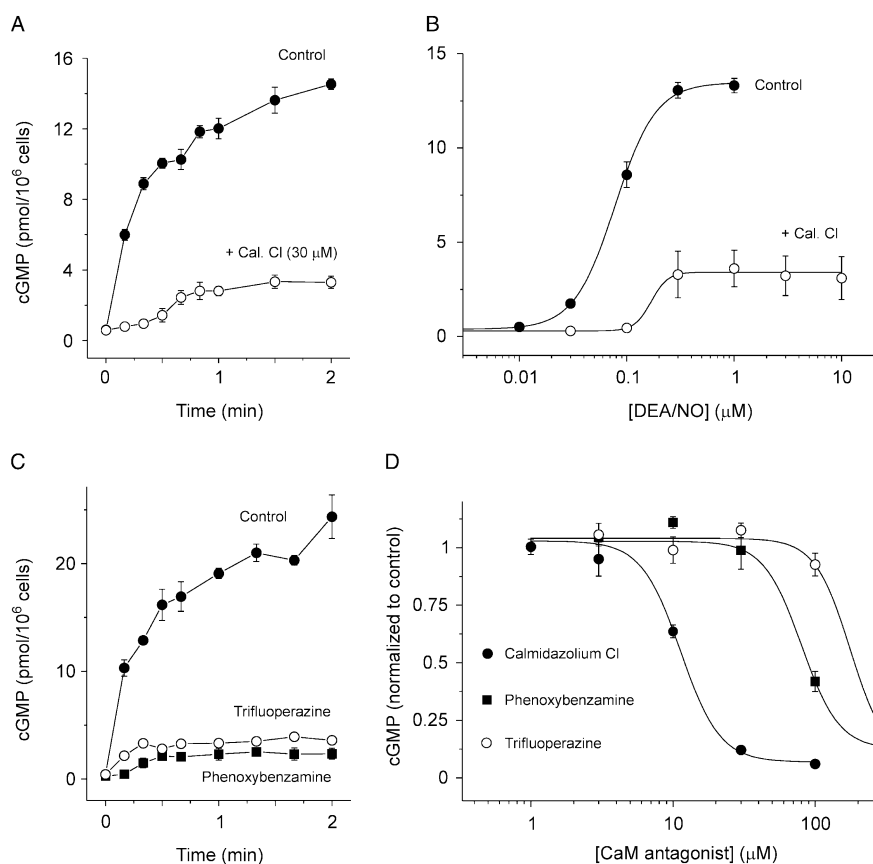


Figure 1 Effect of CaM antagonists on cGMP accumulation in cerebellar cells. (A) Time course of cGMP concentration in untreated cells (control) and cells preincubated for 10 min with calmidazolium chloride (Cal Cl; 30 μ M). 1 μ M DEA/NO was added at 0 s. (B) Concentration–response curves for 2 min exposure to DEA/NO in the presence and absence of 30 μ M calmidazolium. Data were fitted with a logistic equation (Con: EC_{50} = 80 nM, p = 2.3; Cal. Cl: EC_{50} = 170 nM, p = 5.5; see Methods). (C) Time course of cGMP response to 1 μ M DEA/NO in untreated cells and cells pre-incubated for 10 min with trifluoperazine (300 μ M) or phenoxybenzamine (300 μ M). (D) Concentration–response curves for inhibition of cGMP response to 2 min DEA/NO exposure by calmidazolium, phenoxybenzamine and trifluoperazine. Data normalized to control response in each case and fitted with a logistic equation (Cal. Cl: IC_{50} = 11.3 μ M, p = 3.5; phenoxybenzamine: IC_{50} = 80 μ M, p = 3.3; trifluoperazine: IC_{50} = 177 μ M; p = 3.5). CaM, calmodulin; cGMP, 3'-5' cyclic guanosine monophosphate; DEA/NO, 2-(N,N-diethylamino)-diazene-2-oxide.

Calmidazolium (30 μ M) triggered oscillatory increases in cytosolic Ca^{2+} (Figure 2B), confirming that the astrocytes express PLA_2 -stimulated Ca^{2+} entry channels, as previously noted in cerebellar slices (Singaravelu *et al.*, 2006). Ca^{2+} elevation by calmidazolium could be eliminated by pre-incubation of cells for 10 min with the sarco/endoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA) pump inhibitor thapsigargin (2 μ M) and the Ca^{2+} chelator EGTA (4 mM), which transiently emptied internal stores and blocked subsequent Ca^{2+} entry (Figure 2C). This treatment had no effect on the magnitude of DEA/NO-evoked cGMP accumulation after 2 min (Figure 2F). Elimination of Ca^{2+} signalling in this way also failed to prevent the inhibition of DEA/NO-evoked cGMP accumulation by calmidazolium (Figure 2F), indicating that changes in Ca^{2+} concentration were neither necessary nor directly responsible for the inhibitory effect.

Effect of calmidazolium on cGMP degradation

In principle, the calmidazolium-dependent inhibition of cGMP accumulation could result from activation of PDEs,

which hydrolyse cGMP to GMP (Conti and Beavo, 2007), although PDE1 would be inhibited by CaM antagonism and is not apparently expressed at significant levels in these cells (Bellamy and Garthwaite, 2001a).

Quenching free NO by addition of Hb after 2 min of DEA/NO stimulation abruptly halts cGMP synthesis, allowing cellular PDE activity to be measured from the subsequent decline in cGMP concentration (Bellamy and Garthwaite, 2001a). Addition of calmidazolium 5 s before Hb did not increase the rate of cGMP hydrolysis, but instead modestly inhibited the decline in cGMP over time (Figure 3). This result indicates that increased degradation of cGMP cannot account for the inhibitory effect of calmidazolium

Inhibition of NO inactivation by CaM antagonists

Another mechanism which could account for inhibition of cGMP accumulation is that CaM antagonists disrupt the kinetics of NO release from the donor, or enhance inactivation of NO by the cell suspension (Griffiths and Garthwaite, 2001), resulting in a decrease in free NO available to activate sGC.

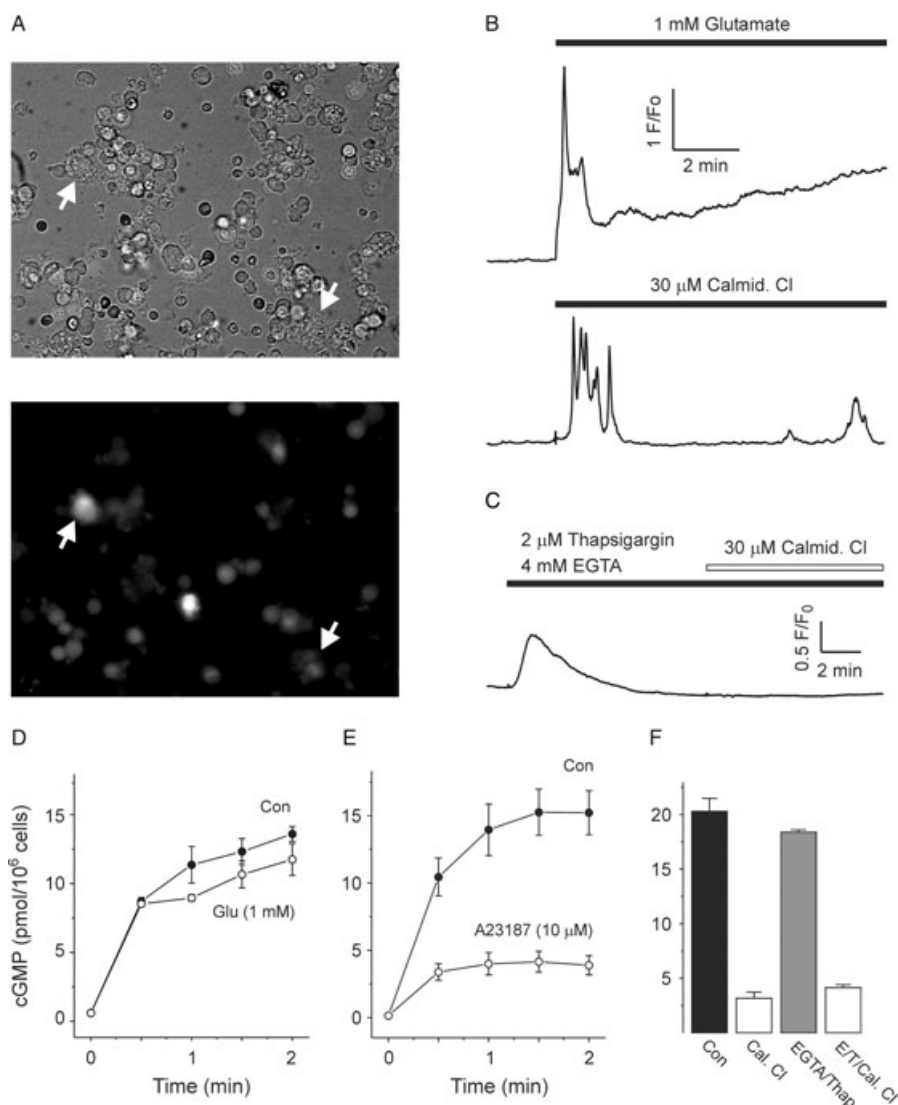


Figure 2 Effect of Ca^{2+} concentration changes on cGMP accumulation. (A) Brightfield (upper panel) and epifluorescent (lower panel) images of cerebellar cells loaded with $2 \mu\text{M}$ Fluo-4 AM. Astrocytes (indicated by arrows) adhere to the coverslip and send out disordered processes, and are readily distinguishable from smaller, more abundant granule neurons. (B) Representative time courses of Fluo-4 fluorescence intensity after addition of 1 mM glutamate and $30 \mu\text{M}$ calmidazolium (Calmid Cl) as indicated. (C) Representative time course from cells incubated with $2 \mu\text{M}$ thapsigargin and 4 mM ethylene glycol-bis(2-aminoethylether)- N,N,N',N' -tetraacetic acid (EGTA) for 10 min before addition of calmidazolium. Note that store depletion and removal of extracellular Ca^{2+} eliminates Ca^{2+} response to calmidazolium. (D) cGMP response to 2 min treatment with $1 \mu\text{M}$ DEA/NO in control cells (Con), and cells pre-incubated (30 s) with 1 mM glutamate (Glu). (E) Time course of cGMP accumulation in cells exposed to $10 \mu\text{M}$ A23187 compared with control cells. (F) cGMP response to 2 min treatment with $1 \mu\text{M}$ DEA/NO in untreated cells (Con), cells preincubated for 10 min with calmidazolium (Cal. Cl), cell preincubated for 10 min with $2 \mu\text{M}$ thapsigargin and 4 mM EGTA to deplete Ca^{2+} (EGTA/Thap), and cells preincubated with calmidazolium for 10 min after depletion of Ca^{2+} (E/T/Cal. Cl). cGMP, 3'-5' cyclic guanosine monophosphate; DEA/NO, 2-(N,N-diethylamino)-diazonolate-2-oxide.

Addition of calmidazolium to buffer before addition of DEA/NO had no effect on subsequent NO release kinetics (Figure 4A), indicating a lack of direct chemical interaction between the compounds. However, pre-incubation of cells with calmidazolium resulted in a lag in NO accumulation in the cell suspension (Figure 4B), the timing of which matched that of the lag in cGMP accumulation (Figure 1A). Thereafter, however, NO concentration rose with a similar time course to untreated cell suspensions, but overshoot the maximal concentration achieved under control conditions (Figure 4B). Trifluoperazine and phenoxybenzamine did not result in a detectable lag in NO accumulation, but were even more effec-

tive than calmidazolium at increasing maximal NO concentration in the cell suspensions (Figure 4C). Furthermore, the relative potencies of the CaM antagonists for increasing free NO concentration were the opposite to that for inhibition of cGMP accumulation (Figure 1D).

Direct inhibition of guanylyl cyclase by calmidazolium

A parsimonious explanation for the inhibition of cGMP accumulation by CaM antagonists is that these compounds directly inhibit sGC activation. In the first instance, we tested this possibility in whole tissue homogenate of rat cerebellum.

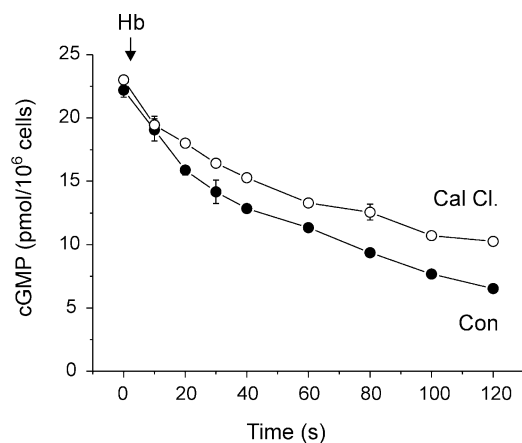


Figure 3 Effect of calmidazolium on cGMP degradation. Cells were pre-incubated with 2 μM DEA/NO for 2 min to allow cGMP accumulation. Calmidazolium (30 μM) or vehicle was added to cells 5 s before 10 μM Hb, which abruptly halts cGMP synthesis. Subsequent decay over 2 min allows cGMP hydrolysis to be measured. cGMP, 3'-5' cyclic guanosine monophosphate; DEA/NO, 2-(N,N-diethylamino)-diazeneolate-2-oxide.

By progressively diluting the concentration of the homogenate, any indirect inhibitory effect of calmidazolium (such as, for example, interaction with Ca^{2+} -free apocalmodulin) should be reduced, whereas a direct effect on sGC should be unaffected. Unexpectedly, we found that 30 μM calmidazolium had no effect on cGMP synthesis when the homogenate was concentrated (1.5–0.5 mg protein·mL⁻¹; Figure 5A), but as the homogenate was diluted, calmidazolium became progressively more effective at inhibiting NO-evoked cGMP accumulation (Figure 5A). The simplest explanation for this observation is that calmidazolium can be sequestered or metabolized by lipid or protein components of homogenized tissue, and that this influence is lost on dilution, revealing the inhibitory effect on sGC.

To further test the hypothesis, we treated purified $\alpha 1\beta 1$ sGC from bovine lung with the antagonists. Calmidazolium (30 μM) substantially inhibited cGMP synthesis by the purified enzyme exposed to a maximal concentration (100 μM) of DEA/NO (Figure 5B). The IC_{50} of calmidazolium for inhibition of purified sGC was ~ 10 μM , indistinguishable from that for cell suspensions (cf. Figures 1D and 5B). Trifluoperazine and phenoxybenzamine were also tested against the purified enzyme at a concentration of 300 μM . Compared with enzyme treated with vehicle alone (1% DMSO; 114 ± 16 μmol cGMP·mg⁻¹, $n = 3$), cGMP production over 10 min was inhibited by both compounds (trifluoperazine: 18.4 ± 2.1 μmol ·mg⁻¹, $n = 3$; phenoxybenzamine: 60.0 ± 8.2 μmol ·mg⁻¹, $n = 3$).

To investigate the mechanism of sGC inhibition, we first tested for a change in the potency of NO in activating sGC using clamped concentrations (Griffiths *et al.*, 2003). Pre-incubation of sGC with 30 μM calmidazolium for 30 s under these conditions resulted in a $\sim 60\%$ reduction in the maximal rate of cGMP synthesis, but no change in NO potency ($\text{EC}_{50} \sim 1.1$ nM), indicating that calmidazolium does not act in a competitive manner at the sGC haem binding site (Figure 6A). This contrasts with the apparent increase in EC_{50} for DEA/NO observed in cells (Figure 1B).

We next tested the hypothesis that as a hydrophobic molecule, calmidazolium could displace the haem moiety from sGC. Detergents such as Tween-20 can strip the haem from sGC in this manner (Roy *et al.*, 2008), rendering the enzyme insensitive to NO. The haem mimetic protoporphyrin IX (PPIX) can substitute for haem and bind to sGC, making it more effective at activating haem-depleted sGC than the native enzyme.

PPIX (1 μM) activated untreated sGC (as supplied by the manufacturer) with around half the effectiveness of DEA/NO (Figure 6B). Pre-treatment of sGC with the detergent Tween-20 (0.5%), followed by filtration to remove the detergent, decreased the effectiveness of DEA/NO for activating cGMP synthesis by 73% (Figure 6B). In contrast, the effectiveness of PPIX for activating sGC was increased 3.2-fold by Tween-20 treatment (Figure 6B). Pre-treatment of sGC with calmidazolium, followed by filtration, caused an 80% inhibition of sGC activation by DEA/NO and a 23% inhibition of activation by PPIX (Figure 6B), indicating that the mechanism of action of calmidazolium does not involve displacement of the haem group. This result also indicates that the inhibitory effect of calmidazolium does not reverse (within the 10 min of this experiment) after filtration.

Finally, we tested calmidazolium for competition with the enzyme substrate, GTP. By measuring cGMP synthesis for 1 min after addition of NO, in the presence of various initial GTP concentrations, we gauged the ability of calmidazolium to alter the maximal rate of cGMP synthesis (V_{max}) and concentration dependence of substrate binding (K_{m}). As calmidazolium concentration increased from 10 to 30 μM , both the V_{max} and the K_{m} of sGC decreased (Figure 6C). This is indicative of an uncompetitive mechanism of enzyme inhibition – as illustrated by parallel lines in a double reciprocal plot (Figure 6D), which is reminiscent of the inhibitory mechanism of Ca^{2+} at sGC (Parkinson *et al.*, 1999).

Discussion and conclusions

Regulation of the NO-cGMP signalling pathway by Ca^{2+} -CaM is well established. CaM was first identified as an activator of PDE1 (Cheung, 1970), and activation of NO synthases by CaM was recognized from the earliest purifications of the enzyme (Bredt and Snyder, 1990; Mayer *et al.*, 1990). Since then, CaM antagonists have been frequently used experimentally to intervene in NO signalling. The known actions of CaM would not, however, predict that its antagonism would lead to the inhibition of cGMP accumulation in cells exposed to an NO donor (Figure 1). This observation led us to investigate the action of common CaM antagonists, principally the widely adopted calmidazolium chloride, on each of the steps in the NO-cGMP signalling pathway in cerebellar astrocytes. Our results show that these compounds inhibit every step of the pathway, resulting in complex disruption of the timing and magnitude of cGMP accumulation.

The role of Ca^{2+} in regulating cellular cGMP dynamics has been much debated. Apart from the activation of PDE1, Ca^{2+} has also been shown to inhibit sGC directly by binding to high and low affinity allosteric sites (Parkinson *et al.*, 1999; Kazerounian *et al.*, 2002), suggesting that Ca^{2+} elevation may

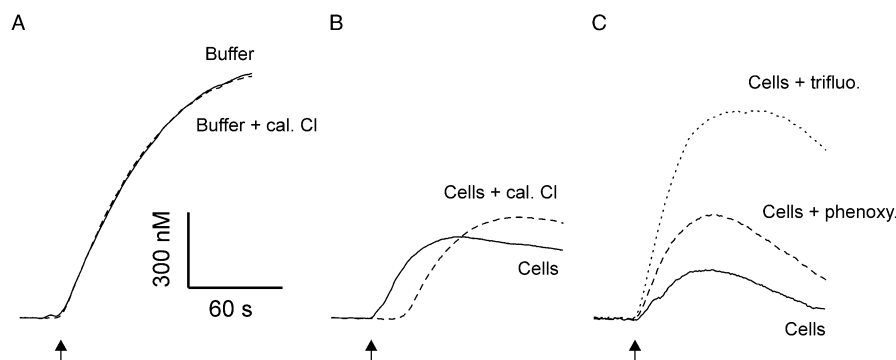


Figure 4 Effect of CaM antagonists on free NO concentration. Representative traces of NO concentration measured with an electrochemical probe in (A) buffer and buffer supplemented with 30 μM calmidazolium (Cal Cl), (B) control cells and cells preincubated with 30 μM calmidazolium, and (C) control cells and cells pre-incubated with 100 μM phenoxybenzamine (phenoxy) or cells pre-incubated with 300 μM trifluoperazine (trifluo). In all cases, 1 μM DEA/NO was added at arrow. CaM, calmodulin; cGMP, 3'-5' cyclic guanosine monophosphate; DEA/NO, 2-(N,N-diethylamino)-diazololate-2-oxide.

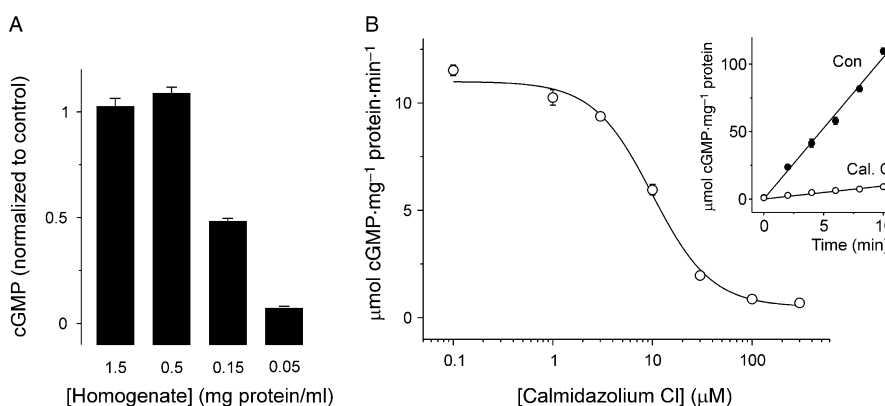


Figure 5 Effect of calmidazolium on homogenate and purified sGC. (A) cGMP accumulation in whole cerebellar homogenate at varying concentrations (expressed in $\text{mg protein}\cdot\text{mL}^{-1}$ as indicated), incubated with 100 μM DEA/NO for 10 min. Homogenate was pre-incubated for 10 min with 30 μM calmidazolium. At each concentration, cGMP response is normalized to the response in homogenate pre-incubated with vehicle alone. (B) Concentration–response curve for inhibition of purified bovine lung $\alpha 1\beta 1$ sGC by calmidazolium. Data are fitted with a logistic equation ($\text{IC}_{50} = 9.9 \mu\text{M}$; $p = 1.5$). Inset shows time course of cGMP synthesis in the presence (Cal. Cl) and absence (Con) of 30 μM calmidazolium chloride. cGMP, 3'-5' cyclic guanosine monophosphate; DEA/NO, 2-(N,N-diethylamino)-diazololate-2-oxide; sGC, soluble guanylyl cyclase.

counter cGMP signalling by both accelerating degradation and retarding synthesis. This potential for crosstalk is of particular interest with regards to astrocytes, which are known to express numerous receptors coupled to Ca^{2+} elevation (Verkhatsky *et al.*, 1998; Haydon, 2001). Furthermore, calmidazolium triggers Ca^{2+} entry in cerebellar astrocytes (Singaravelu *et al.*, 2006) by antagonism of CaM-dependent inhibition of PLA_2 (Harper and Daly, 2000; Jan and Tseng, 2000; Peppiatt *et al.*, 2004; Smani *et al.*, 2004), an observation we reproduced in freshly dissociated cerebellar cells (Figure 2B). We therefore explored the possibility that increased Ca^{2+} concentration could inhibit sGC, and that this was the means by which calmidazolium was acting. However, mobilization of Ca^{2+} by several neurotransmitter receptor agonists had no influence on the amplitude of cGMP responses evoked by DEA/NO – inhibition was only observed when cells were permeabilized with the Ca^{2+} ionophore A23187 (Figure 2E) – and depletion of Ca^{2+} from internal stores and the extracellular space did not alter the cGMP response to NO, nor prevent the inhibition of cGMP accumulation by calmidazolium (Figure 2C,F). These results

indicate that the inhibitory effect of calmidazolium on cGMP accumulation does not depend on Ca^{2+} signalling, with or without CaM. The apparent insensitivity of NO-cGMP signalling to changes in Ca^{2+} also suggests that Ca^{2+} elevation by physiological mechanisms does not result in the inhibition of sGC activity in intact cerebellar astrocytes. It therefore seems unlikely that the dynamic Ca^{2+} signalling observed in astrocyte networks *in vivo* will alter the cells' responsiveness to NO.

Further investigation revealed little impact of calmidazolium on cGMP breakdown by PDEs, consistent with an earlier report that concluded these cells have negligible PDE1 activity (Bellamy and Garthwaite, 2001a). However, two CaM-independent, pharmacological effects of CaM antagonists on NO signalling were identified: inhibition of NO inactivation by the cell suspension, and direct inhibition of sGC.

NO inactivation under experimental conditions can result from numerous mechanisms, including generation of superoxide by buffers, reaction with cellular haemoproteins, reaction with cytochrome P450 and reaction with lipid peroxyl radicals (Martin *et al.*, 1985; Crow and Beckman, 1995;

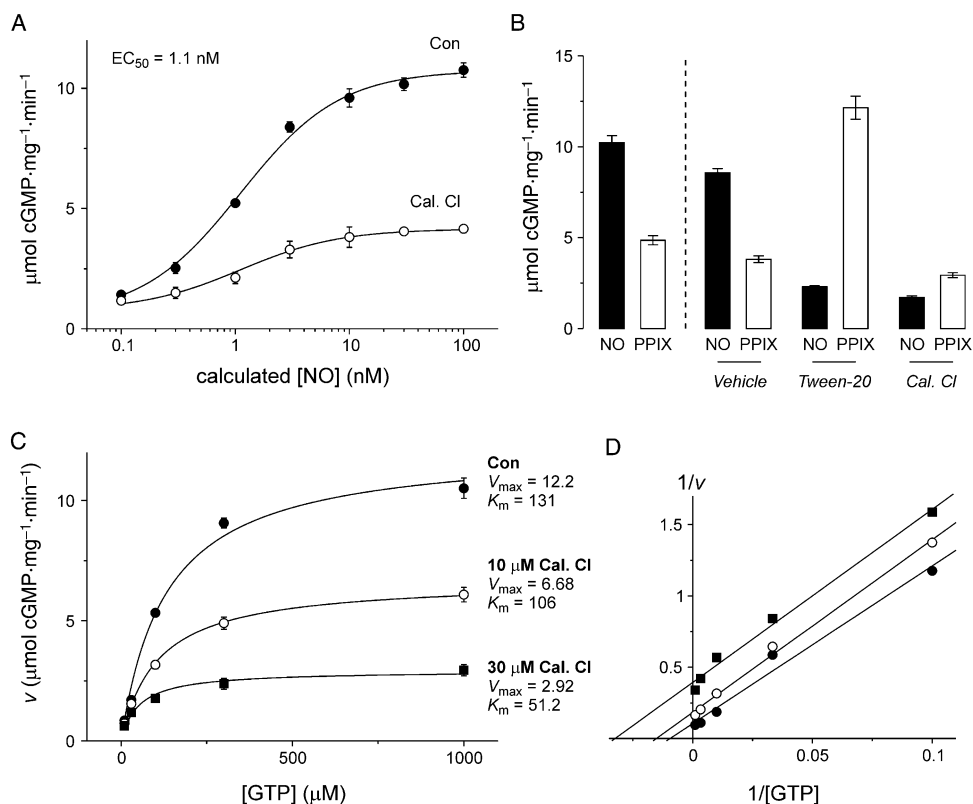


Figure 6 Mechanism of inhibition of purified sGC by calmidazolium. (A) Concentration–response of purified sGC to clamped nitric oxide (NO) concentrations (calculated from concentration of SPER/NO, as described in Griffiths *et al.*, 2003), after 30 s pre-incubation with vehicle (Con) or 10 μM calmidazolium (Cal. Cl). Both datasets were fitted with a logistic equation, with $EC_{50} = 1.1 \text{ nM}$, $p = 1$. (B) Response of sGC to 10 min incubation with DEA/NO (100 μM ; NO) and protoporphyrin IX (1 μM ; PPIX), under standard experimental conditions (columns to left of dashed line) and after 10 min pre-incubation with buffer + 1% DMSO (vehicle), 0.5% Tween-20 (Tween-20) or 30 μM calmidazolium chloride (Cal. Cl), followed by filtration and resuspension in normal buffer. (C) Concentration response of sGC activated by 100 μM DEA/NO to GTP, in the absence (Con) or presence of calmidazolium Cl (Cal. Cl) at 10 and 30 μM . Data were fitted with the Michaelis–Menten equation. Values for V_{max} (in $\mu\text{mol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$) and K_m (in μM) were as indicated. (D) Data from panel c as a double reciprocal plot, to illustrate parallel lines indicative of uncompetitive inhibition. DEA/NO, 2-(N,N-diethylamino)-diazeneolate-2-oxide; DMSO, dimethyl sulphoxide; sGC, soluble guanylyl cyclase; SPER/NO, (Z)-1-{N-[3-aminopropyl]-N-[4-(3-aminopropylammonio)butyl]-amino}-diazene-1-ium-1,2-diolate.

Keynes *et al.*, 2003; 2005; Hall *et al.*, 2009). The latter mechanism has been found to predominate in cell suspensions and homogenates under the conditions adopted in this study (Keynes *et al.*, 2005). CaM antagonists have previously been identified as inhibitors of lipid peroxidation (Hara *et al.*, 1992), which could account for the reduction of NO inactivation observed in our experiments. How calmidazolium can generate a biphasic change in free NO concentration – an initial lag in release, followed by inhibition of inactivation – is not easily explained. The ‘NONOate’ donors release NO spontaneously in a temperature and pH-dependent manner (Morley and Keefer, 1993), and so inhibition of a metabolic process needed to trigger release (akin to that for nitroglycerin; Chen *et al.*, 2002) can be ruled out. The generation of a saturable sink for NO by calmidazolium – by, for example, generation of radical species – could account for the lag in NO accumulation, although no obvious mechanism presents itself. Nevertheless, this peculiar effect does account for the disruption in the kinetics of cGMP accumulation caused by calmidazolium (Figure 1A), and probably also underlies the change in the shape and EC_{50} of the concentration–response curve for 2 min DEA/NO exposure (Figure 1B).

A previous study has reported that NO-dependent cGMP accumulation in pancreatic acinar cells was inhibited by several CaM antagonists (Gukovskaya and Pandol, 1995). In particular, the authors noted that W-7 inhibited cGMP synthesis in acinar cell lysates, an effect that varied with Ca^{2+} concentration (cf. our Figure 2F), but was not reversed by addition of excess CaM. The interpretation of Gukovskaya *et al.* was that the CaM antagonists may work through an alternative Ca^{2+} binding protein, which acted as an sGC inhibitor. The authors did not rule out direct inhibition of sGC, however, which would be consistent with our observation of inhibition of purified sGC by calmidazolium. Confusingly, we have been unable to reproduce the inhibition of cGMP synthesis by W-7 in cerebellar homogenate or with purified bovine lung sGC (up to 300 μM ; data not shown). This tissue-specific discrepancy is surprising, but may result from variable potency of the drugs in different cellular contexts (see Figure 5A). In contrast, calmidazolium inhibited purified sGC with similar potency to its effect on cerebellar astrocytes (Figure 1B cf Figure 5B), and both phenoxybenzamine and trifluoperazine were also effective (at 300 μM).

The mechanism of inhibition by calmidazolium did not depend on competition with NO for the haem binding site (Figure 6A), or on the loss of haem from the receptor protein (Figure 6B). Calmidazolium also inhibited sGC that had been depleted of haem and stimulated by the haem mimetic protoporphyrin IX (Figure 6B). An analysis of the effect of calmidazolium on substrate (GTP) concentration dependence revealed that the CaM antagonist acts as an uncompetitive inhibitor of sGC – that is, the inhibitor acts selectively on the substrate-enzyme complex (Figure 6C,D). A straightforward interpretation of this result is that the conformational change in the active site associated with substrate binding enables the binding of calmidazolium, which decreases the rate of catalysis. The inhibition was found to be essentially irreversible, at least by filtration and resuspension of the receptor protein (Figure 6B), which may reflect a lasting disruption of protein structure, or a very tight association of calmidazolium with sGC, which survives filtration. The latter interpretation seems incompatible with the relatively modest potency of the drug ($EC_{50} \sim 0 \mu\text{M}$), but detailed binding kinetics has not been determined.

CaM-independent effects of calmidazolium have been observed in other systems. For example, calmidazolium has been reported to facilitate steroidogenesis in Leydig and adrenocortical cells in a Ca^{2+} -independent manner (Choi and Cooke, 1992), bind to peripheral benzodiazepine receptors (Zisterer and Williams, 1997), inhibit plasma membrane Ca^{2+} ATPases (Gietzen *et al.*, 1982) and inhibit IP_3 receptors (Khan *et al.*, 2001). Of particular note, calmidazolium has a bimodal effect on some isoforms of Ca^{2+} -CaM activated adenylyl cyclase (AC), being stimulatory at lower concentrations and inhibitory at higher concentrations (Haunso *et al.*, 2003). The inhibitory effect was preserved in soluble recombinant fusion proteins of the catalytic subunits of AC and arose from direct binding, but in contrast to our findings with sGC, the inhibitory mechanism at AC was non-competitive (Haunso *et al.*, 2003). It does not appear, therefore, that there is a common binding site for calmidazolium in the catalytic cores of AC and sGC, despite the similarities between the enzymes' catalytic mechanisms. The potency of calmidazolium for inhibition of these diverse targets, and for antagonising CaM, varies substantially between experimental preparations. Variation in the concentration of CaM, the binding of CaM to target proteins, the relative volumes of lipid and aqueous phases, and potential metabolism or sequestration of the drug (Figure 5A), can all influence the IC_{50} of a given target. As such, in the concentration range typically used (1–100 μM), calmidazolium is likely to act on numerous targets with overlapping concentration–response curves. Selective inhibition therefore appears difficult to achieve with confidence without detailed characterization of targets within the system of interest.

With respect to the binding site of calmidazolium on sGC, it is noteworthy that a low-affinity Ca^{2+} binding site has been described that also mediates uncompetitive inhibition of the enzyme (Parkinson *et al.*, 1999). It will be worth exploring the potential for competition between Ca^{2+} and calmidazolium for binding at this site, as it may represent a new pharmacological target for small molecule inhibitors. Currently, the most widely applied inhibitor of sGC is 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), which

binds to the receptor haem site and oxidizes the ferrous iron centre, greatly decreasing NO potency (Garthwaite *et al.*, 1995; Bellamy and Garthwaite, 2002). Because of a similar interaction with haemoglobin, the bioavailability of ODQ *in vivo* is limited. Development of new compounds active at a different site, such as that putatively engaged by calmidazolium, would be advantageous both as experimental tools, and, in principle, as pharmaceutical agents.

In conclusion, these previously unrecognized pharmacological actions of calmidazolium, phenoxybenzamine and trifluoperazine highlight the need for caution in the use of CaM antagonists in a cellular context. For any physiological process in which NO signalling plays a role, clear interpretation of the impact of CaM antagonists on the experimental system will be challenging.

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Statement of conflicts of interest

None.

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