British Journal of Pharmacology (2002) 137, 139–145

www.nature.com/bjp

The smooth muscle relaxant effect of hydrogen sulphide *in vitro*: evidence for a physiological role to control intestinal contractility

[1]B. Teague, [1]S. Asiedu & *,[1]P.K. Moore

[1]Centre for Cardiovascular Biology and Medicine, King's College, University of London, London SE1 1UL

1 Sodium hydrogen sulphide (NaHS), a donor of hydrogen sulphide (H_2S), produced dose-related relaxation of the rabbit isolated ileum (EC_{50}, 76.4 ± 7.9 μM) and rat vas deferens (EC_{50}, 64.8 ± 5.4 μM) and reduced ACh-mediated contraction of the guinea-pig isolated ileum.

2 NaHS also reduced the response of the guinea-pig (EC_{50}, 80.0 ± 5.7 μM) and rat (EC_{50}, 108.2 ± 11.2 μM) ileum preparations to electrical stimulation of the intramural nerves. In guinea-pig ileum this effect was spontaneously reversible and mimicked by sodium nitroprusside (SNP, EC_{50}, 2.1 μM). Combination of NaHS (20 μM) with SNP (0.5 μM) produced a greater than additive inhibition of the twitch response of the ileum to electrical stimulation.

3 The inhibitory effect of NaHS on the field-stimulated guinea-pig ileum was unaffected by pretreatment with L-NAME (100 μM), indomethacin (10 μM), naloxone (1 μM) or glibenclamide (100 μM). Furthermore, NaHS (200 μM) did not affect the contractile response of the ileum to KCl (10 to 60 mM).

4 Propargylglycine (PAG, 1 mM) and β-cyanoalanine (BCA, 1 mM) (inhibitors of cystathionine-γ-lyase) but not aminooxyacetic acid (AOAA, 1 mM) (inhibitor of cystathionine-β-synthetase) caused a slowly developing increase in the contraction of the guinea-pig ileum to field stimulation. This effect was reversed by cysteine (1 mM).

5 These results show that NaHS relaxes gastrointestinal and urogenital smooth muscle and suggest that H_2S is responsible for these effects. The possibility that endogenous H_2S, formed as a consequence of activation of intramural nerves, plays a part in controlling the contractility of the guinea-pig ileum is discussed.

British Journal of Pharmacology (2002) **137,** 139–145. doi:10.1038/sj.bjp.0704858

Keywords: Hydrogen sulphide; ileum; acetylcholine; vas deferens; propargylglycine; β-cyanoalanine; cysteine

Abbreviations: BCA, β-cyanoalanine; CBS, cystathionine-β-synthetase; CO, carbon monoxide; CSE, cystathionine-γ-lyase; H_2S, hydrogen sulphide; NaHS, sodium hydrogen sulphide; NO, nitric oxide; PAG, propargylglycine

Introduction

Whilst the pharmacology of gaseous mediators such as nitric oxide (NO) and carbon monoxide (CO) has been extensively studied over many years, the profile of biological activity of another such biologically active gas, hydrogen sulphide (H_2S), has received scant attention.

H_2S is found in the atmosphere (about 1 $\mu g\,m^{-3}$, Air Quality Guidelines for Europe, World Health Organization, 1987) as well as in foodstuffs such as dairy products and cooked meats (Kraft *et al*., 1956). In the environment, H_2S is a chemical hazard associated with natural gas production, sewage treatment and is a bi-product of certain types of paper and pulp production (Guidotti, 1996). It is a product of bacterial (Pitcher *et al*., 2000) and helminth (Goffredi *et al*., 1997) metabolism and, perhaps more significantly, also occurs naturally in mammals. Thus, H_2S can be found in (concentration of 1.1 μM in man, Suarez *et al*., 1998), and indeed accounts for, the characteristic odour of flatus. H_2S also occurs in human faeces (e.g. Florin *et al*., 1991). In both cases, the H_2S detected is probably of bacterial origin. However, the *in situ* formation of H_2S by mammalian cells has also been noted. For example, relatively high concentrations of H_2S occur in rat (50 μM; Zhao *et al*., 2001) and human (10–100 μM; Richardson *et al*., 2000) blood and in rat brain homogenates (60–150 μM, Warenycia *et al*., 1989). Furthermore, both renal cortical tubule cells (Stipanuk *et al*., 1990) and colonic enterocytes (Coloso & Stipanuk, 1989) synthesise H_2S.

The formation of H_2S by mammalian cells is most probably accounted for by two enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS). These occur in bacteria and mammals and have been known for many years to catalyse the final step in a transsulphuration pathway by which methionine and cysteine are metabolically interchanged (Finkelstein & Martin, 1984). Two decades ago, Stipanuk & Beck (1982) reported that CSE was able to utilize cysteine (in addition to cystathionine) as a substrate to form thiocysteine and H_2S. Since CSE occurs in several mammalian (including human) cells and tissues it seems reasonable to propose that this enzyme may account for, or at least contribute to, the presence of H_2S in mammalian blood and organ homogenates.

The profile of pharmacological activity of H_2S is not entirely clear. Administered acutely, and at high concentrations, H_2S is toxic by complexing with the Fe^{3+} of mitochondrial cytochrome oxidase thereby blocking cellular

*Author for correspondence; E-mail: phillip.moore@kcl.ac.uk

oxidative metabolism (Gosselin *et al*., 1984). Other possible targets include carbonic anhydrase (Nicholson *et al*., 1998) and tyrosine aminotransferase (Hargrove, 1988), both of which are inhibited by H_2S. Interestingly, at lower (more physiological) concentrations, H_2S has recently been shown to exert a range of biological effects. For example, it relaxes isolated vascular (Zhao *et al*., 2001), reproductive (Hayden *et al*., 1989; Sidhu *et al*., 2001) and gastrointestinal (Hosoki *et al*., 1997) smooth muscle preparations *in vitro*. In the central nervous system, H_2S also enhances NMDA receptor-mediated currents and facilitates long-term potentiation (LTP) in the hippocampus (Abe & Kimura, 1996) as well as hyperpolarizing brainstem neurones (Kombian *et al*., 1993) and inhibiting corticotrophin releasing factor (CRF) efflux from the hypothalamus (Navarra *et al*., 2000).

With this in mind, we have now investigated the effect of H_2S (as sodium hydrogen sulphide, NaHS) on contractions of guinea-pig, rat and rabbit ileum and rat vas deferens to appropriate drugs and/or to electrical (field) stimulation of the intramural nerves. In addition, using inhibitors of CSE and CBS, we have obtained evidence of a role for endogenous H_2S in the regulation of gut contractility in the guinea-pig ileum *in vitro*.

Methods

Guinea-pigs (male, Dunkin-Hartley, 500–700 g), rats (Wistar, male, 200–250 g) and rabbits (New Zealand White, male 2.5–3.5 kg) were used in this study. Guinea-pigs and rats were killed by cervical dislocation and exsanguination. Rabbits were killed by an overdose of sodium pentobarbitone (60 mg kg^{-1}) administered *via* a marginal ear vein. All experiments were conducted under the authority of the UK Animals (Scientific Procedures) Act (1986).

Pharmacological preparations were rapidly dissected and mounted in 25 ml organ baths containing warmed (37°C) and oxygenated (95% O_2: 5% CO_2) Krebs' solution (composition, mM: NaCl 118, KCl 5.4, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$, 2.5, glucose 11.1, pH 7.4). Changes in tension were recorded using Grass-FT03 force transducers connected to a Devices pen recorder.

After equilibration (60 min), preparations were exposed to ACh (88 nM, EC_{70} from preliminary experiments, not shown), KCl (10 or 60 mM) or were electrically stimulated by means of parallel platinum electrodes positioned on either side of the tissue and connected to a square wave stimulator (Harvard Apparatus Ltd.). For guinea-pig ileum and rat vas deferens, the following stimulation parameters were used: 0.1 Hz (frequency), 0.5 ms (pulse width) and 80 V. A higher frequency of stimulation (5 Hz) was used for rat ileum preparations due to the spontaneous activity of these preparations which obscured contractions at lower frequencies.

In some experiments, field-stimulated guinea-pig ileum preparations were exposed to graded concentrations of sodium hydrogen sulphide (NaHS, 1–1000 μM) or sodium nitroprusside (SNP, 0.1–100 μM) in the presence or absence of propargylglycine (PAG, 1 mM), β-cyanoalanine (β-CA, 1 mM; both inhibitors of cystathionine-γ-lyase, Reed, 1995; Uren *et al*., 1978), amino-oxyacetic acid (AOAA, 1 mM; inhibitor of cystathionine-β-synthetase, Braunstein *et al*., 1971), cysteine hydrochloride or base (1 mM) or appropriate concentrations (derived from similar tissue bath experiments reported in the literature, see references indicated below) of the nitric oxide synthase inhibitor (L-N^G nitroarginine methyl ester, L-NAME, 100 μM; Moore *et al*., 1990), cyclo-oxygenase inhibitor, indomethacin (10 μM; Rodriguez-Martinez *et al*., 1998), opioid antagonist, naloxone (1 μM; Rizzi *et al*., 2001), or the K_{ATP} channel blocker, glibenclamide (100 μM; Zhao *et al*., 2001). In all cases, tissues were exposed to the inhibitor for 60 min prior to further testing. Control tissues were exposed to an appropriate volume (1.0 ml per l Krebs) of vehicle (0.9% w v^{-1} NaCl i.e. saline or DMSO) for the same time period. In some experiments, electrically stimulated guinea-pig ileum preparations were exposed to a mixture of NaHS (20 μM) and SNP (0.5 μM). In this case, drugs were added to the bath simultaneously.

Results show mean ± s.e.mean with the number of observations indicated in parenthesis. Statistical analysis of differences between multiple sets of data was determined using ANOVA followed by *post-hoc* Dunnett's test. For comparison between two data sets unpaired Student's *t*-test was used. In both cases, a P value less than 0.05 was taken to indicate statistical significance. All drugs were purchased from Sigma-Aldrich Ltd. Stock solutions of indomethacin and glibenclamide were dissolved in DMSO. All other drugs were dissolved in saline. Solutions of NaHS and SNP were prepared fresh on the morning of each experiment and kept stoppered on ice.

Results

Effect of NaHS on gastrointestinal and urogenital smooth muscle in vitro

Addition of NaHS (10–2000 μM) to the bath produced dose-related inhibition of the spontaneous, pendular contractions of the isolated rabbit ileum preparation (Figure 1A). The EC_{50} for NaHS was 76.4 ± 7.9 μM with an E_{max} of 70.0 ± 6.5% (both $n=5$). NaHS (80 μM) exhibited no demonstrable effect on resting tone of the guinea-pig, but did reduce the contractile response of this tissue to exogenous ACh (88 nM; Figure 1B). The smooth muscle relaxant effect of NaHS (determined as inhibition of the ACh-induced contraction) in the guinea-pig ileum was time-dependent, with a decline in activity as the pre-incubation period was increased (Figure 1B).

NaHS (1–1000 μM) also produced a rapidly developing (commencing within a few seconds) and dose-dependent inhibition of the response of the guinea-pig ileum to electrical field stimulation (Figure 2A). The twitch response was completely abolished at a concentration of 400 μM and the calculated EC_{50} was 62.6 ± 4.7 μM ($n=8$). In some experiments, the effect of NaHS (80 μM) on the twitch response was monitored in the absence of drug washout in order to determine the longevity of the response. The inhibitory effect of NaHS was observed to be relatively short-lived reversing spontaneously within 150–180 s. For comparison, sodium nitroprusside (SNP) produced qualitatively similar results but was approximately 30 times more potent than NaHS (EC_{50}, 2.1 ± 0.08 μM, $n=6$) (Figure 2A). Interestingly, the combination of low concentrations of NaHS (20 μM) and SNP

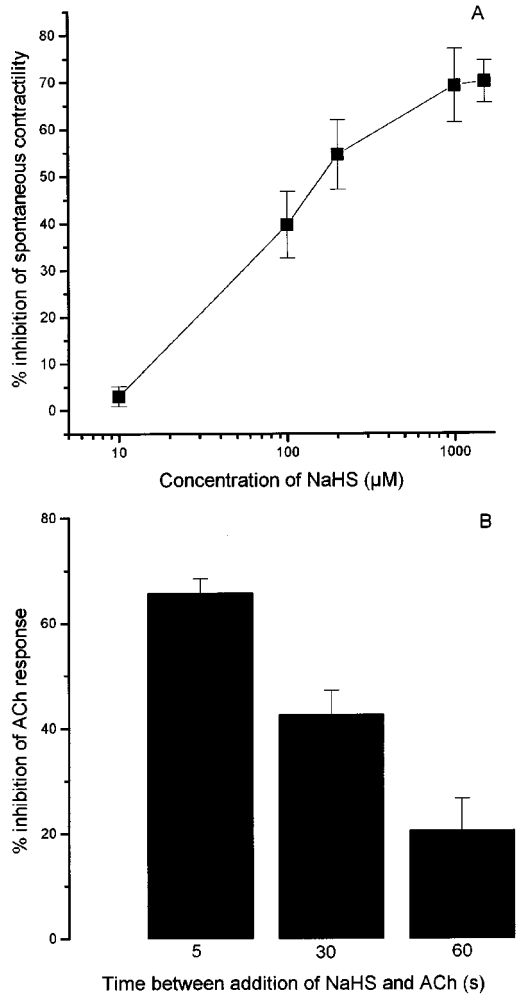

Figure 1 (A) Relaxant effect of NaHS on the rabbit isolated ileum. Results show per cent inhibition of spontaneous contractility and are mean ± s.e.mean, $n=5$. (B) Time-dependent inhibition of the ACh-induced contraction of the guinea-pig isolated ileum to NaHS. ACh (88 nM, EC_{70} determined in preliminary experiments) was added to the organ bath at timed intervals (5–60 s) after prior application of NaHS (80 μM). Results show per cent reduction in ACh response and are mean ± s.e.mean.

(0.5 μM) resulted in a degree of inhibition of the twitch response of the guinea-pig ileum which was greater than that expected from the simple additive effect of the two drugs (Figure 2B).

A number of experiments were carried out in an attempt to determine the mechanism of action of NaHS in the guinea-pig ileum. Thus, the inhibitory effect of NaHS in the field-stimulated guinea-pig ileum was unaltered by preincubation of tissues for 60 min with L-NAME (100 μM), indomethacin (10 μM), naloxone (1 μM) or glibenclamide (100 μM) or the appropriate vehicle (saline or DMSO) (Figure 2C). In separate experiments, the effect of a concentration of NaHS (200 μM), which produced >90% inhibition of the response to electrical stimulation, was assessed on contractions of the guinea-pig ileum to 'low' (10 mM) and 'high' (60 mM) concentrations of KCl. Preincubation of tissues with NaHS for 30 s did not affect the responsiveness of the tissue to either concentration of KCl (Figure 2D).

NaHS (10–1000 μM) also produced dose-dependent inhibition of the contractile response of the field-stimulated rat vas deferens and rat ileum (Figure 3). The EC_{50} values for NaHS in these tissues were 108.2 ± 11.2 μM ($n=5$) and 64.8 ± 5.4 μM ($n=8$) respectively. Like the guinea-pig ileum, responses to NaHS were rapid in onset and spontaneously reversible (about 180 s) without washout. Unlike the guinea-pig ileum preparation, high concentrations of NaHS failed to bring about complete abolition of the response to field stimulation in these tissues (E_{max} values of 60.0 ± 10.2% and 60.6 ± 6.8% inhibition, $n=5-8$, respectively).

Effect of inhibitors of H_2S synthesis on responses of the field-stimulated guinea-pig ileum

Preincubation of field-stimulated guinea-pig ileum preparations with propargylglycine (PAG; 1 mM) or β-cyanoalanine (BCA; 1 mM) led to a slowly developing increase in the twitch contraction (Figure 4). The enhanced response was first apparent following exposure to either drug for 15 min, and thereafter reached a plateau at 60 min, at which time the contraction to field stimulation was increased by 43.0 ± 3.0%, (PAG, $n=11$) or 31.2 ± 8.1%, (BCA, $n=6$). Exposure (60 min) of ileum preparations to BCA (1 mM) did not affect contractions due to exogenous ACh (inset to Figure 4). In addition, no significant increase in twitch response was observed in the 60 min period after injection into the bath of either saline (0.1 ml) or amino-oxyacetic acid (AOAA, 1 mM) (Figure 4).

In preliminary experiments, cysteine hydrochloride (1 mM) added to the organ bath resulted in a prompt (within 10 s) and complete inhibition of the twitch response of the guinea-pig isolated ileum to field stimulation. Subsequent investigation revealed that this effect was due to the highly acid pH (i.e. 1.5) of the stock drug solution used and did not occur when pH-neutral cysteine base (1 mM) was substituted.

In subsequent work the ability of cysteine base to reverse the pro-contractile effect of PAG (1 mM) and β-CA (1 mM) in the field-stimulated guinea-pig ileum preparation was assessed. For these experiments, cysteine (1 mM) or saline (0.1 ml) was added to field-stimulated guinea-pig ileum preparations which had been pre-exposed (60 min) to PAG or β-CA and in which contractility was consequently increased by about 50%. Addition of cysteine (but not saline) in these circumstances resulted in a slowly developing decline in the size of the β-CA-augmented twitch response with complete reversal to control levels occurring within 60 min (Figure 5). It should be noted that cysteine did not affect responses of the electrically stimulated guinea-pig ileum in the absence of CSE inhibitor. Identical treatment of guinea-pig ileum preparations with either cysteine (1 mM) or saline did not significantly reverse the pro-contractile effect of PAG (1 mM) (Figure 5).

Discussion

Recent studies have revealed that NaHS relaxes precontracted rat aortic rings (Zhao *et al.*, 2001) and uterine strips (Hayden *et al.*, 1989; Sidhu *et al.*, 2001). NaHS was used in these particular studies, and in the present experiments, since it dissociates into hydrosulphide anion (HS^-) which then

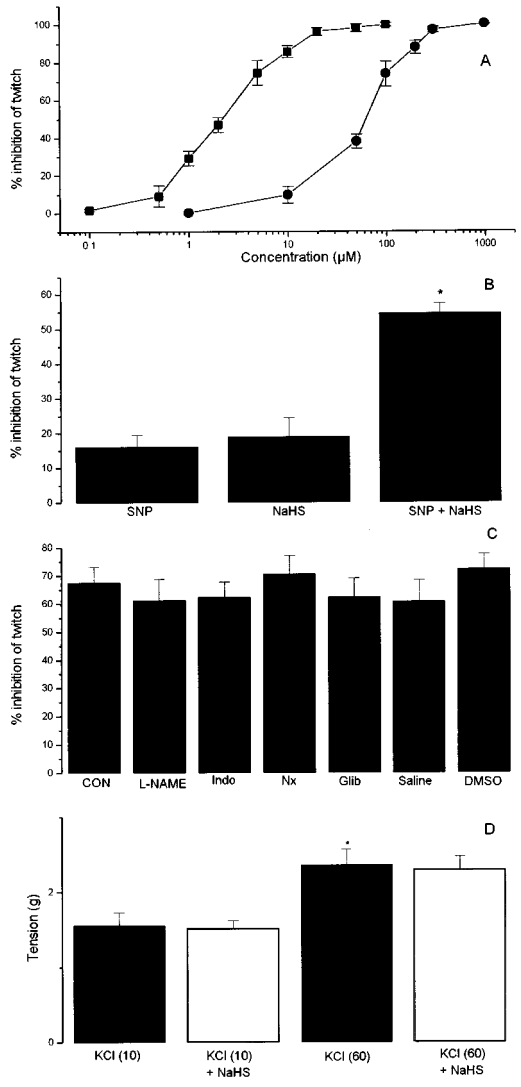

Figure 2 (A) Inhibition of the twitch response of the guinea-pig isolated ileum to electrical stimulation by NaHS and SNP. Results show peak relaxant effect and are mean ± s.e.mean, $n=8$ (NaHS) and $n=6$ (SNP). (B) Effect of SNP (0.5 μM), NaHS (20 μM) alone and in combination (SNP + NaHS) on the response to electrical stimulation of the guinea-pig ileum. Results show per cent inhibition of twitch response and are mean ± s.e.mean, $n=6$, $^*P<0.05$ c.f. either SNP or NaHS (ANOVA plus *post-hoc* Dunnett's test). (C) Effect of NaHS (80 μM) on the response of the guinea-pig ileum to electrical stimulation in the presence of L-NAME (100 μM), indomethacin (Indo, 10 μM), naloxone (Nx, 1 μM), glibenclamide (Glib, 100 μM), saline (1 ml l^{-1} or DMSO (1 ml l^{-1}). CON represents the response to NaHS prior to drug or vehicle administration. Inhibitors were left in contact with the tissue for 60 min before further testing. Results show per cent inhibition of twitch response and are mean ± s.e.mean, $n=4-8$, ($P>0.05$). (D) Effect of a high concentration of NaHS (200 μM) on contractions of the guinea-pig ileum due to KCl at 'low' concentration (10 mM) or 'high' concentration (60 mM). The interval between administration of NaHS and KCl was 30 s. Results show tension developed (g) and are mean ± s.e.mean, $n=5$, $^*P>0.05$, c.f. KCl (10 mM).

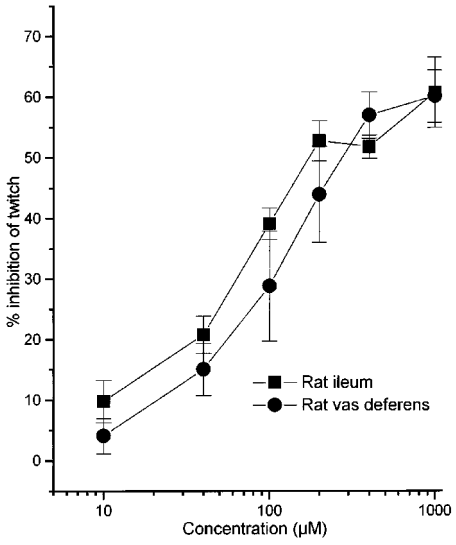

Figure 3 Relaxant effect of NaHS on the rat isolated ileum and vas deferens. Results show per cent inhibition of response to electrical stimulation at a frequency of either 0.1 Hz (vas deferens) or 5 Hz (rat ileum) and are mean ± s.e.mean, $n=5-7$.

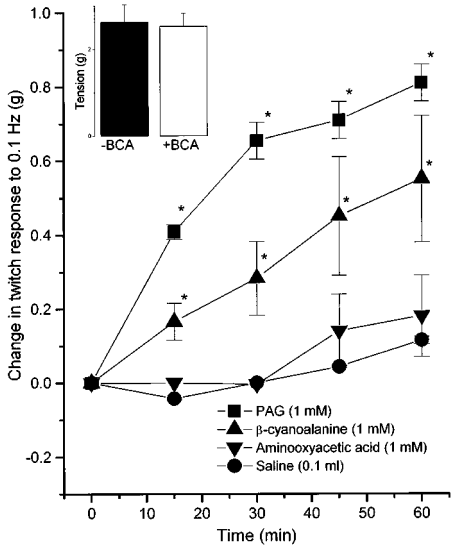

Figure 4 Effect of BCA and PAG (both 1 mM, inhibitors of cystathione-γ-lyase, CES), aminooxyacetic acid (1 mM, inhibitor of cystathionine-β-synthetase, CBS) and vehicle (saline, 0.1 ml) on the twitch response of the isolated guinea-pig ileum to electrical stimulation. Results show per cent change in response to electrical stimulation (0.1 Hz) with time and are mean ± s.e.mean, $n=6$ (BCA) and $n=11$ (PAG), $^*P<0.05$ (c.f. prior to drug treatment, ANOVA plus *post-hoc* Dunnett's test). Inset shows the effect of pretreatment (60 min) of the guinea-pig ileum with BCA (1 mM; + BCA) or saline (0.1 ml; − BCA) on the response to ACh (88 nM, EC_{70}). Results show tension developed (g) and are mean ± s.e.mean, $n=5-11$, $P>0.05$, Student's *t*-test).

reacts with H^+ to form H_2S. As such, NaHS provides a useful experimental source of H_2S.

We report here that NaHS causes dose-related inhibition of the response of the rat and guinea-pig ileum and the rat vas deferens to electrical stimulation, reduces the spontaneous contractility of the rabbit ileum and inhibits contractions of the guinea-pig ileum due to ACh. Importantly, the smooth muscle relaxant effect of exogenous NaHS in these various preparations occurs, for the most part, over a dose range which is similar to the H_2S concentration found naturally in both rat (i.e. 50 μM; Zhao *et al.*, 2001) and human (Richardson *et al.*, 2000; 10 – 100 μM) blood. It should be

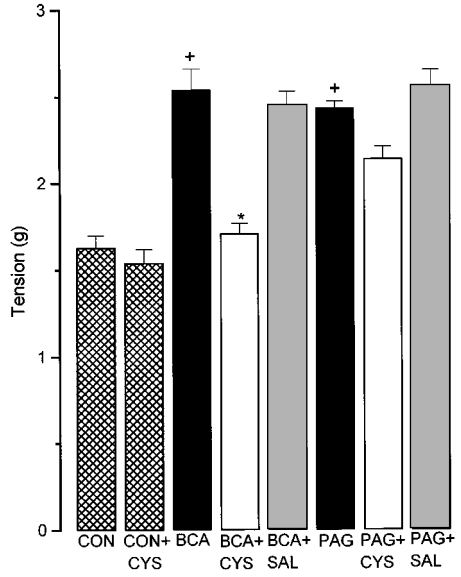

Figure 5 Effect of cysteine base (CYS, 1 mM); or saline (SAL; 0.1 ml) on the response of the isolated guinea-pig ileum to electrical stimulation before (hatched columns) and after exposure (60 min) to either BCA or PAG (both 1 mM). CON represents data from tissues prior to drug exposure. CON + CYS indicates contractions following 60 min incubation with cysteine. Results show tension developed (g) per twitch and are mean ± s.e.mean, $n=6$, $+P<0.05$ c.f. CON, $*P<0.05$ c.f. BCA.

noted that, at physiological pH, H_2S in solution is approximately 30% intact with the remaining 70% present as HS^- anion (US National Research Council, 1979). Whether HS^- anions contribute to the pharmacological effects of NaHS observed in this, or other studies, is not known. However, assuming that H_2S is the pharmacologically active moiety it is conceivable that the present experiments may underestimate the potency of NaHS in molar terms.

The ability of NaHS to inhibit the response of the ileum to both electrical stimulation and to exogenous ACh is relatively short-lived. It is possible that this reflects either an inherent instability of H_2S in the experimental conditions employed, or its escape from the organ bath by diffusion, or rapid enzymatic catabolism of H_2S. In the latter case, both thiol S-methyltransferase and rhodanese occur in human small intestine (Picton *et al*., 2002) and have been reported to break down H_2S (Weisiger *et al*., 1980).

An additional point of interest in the present study is that NaHS mimics the effect of SNP (albeit with reduced potency) in producing spontaneously reversible and dose-related inhibition of contractions of the guinea-pig ileum to field stimulation. The combination of low doses of NaHS and SNP resulted in a disproportionately greater inhibition of the response to electrical stimulation than might be expected from addition of either drug alone. A synergistic vasorelaxant effect of NaHS and NO has also been noted in isolated rat aorta (Hosoki *et al*., 1997). This aspect of the effect of H_2S on gastrointestinal smooth muscle requires further study.

The mechanism of the smooth muscle relaxant effect of H_2S in the field-stimulated guinea-pig ileum has been examined in some detail. Pretreatment of ileum preparations with indomethacin, L-NAME or naloxone at doses used previously by other researchers to inhibit cyclo-oxygenase, nitric oxide synthase (NOS) enzyme activity or antagonize opioid receptors in isolated tissues failed to influence the effect of NaHS on contractions due to electrical stimulation. Thus, it may be concluded that the release of prostanoids, NO or endogenous opioids does not contribute to the inhibitory effect of NaHS in the field-stimulated guinea-pig ileum.

In a recent study, Zhao *et al*. (2001) reported that glibenclamide antagonized the relaxant effect of NaHS in the phenylephrine-precontracted rat aorta thereby concluding that H_2S opens K_{ATP} channels in vascular smooth muscle cells to bring about smooth muscle relaxation. A similar mechanism may therefore underlie the relaxant effect of H_2S in nonvascular smooth muscle. Indeed, cromakalim has previously been shown to relax guinea-pig ileum in a glibenclamide-sensitive manner (Sun & Benishin, 1994) indicating the presence of operational K_{ATP} channels in this preparation. However, in the present experiments, glibenclamide did not affect the response of the electrically stimulated ileum to NaHS suggesting that K_{ATP} channels are most probably not involved. To investigate this possibility in more detail, additional experiments were carried out in which the relaxant effect of NaHS was assessed on contractions of the guinea-pig ileum to both low (10 mM) and high (60 mM) concentrations of KCl. Activation of smooth muscle K_{ATP} channels would be expected to result in an inhibition of the response to low (but not high) concentrations of KCl whereas inhibition of L-type Ca^{2+} channels would be expected to reduce the response to both concentrations of KCl. NaHS, at a concentration (200 μM), which caused near maximal inhibition of the response to electrical stimulation in this preparation, failed to affect the contractile response to KCl at either concentration. Again, in an analogous experiment, Zhao *et al*. (2001) noted that NaHS preferentially inhibited contractions of the rat aorta to a 'low' concentration of KCl (in this case, 20 mM) with very much less effect on the response to 'high' KCl (in this case, 100 mM).

Accordingly, we consider it unlikely that activation of K_{ATP} channels (or indeed inhibition of L-type Ca^{2+} channels) accounts for the relaxant effect of NaHS in the guinea-pig ileum. It is clear that H_2S relaxes guinea-pig ileum (this study) and rat aorta (Zhao *et al*., 2001) by different mechanisms. The precise nature of these mechanisms and the manner by which H_2S and NO interact at the cellular level remains to be clarified.

In addition to characterizing the effect of exogenous H_2S on the response of several nonvascular smooth muscle preparations, the present study also provides evidence for endogenous production of this mediator by the electrically stimulated guinea-pig ileum. To the best of our knowledge this is the first demonstration of a biological effect of naturally produced H_2S and, as such, the results on which this conclusion is based warrant very careful scrutiny.

The profile of biological activity of the CSE and CBS inhibitors used in this study is of particular interest. Of the inhibitors employed, PAG has been most studied. This compound causes an irreversible, mechanism-based inhibition of CSE enzyme activity *in vitro* (Johnston *et al*., 1979) and, when administered to rats, produces an almost complete inhibition of liver CSE enzyme activity (measured *ex vivo*) (Porter *et al*., 1996; Uren *et al*., 1978) as well as elevated

brain cystathionine concentration (Yu *et al*., 2000). Despite assertions to the contrary (e.g. Hosoki *et al*., 1997) it would therefore appear that PAG is well absorbed and readily crosses biological membranes (see also review by Reed, 1995). Of the other compounds used in the present experiments, AOAA is a potent and reversible inhibitor of CBS (Braunstein *et al*., 1971) but, in addition, also inhibits other pyridoxal phosphate-dependent enzymes notably GABA transaminase (Loscher, 1981) and glutamic acid decarboxylase (Hamel *et al*., 1982). Less is known of the pharmacology of β-CA although this compound has been reported to cause potent and reversible inhibition of CSE activity (Uren *et al*., 1978; Pfeffer & Ressler, 1967).

Of the drugs tested, the CSE inhibitors, PAG and β-CA, but not the CBS inhibitor, AOAA, produced a slowly-developing increase in contractile response of the ileum to field stimulation. Bearing in mind the relative lack of information concerning the pharmacology of these inhibitors (see above) the possibility that one or more of these compounds may affect the guinea-pig ileum by mechanism(s) which are unrelated to inhibition of CSE or CBS (and thus of H_2S) cannot be excluded. However, the finding that BCA exposure does not influence responses of the ileum to applied ACh excludes, for example, a non-selective post-junctional effect of this compound to augment smooth muscle responsiveness as well as an anti-cholinesterase action.

Perhaps more convincingly, cysteine (precursor for H_2S biosynthesis by CSE) completely reversed the BCA-mediated but failed to affect the PAG-mediated increase in contractile response of the ileum to electrical stimulation. The difference in susceptibility of the two inhibitors with respect to cysteine reversal may reflect the manner in which they interact with CSE i.e. competitive (with substrate) for BCA, irreversible for PAG. In separate experiments, cysteine (at the same concentration and over the same time course) did not affect the contractility of the ileum to electrical stimulation in the absence of H_2S biosynthesis.

Based on these various experimental observations, we therefore propose the following working hypothesis: (1) electrical stimulation of the guinea-pig ileum triggers the formation of H_2S by CSE (but not CBS); and (2) the H_2S which is formed, possibly acting in concert with NO, acts on ileal smooth muscle cells to cause relaxation by an, as yet, unidentified mechanism.

Clearly, a number of questions remain to be resolved. For example, information about the precise cellular site of H_2S biosynthesis in the ileum (e.g. smooth muscle cells, nerves) is lacking. That BCA does not affect the response of the ileum to exogenous ACh suggests that CSE activity is a direct consequence of electrical activity in intramural nerves in this tissue rather than a result of smooth muscle contraction *per se*. This would imply a neuronal localization of CSE. In this context, (Eto *et al*., 2002) have recently reported that electrical stimulation of mouse cerebral cortical slices also triggers H_2S formation suggesting that H_2S is formed within neurones. In this case, the particular enzyme involved is likely to be CBS and not CSE. It is also not clear whether analogous release of H_2S occurs *in vivo* and, if it does, to what extent (if any) does H_2S contribute to the regulation of gastrointestinal contractility in the whole animal?

Whilst the present data go some way towards characterizing the pharmacological effects of H_2S, further research will clearly be required to determine the precise physiological significance (if any) of this novel, gaseous mediator. Nevertheless, the present data suggest a hitherto unappreciated role for, most probably neuronally-derived H_2S, in the regulation of autonomic transmission in the guinea-pig ileum.

References

ABE, K. & KIMURA, H. (1996). The possible role of hydrogen sulfide as an endogenous neuromodulator. *J. Neurosci.,* **16,**1066 – 1071.

BRAUNSTEIN, A.E., GORYACHENKOVA, E.V., TOLOSA, E.A., WILLHARDT, I.H. & YEFREMOVA, L.L. (1971). Specificity and some other properties of liver serine sulphhydrase: evidence for its identity with cystathionine-β-synthase. *Biochim. Biophys. Acta.,* **242,** 247 – 260.

COLOSO, R.M. & STIPANUK, M.H. (1989). Metabolism of cysteine in rat enterocytes. *J. Nutr.,* **119,** 1914 – 1924.

ETO, K., OGASAWARA, M., UMEMURA, K., NAGAI, Y. & KIMURA, H. (2002). Hydrogen sulphide is produced in response to neuronal excitation. *J. Neurosci.,* **22,** 3386 – 3391.

FINKELSTEIN, J.D. & MARTIN, J.J. (1984). Methione metabolism in mammals. Distribution of homocysteine between competing pathways. *Biochem. Biophys. Res. Commun.,* **118,** 14 – 19.

FLORIN, T.H. (1991). Hydrogen sulfide and total acid-volatile sulfides in faeces, determined with a direct spectrophotometric method. *Clin. Chim. Acta.,* **196,** 127 – 134.

GOFFREDI, S.K., CHILDRESS, J.J., DESAULNIERS, N.T. & LALLIER, F.H. (1997). Sulfide acquisition by the vent worm *Riftia Pachyptila* appears to be via uptake of HS^- rather than H2S. *J. Exp. Biol.,* **200,** 2609 – 2616.

GOSSELIN, R.E., SMITH, R.P. & HODGE, H.C. (1984). Hydrogen sulfide: In: *Clinical Toxicology of Commercial Products,* Baltimore, USA: Williams and Wilkins Ltd.

GUIDOTTI, T.L. (1996). Hydrogen sulphide. *Occup. Med.,* **46,** 367 – 371.

HAMEL, E., KRAUSE, D.N. & ROBERTS, E. (1982). Characterization of glutamic acid decarboxylase activity in cerebral blood vessels. *J. Neurochem.,* **39,** 842 – 849.

HARGROVE, J.L. (1988). Persulfide generated from L-cysteine inactivates tyrosine aminotransferase. Requirement for a protein with cysteine oxidase activity and gamma cysthionase. *J. Biol. Chem.,* **263,** 17262 – 17269.

HAYDEN, L.J., FRANKLIN, K.J, ROTH, S.H. & MOORE, G.J. (1989). Inhibition of oxytocin-induced but not angiotensin-induced rat uterine contractions following exposure to sodium sulfide. *Life Sci.,* **45,** 2557 – 2560.

HOSOKI, R., MATSUKI, N. & KIMURA, H. (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophys. Res. Commun.,* **237,** 527 – 531.

JOHNSTON, M., JANKOWSKI, D., MARCOTTE, P., TANAKA, H., ESAKI, N., SODA, K. & WALSH, C. (1979). Suicide inactivation of bacterial cystathionine gamma synthase and methionine gamma lyase during processing of L-propargylglycine. *Biochemistry,* **18,** 4690 – 4701.

KOMBIAN, S.B., REIFFENSTEIN, R.J. & COLMERS, W.F. (1993). The actions of hydrogen sulphide on dorsal raphe serotonergic neurons *in vitro*. *J. Neurophysiol.,* **70,** 81 – 96.

KRAFT, A.A., BRANT, A.W. & AYRES, J.C. (1956). Detection of hydrogen sulphide in packaged meats and in broken-out shell eggs. *Food Technology,* **10,** 443 – 444.

LOSCHER, W. (1981). Effect of inhibitors of GABA aminotransferase on the metabolism of GABA in brain tissue and synaptosomal fractions. *J. Neurochem.,* **36,** 1521 – 1527.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). L-NG-nitro arginine (L-NOARG)- a novel, L-arginine reversible inhibitor of endothelium-dependent vasodilatation. *Br. J. Pharmacol.,* **99,** 408 – 412.

NAVARRA, P., DELLO RUSSO, C., MANCUSO, C., PREZIOSI, P. & GROSSMAN, A. (2000). Gaseous neurotransmitters in the control of the neuroendocrine stress axis. *Ann. N.Y. Acad. Sci.,* **917,** 638 – 646.

NICHOLSON, R.A., RITH, S.H., ZHANG, A., BROCKES, J., SKRAJNY, B. & BENNINGTON, R. (1998). Inhibition of respiratory and bioenergetic mechanisms by hydrogen sulphide in mammalian brain. *J. Toxicol. Environ. Health,* **54,** 491 – 507.

PFEFFER, M. & RESSLER, C. (1967). Beta-cyanoalanine, an inhibitor of rat liver cystathionase. *Biochem. Pharmacol.,* **242,** 2299 – 2308.

PICTON, R., EGGO, M.C., MERRILL, G.A., LANGMAN, M.J.S. & SINGH, S. (2002). Mucosal protection against sulphide: importance of the enzyme rhodanese. *Gut,* **50,** 201 – 205.

PITCHER, M.C.L., BEATTY, E.R. & CUMMINGS, J.H. (2000). The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. *Gut,* **46,** 64 – 72.

PORTER, D.W., NEALLEY, E.W. & BASKIN, S.L. (1996). *In vivo* detoxification of cyanide by cystathionase gamma-lyase. *Biochem. Pharmacol.,* **27,** 941 – 944.

REED, D.J. (1995). Cystathionine. *Meth. In Enzymol.,* **252,** 92 – 102.

RICHARDSON, C.J., MAGEE, E.A. & CUMMINGS, J.H. (2000). A new method for the determination of sulphide in gastrointestinal contents and whole blood by microdistillation and ion chromatography. *Clin. Chim. Acta,* **293,** 115 – 125.

RIZZI, D., BIGONI, R., RIZZI, A., JENCK, F., WICHMANN, J., GUERRINI, R., REGOLI, D. & CALO, G. (2001). Effects of Ro 64-6198 in nociceptin/orphanin FQ-sensitive isolated tissues. *Naunyn Schmiedebergs Arch. Pharmacol.,* **363,** 551 – 555.

RODRIGUEZ-MARTINEZ, M.A., GARCIA-COHEN, E.C., BAENA, A.B., GONZALEZ, R., SALAICES, M. & MARIN, J. (1998). Contractile responses elicited by hydrogen peroxide in aorta from normotensive and hypertensive rats. Endothelial modulation and mechanisms involved. *Br. J. Pharmacol.,* **125,** 1329 – 1335.

SIDHU, R., SINGH, M., SAMIR, G. & CARSON, R.J. (2001). L-cysteine and sodium hydrosulphide inhibit spontaneous contractility of isolated pregnant rat uterine strips *in vitro*. *Pharmacol. Toxicol.,* **88,** 198 – 203.

STIPANUK, M.H. & BECK, P.W. (1982). Characterisation of the enzymatic capacity for cysteine desulphydration in liver and kidney of the rat. *Biochem. J.,* **206,** 267 – 277.

STIPANUK, M.H., DE LA ROSA, J. & HIRSCHBERGER, L.L. (1990). Catabolism of cysteine by rat renal cortical tubules. *J. Nutr.,* **120,** 450 – 458.

SUAREZ, F.L., SPRINGFIELD, J. & LEVITT, M.D. (1998). Identification of gases responsible for the odour of human flatus and evaluation of a device purported to reduce this odour. *Gut,* **43,** 100 – 104.

SUN, Y.D. & BENISHIN, C.G. (1994). K^+ channel openers relax longitudinal muscle of guinea pig ileum. *Eur. J. Pharmacol.,* **271,** 453 – 459.

UREN, J.R., RAGIN, R. & CHAYKOVSKY, M. (1978). Modulation of cysteine metabolism in mice – effects of propargylglycine and L-cysteine-degrading enzymes. *Biochem. Pharmacol.,* **27,** 2807 – 2814.

US NATIONAL RESEARCH COUNCIL. (1979). *Subcommittee on hydrogen sulphide*. Baltimore, USA: University Park Press.

WARENYCIA, M.W., STEELE, J.A., KARPINSKI, E. & REIFFENSTEIN, R.J. (1989). Hydrogen sulfide in combination with taurine or cysteic acid reversibly abolishes sodium currents in neuroblastoma cells. *Neurotoxicology,* **10,** 191 – 199.

WEISIGER, R.A., PINKUS, L.M. & JAKOBY, W.B. (1980). Thiol S-methyltransferase: suggested role in detoxication of intestinal hydrogen sulfide. *Biochem. Pharmacol.,* **29,** 2885 – 2887.

WORLD HEALTH ORGANIZATION. (1987). *Air Quality Guidelines for Europe*. Copenhagen: WHO Regional Office for Europe.

YU, S., SUGAHARA, K., NAKAYAMA, K., AWATA, S. & KODAMA, H. (2000). Accumulation of cystathionine, cystathionine ketimine, and pehydro-1,4-thiazepine-3,5-dicarboxylic acid in whole brain and various regions of the brain of D,L-propylargylglycine-treated rats. *Metabolism,* **49,** 1025 – 1029.

ZHAO, W., ZHANG, J., LU, Y. & WANG, R. (2001). The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J.,* **20,** 6008 – 6016.

(Received May 20, 2002
Revised June 26, 2002
Accepted June 27, 2002)