Hydrogen Sulfide Mediated Inhibitory Neurotransmission to the Pig Bladder Neck: Role of K_{ATP} Channels, Sensory Nerves and Calcium Signaling

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Purpose: Because neuronal released endogenous H_2S has a key role in relaxation of the bladder outflow region, we investigated the mechanisms involved in H_2S dependent inhibitory neurotransmission to the pig bladder neck.

Materials and Methods: Bladder neck strips were mounted in myographs for isometric force recording and simultaneous measurement of intracellular Ca^2 and tension.

Results: On phenylephrine contracted preparations electrical field stimulation and the H_2S donor GYY4137 evoked frequency and concentration dependent relaxation, which was reduced by desensitizing capsaicin sensitive primary afferents with capsaicin, and the blockade of adenosine 5=-triphosphate dependent K channels, cyclooxygenase and cyclooxygenase-1 with glibenclamide, indomethacin and SC560, respectively. Inhibition of vanilloid, transient receptor potential A1, transient receptor potential vanilloid 1, vasoactive intestinal peptide/pituitary adenylyl cyclase-activating polypeptide and calcitonin gene-related peptide receptors with capsazepine, HC030031, AMG9810, $PACAP_{6-38}$ and $CGRP_{8-37}$, respectively, also decreased electrical field stimulation and GYY4137 responses. H_2S relaxation was not changed by guanylyl cyclase, protein kinase A, or Ca^2 activated or voltage gated K channel inhibitors. GYY4137 inhibited the contractions induced by phenylephrine and by K enriched (80 mM) physiological saline solution. To a lesser extent it decreased the phenylephrine and K induced increases in intracellular Ca^2 .

Conclusions: H_2S produces pig bladder neck relaxation via the activation of adenosine 5=-triphosphate dependent K channel and by smooth muscle intracellular Ca^2 desensitization dependent mechanisms. H_2S also promotes the release of sensory neuropeptides and cyclooxygenase-1 pathway derived prostanoids from capsaicin sensitive primary afferents via transient receptor potential A1, transient receptor potential vanilloid 1 and/or related ion channel activation.

Key Words: urinary bladder; muscle, smooth; hydrogen sulfide; potassium channels; synaptic transmission

Abbreviations and Acronyms

4-AP 4-aminopyridine
AM acetoxymethyl ester
ATP adenosine 5=-triphosphate
$[Ca^2]_i$ intracellular Ca^2
CGRP calcitonin gene-related peptide
COX cyclooxygenase
CSE cystathionine -lyase
CSPA capsaicin sensitive primary afferent
Emax maximum response
K_{ATP} ATP dependent K
K_{Ca} Ca^2 activated K
KPSS potassium rich PSS
K_V voltage gated K
L-NOARG N^G-nitro-L-arginine
MLCP myosin light chain phosphatase
NO nitric oxide
PACAP pituitary adenylyl cyclase activating polypeptide
PKA protein kinase A
PSS physiological saline solution
$TRPA_1$ transient receptor potential A1
$TRPV_1$ transient receptor potential vanilloid 1
VOC voltage gated Ca^2
VPAC vasoactive intestinal peptide receptor

HYDROGEN sulfide is considered the third endogenous gaseous transmitter besides NO and CO.[1,2] Endogenous H_2S is synthesized from L-cysteine by the action of the pyridoxal-5=-phosphate dependent enzymes cystathionine -synthase or CSE.[1–4] In the lower urinary tract H_2S donors produce smooth muscle contraction and/or relaxation. Thus, in rat detrusor the H_2S donor NaHS produces contraction by CSPA stimulation, producing the release of tachykinins such as substance P or neurokinin A.[5] $TRPA_1$ immunoreactivity was noted in nerve fibers in suburothelial and muscular layers in the human urethra, where NaHS produces smooth muscle relaxation.[6]

The bladder neck is part of the urine bladder outflow region in which NO[7] and several nonNO mediators such as ATP,[8] peptides such as vasoactive intestinal peptide, $PACAP_{38}$[9] and CGRP,[10] and an unidentified nerve dependent component[11] are involved in inhibitory transmission. Recently, a key role for H_2S endogenously generated from L-cysteine by CSE was unmasked as a signaling gaseous molecule in inhibitory neurotransmission to the pig bladder neck.[12]

Knowledge of the mechanisms involved in the control of bladder neck smooth muscle tone is essential to provide therapeutic agents to relax the bladder outlet region during the voiding phase under pathophysiological conditions, such as urinary obstruction symptoms associated with benign prostatic hypertrophy.[13] Thus, we investigated the mechanisms involved in H_2S relaxation to the pig bladder neck.

MATERIALS AND METHODS

Myographs for Isometric Force Recording

Adult pigs (25 males and 36 females) with no urinary tract lesions were selected from the local slaughterhouse. Bladders were removed immediately after sacrifice and kept in chilled PSS at 4C. Bladder neck strips were dissected transversely to 4 to 6 mm long 2 to 3 mm wide. They were suspended horizontally with 1 end connected to an FT03C isometric force transducer (Grass Instruments, West Warwick, Rhode Island). The other end was connected to a m screw in 5 ml organ baths containing 37C PSS continuously gassed with carbogen (95% O_2 and 5% CO_2) to a final pH of 7.4. The signal was continuously recorded on a Multi Corder MC6621 polygraph (Graphtec, Jessup, Maryland). Passive tension (2.0 gm) was applied to the strips and they were allowed to equilibrate for 60 minutes.[10]

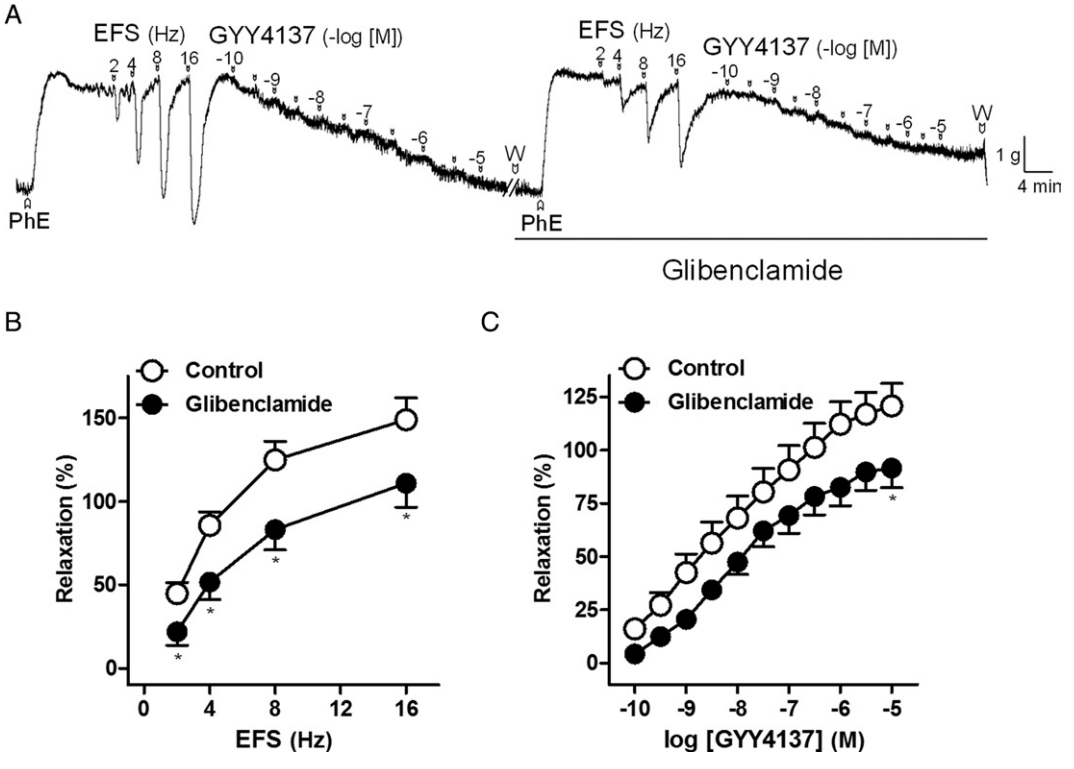

Figure 1. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of K_{ATP} channel blocker glibenclamide (1 M) on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W,* wash. *g,* gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in glibenclamide absence (*Control*) and presence. Results represent mean SEM of 8 preparations from total of 4 pigs. Asterisk indicates paired t test p 0.05 vs control.

Strip contractile ability was determined by exposing them to 124 mM KPSS. In electrical field stimulation experiments noradrenergic neurotransmission, muscarinic receptors and NO synthase were blocked by preincubation with guanethidine (10 M), atropine (0.1 M) and L-NOARG (100 M) for 1 hour. Solution was replaced every 20 minutes. These drugs were present throughout the experiment. In strips precontracted with 1 M phenylephrine we performed electrical field stimulation by delivering rectangular pulses (duration 1 millisecond, 2 to 16 Hz and 20-second trains with constant current output adjusted to 75 mA) at 4-minute intervals using a CS20 stimulator (Cibertec, Barcelona, Spain). A first control response curve was obtained to electrical field stimulation or to addition of the H_2S donor GYY4137 (0.1 nM to 10 M). Electrical field stimulation and GYY4137 relaxations were achieved under matched precontraction levels and these responses did not significantly differ in preparations from male or female pigs.[12] Bath solution was then changed every 15 minutes for a total of 90 minutes. Preparations were incubated with the specific treatments for 30 minutes and a second relaxation curve was constructed.

Tension and $[Ca^2]_i$ Simultaneous Measurement

Tension and $[Ca^2]_i$ were simultaneously measured in intact bladder neck strips by fura-2 AM fluorescence, as previously described.[13] Preparations were loaded in the dark in PSS containing 8 M fura-2 AM and 0.05% Cremophor® EL for 2 hours at 37C. They were washed 3 times in PSS and the solution was changed to PSS with fresh fura-2 AM after 45 minutes.

The myograph chamber was mounted on an inverted microscope (Carl Zeiss, Jena, Germany) equipped for dual excitation wavelength fluorimetry with a Deltascan™ device. Strips were illuminated with alternating 340 and 380 nm light. The intensity of emitted fluorescence was collected at a wavelength of 510 nm using a photomultiplier and monitored together with tension.

At the end of each experiment Ca^2 insensitive signals were determined after quenching with Mn^2 . Values were subtracted from those obtained during the experiment. The ratio of fluorescence at 340 and 380 nm (F_{340}/F_{380}), corrected for autofluorescence, was considered a measure of $[Ca^2]_i$. Mean SEM minimum and maximum ratios were 0.59 0.08 and 0.95 0.11, respectively.

Drugs and Solutions

The drugs used were AMG9810, 4-AP, atropine, guanethidine, indomethacin, L-NOARG, phenylephrine (Sigma®), apamin, $CGRP_{8-37}$, capsaicin, glibenclamide, GYY4137, HC030031, iberiotoxin, KT5720, NS398, ODQ, $PACAP_{6-38}$, SC560 and TRAM34 (Tocris Bioscience, Bristol, United Kingdom). We dissolved AMG9810, $CGRP_{8-37}$, glibenclamide, GYY4137, HC030031, KT5720, NS398, ODQ, $PACAP_{6-38}$, SC560 and TRAM34 in dimethyl sulfoxide.

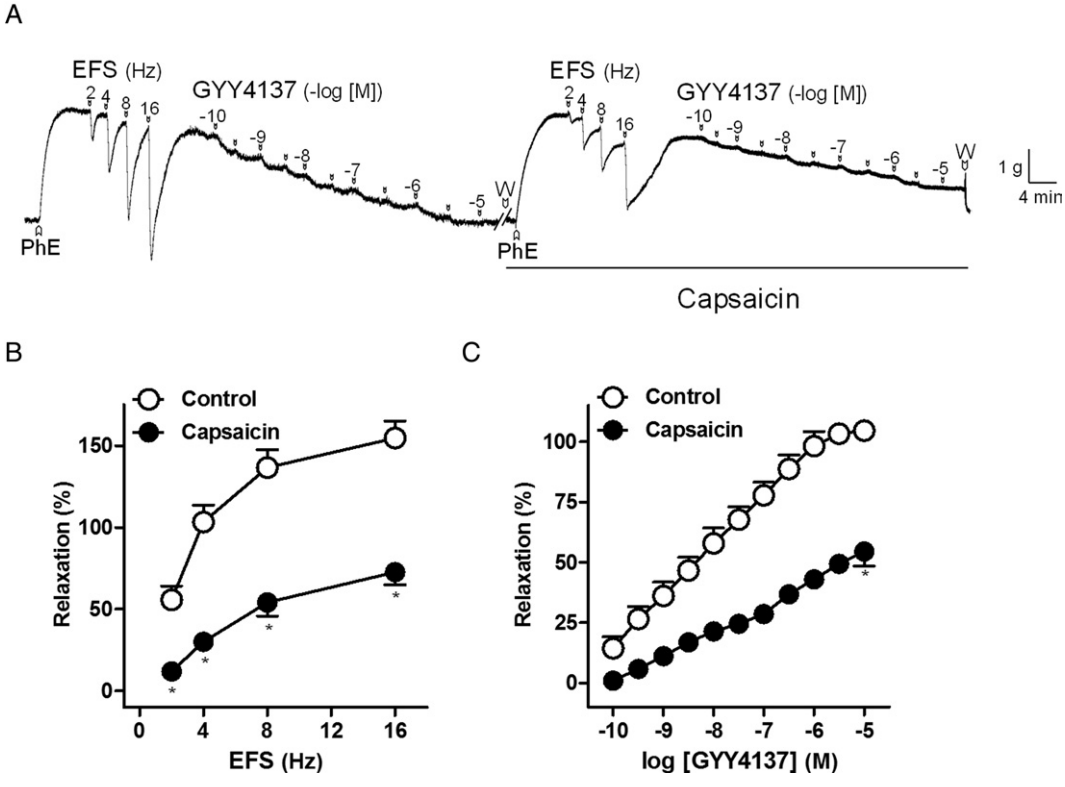

Figure 2. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of 10 M of CPSA neurotoxin capsaicin on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W,* wash. *g,* gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in capsaicin absence (*Control*) and presence. Results represent mean SEM of 8 preparations from total of 4 pigs. Asterisk indicates paired t test p 0.05 vs control.

Indomethacin was dissolved in ethanol. The other drugs were dissolved in distilled water. Solvents had no effect on the contractility of bladder neck preparations.

PSS was composed of 119 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 24.9 mM $NaHCO_3$, 11 mM glucose, 1.5 mM $CaCl_2$, 1.2 mM KH_2PO_4 and 0.027 mM ethylenediaminetetraacetic acid. The solution was maintained at 37C and continuously gassed with 95% O_2 and 5% CO_2 to maintain pH 7.4.

Calculations and Statistical Analysis

Sensitivity to GYY4137 is expressed as pD_2, where pD_2 –log EC_{50} and EC_{50} is the agonist concentration needed to produce the half maximal response. pD_2 was estimated by computerized nonlinear regression analysis using Prism®. Results are shown as the percent reversal of the phenylephrine or KPSS induced contraction and represent the mean SEM of the number of preparations (1 or 2 strips per animal). Differences were analyzed by the Student t test for paired observations with differences considered significant at p 0.05.

RESULTS

Relaxation to Electrical Field Stimulation and GYY4137

Urothelium denuded strips of pig bladder neck (108 preparations from a total of 61 pigs) were allowed to equilibrate to a mean passive tension of 1.9 0.2 gm. Phenylephrine (1 M) induced a mean sustained contraction above basal tension of 3.3 0.1 gm in 107 preparations.

To assess NO independent neurogenic relaxation, strips were pretreated with the NO synthase inhibitor L-NOARG. Under these conditions, electrical field stimulation (2 to 16 Hz) evoked reproducible frequency dependent relaxations (at 16 Hz mean maximal relaxation mean 114% 9% of the phenylephrine induced contraction in 104 preparations from a total of 57 pigs). GYY4137 (0.1 nM to 10 M) induced potent concentration dependent bladder neck relaxations in 104 preparations (mean pD_2 and Emax 7.9 0.1 and 103% 5%, respectively).

Effects

Soluble guanylyl cyclase, PKA and K channel blockade on electrical field stimulation and GYY4137 relaxation. To investigate the role of guanylyl cyclase and PKA on relaxations elicited by H_2S, we constructed curves to electrical field stimulation and GYY4137 in the absence or presence of ODQ (5 M) or KT5720 (3 M), which are blockers of soluble guanylyl cyclase and PKA, respectively. This failed

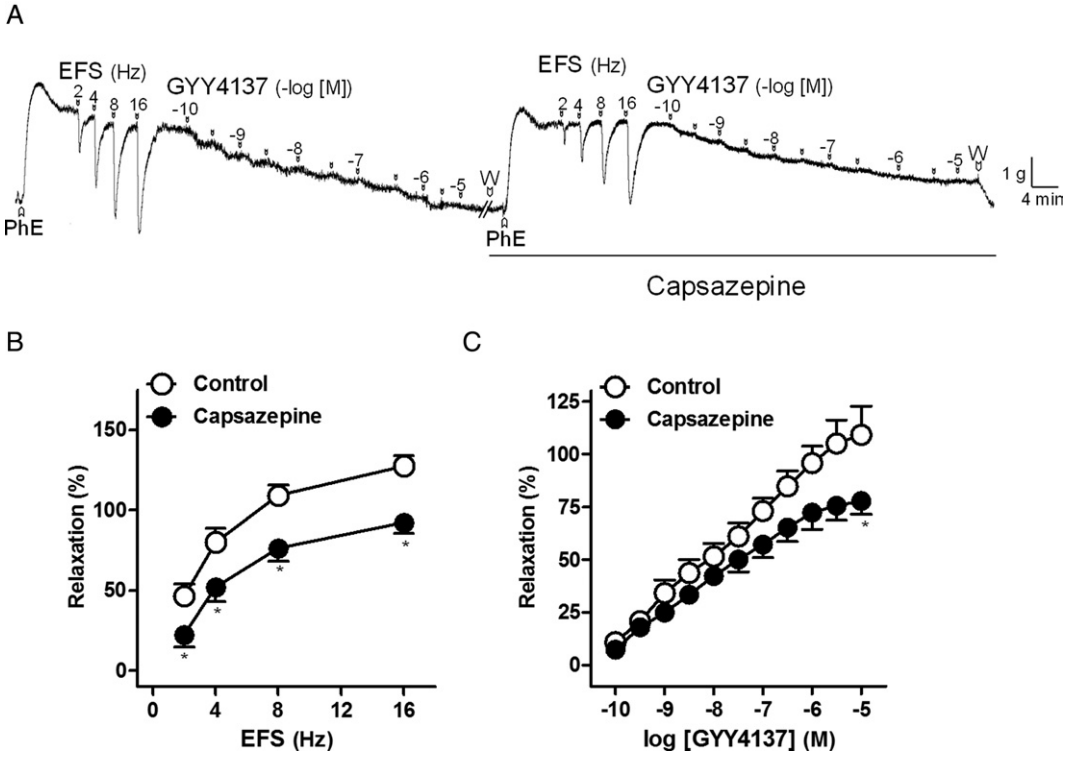

Figure 3. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of 10 M of vanilloid receptor antagonist capsazepine on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W*, wash. *g*, gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in capsazepine absence (*Control*) and presence. Results represent mean SEM of 8 preparations from total of 4 pigs. Asterisk indicates paired t test p 0.05 vs control.

to modify electrical field stimulation or GYY4137 relaxations.

To study the role of K channels in H_2S responses, we used high (80 mM) extracellular K concentrations, which induced a mean sustained tone of 2.1 0.1 gm in 7 preparations. GYY4137 induced a concentration dependent relaxation on 80 mM KPSS precontracted strips, which was lower than that on 1 M phenylephrine contracted strips in 7 preparations each (mean Emax 85% 7% and 61% 7% for phenylephrine and KPSS, respectively, vs control paired t test p 0.05). Together with the fact that the K_{ATP} channel inhibitor glibenclamide (1 M) reduced electrical field stimulation and GYY4137 relaxation (fig. 1), this suggests the involvement of K_{ATP} channels in H_2S responses.

Iberiotoxin (100 nM), TRAM34 (20 nM) and apamin (0.5 M), which are blockers of large, intermediate and small K_{Ca} channels, respectively, failed to modify relaxations to electrical field stimulation or GYY4137, initially ruling out a role for these channels. In 7 preparations from a total of 4 pigs the K_V channel inhibitor 4-AP (1 mM) potentiated relaxations to electrical field stimulation (at 16 Hz mean response 117% 12% and 142% 12% in the absence and presence of 4-AP, respectively, vs control paired t test p 0.05). However, this did not change GYY4137 relaxations.

CSPA desensitization, and blockade of $TRPA_1$, $TRPV_1$, VPAC and CGRP receptors, and COX on electrical field stimulation and GYY4137 relaxation. To study the mediation of CSPAs, and VPAC and CGRP receptors in H_2S relaxations, we constructed curves to electrical field stimulation and GYY4137 in the absence and presence of the CSPA neurotoxin capsaicin (10 M), and capsazepine (10 M), HC030031 (60 M), AMG9810 (10 M) (figs. 2 to 5), vanilloid, $TRPA_1$ and $TRPV_1$ receptor antagonists, respectively, which decreased electrical field stimulation and GYY4137 responses. $PACAP_{6-38}$ (3 M), $CGRP_{8-37}$ (3 M), VPAC and CGRP receptor antagonists reduced responses to electrical field stimulation in 8 preparations from a total of 4 pigs (at 16 Hz mean relaxation 103 10 and 73 5 in the absence and presence of $PACAP_{6-38}$, and 97 7 and 72 10 in the absence and presence of $CGRP_{8-37}$, respectively, vs control paired t test p 0.05). In 8 preparations from a total of 4 pigs they also decreased responses to GYY4137 (mean Emax 120% 9% and 104% 7% in the absence and presence of $PACAP_{6-38}$, and 114% 45% and 93% 8% in the

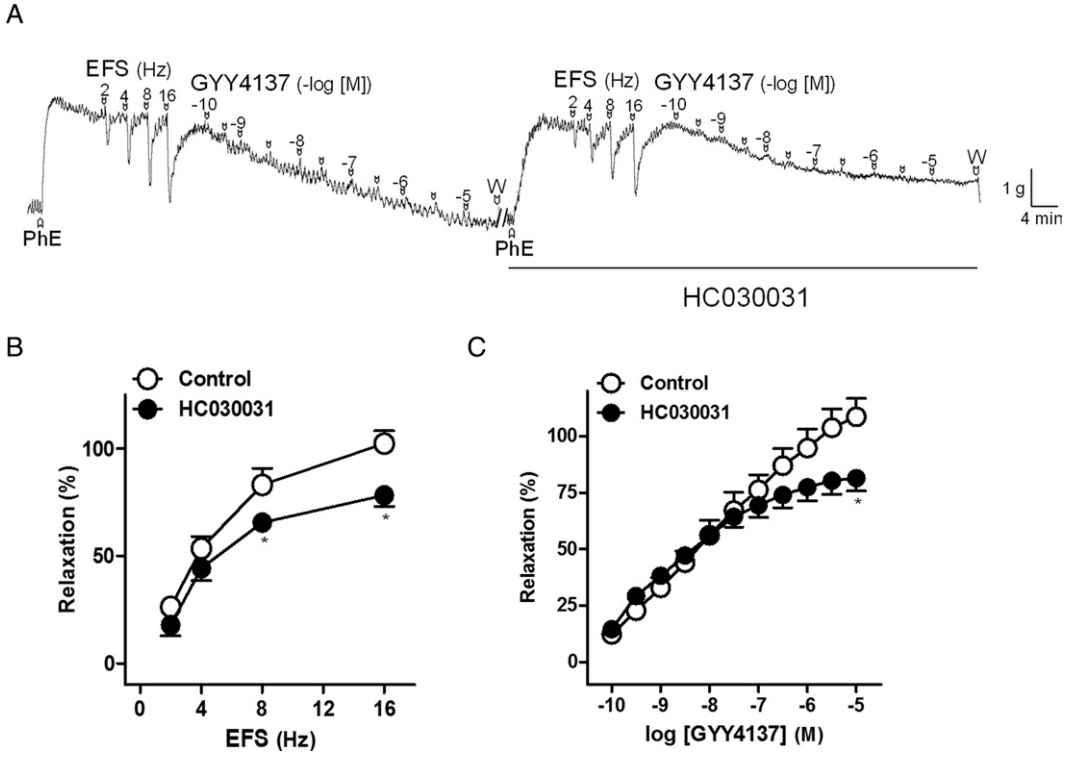

Figure 4. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of 60 M of $TRPA_1$ selective antagonist HC030031 on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W*, wash. *g*, gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in HC030031 absence (*Control*) and in presence. Results represent mean SEM of 7 preparations from 4 pigs. Asterisk indicates paired t test p 0.05 vs control.

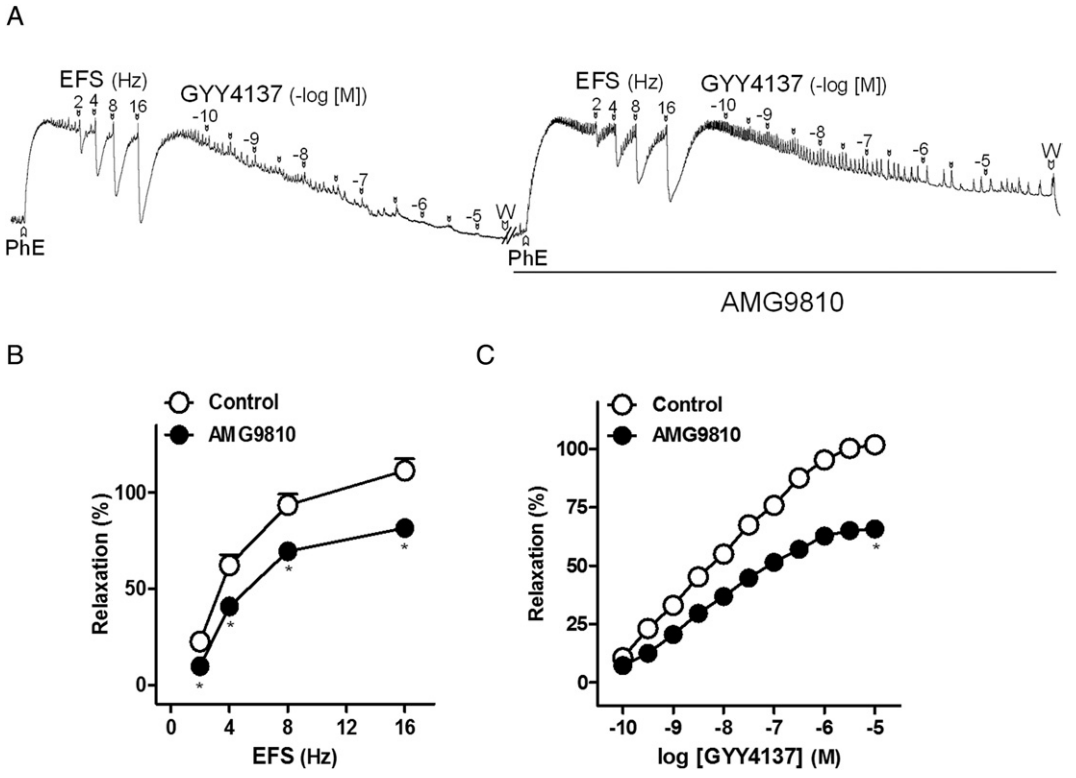

Figure 5. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of 10 M of $TRPV_1$ selective antagonist AMG9810 on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W*, wash. g, gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in AMG9810 absence (*Control*) and presence. Results represent mean SEM of 8 preparations from total of 4 pigs. Asterisk indicates paired t test p 0.05 vs control.

absence and presence of $CGRP_{8-37,}$ respectively, vs control paired t test p 0.05). Results suggest the release by H_2S of $PACAP_{38}$ and CGRP from CSPAs via $TRPA_1$, $TRPV_1$ and/or related ion channel activation.

Indomethacin (3 M) and SC560 (1 M), which are inhibitors of COX and COX-1, respectively, reduced electrical field stimulation or GYY4137 relaxations, while NS398 (1 M), a COX-2 selective inhibitor, did not change these responses (figs. 6 and 7). This suggests that COX-1 pathway derived prostanoids from CSPAs are also involved.

GYY4137 Induced Tension and Intracellular Ca^2 Changes

Mean basal F_{340}/F_{380} was 0.98 0.04 in 4 preparations from a total of 4 pigs. Stimulating 4 bladder neck strips each with phenylephrine (1 M) and KPSS (80 mM) induced simultaneous increases in tension (mean 0.9 0.2 and 0.5 0.1 gm) and in $[Ca^2]_i$ (mean $[F_{340}/F_{380}]$ 0.27 0.05 and 0.18 0.04, respectively, fig. 8). GYY4137 (1 nM, and 0.1 and 10 M) inhibited the tension induced by phenylephrine or KPSS and to a lesser extent the increases in $[Ca^2]_i$ evoked by each agent (fig. 8, *A* to *C* and *E* to *G*). The relationship of $[Ca^2]_i$ to tension for GYY4137 in phenylephrine and KPSS precontracted strips showed small dependence on $[Ca^2]_i$ decreases in H_2S donor relaxations (fig. 8, *D* and *H*), suggesting Ca^2 desensitization mechanisms.

DISCUSSION

Our results suggest that H_2S induces smooth muscle relaxation via K_{ATP} channel activation and $[Ca^2]_i$ desensitization mechanisms with a minor role for extracellular Ca^2 entry inhibition. H_2S also promotes the release of inhibitory neuropeptides, such as $PACAP_{38}$ or CGRP, as well as COX-1 derived prostanoids from CSPAs via the activation of $TRPA_1$, $TRPV_1$ and/or related ion channels in sensory nerves.

We recently reported an essential role for endogenous H_2S released from intramural nerves as a powerful signaling gaseous molecule in NO independent transmission in the bladder neck.[12] Rich expression of the H_2S synthesis enzyme CSE was noted in nerve fibers widely distributed in the smooth muscle layer.[12] The H_2S synthetic pathway is intermingled with the NO pathway. In fact, in

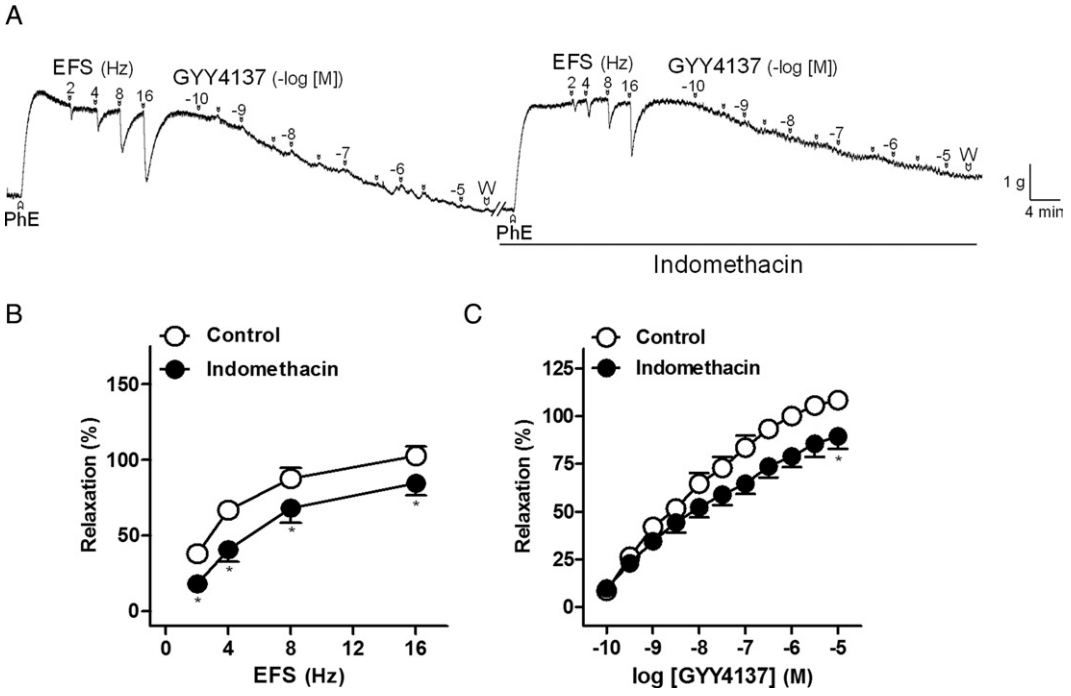

Figure 6. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of 3 M of COX inhibitor indomethacin on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W,* wash. *g,* gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in indomethacin absence (*Control*) and presence. Results represent mean SEM of 6 preparations from total of 3 pigs. Asterisk indicates paired t test p 0.05 vs control.

vascular smooth muscle cells CSE expression and activity are up-regulated by NO.[14] Since NO and H_2S have a key role in bladder neck inhibitory neurotransmission, the arrival of action potentials could favor NO and H_2S synthesis and release from motor nerve endings. Relaxations to electrical field stimulation or the H_2S donor GYY4137 showed high relaxant potency in the bladder neck vs that in rat aorta (pD_2 8 vs 4).[15] The fact that such relaxations were achieved in urothelium denuded bladder neck strips pretreated with the NO synthesis enzyme inhibitor L-NOARG suggests that H_2S produces smooth muscle relaxation via urothelium or NO independent mechanisms.[12]

In the pig bladder neck NO dependent neuronal relaxation is produced in part via guanylyl cyclase dependent mechanisms.[7] Like neuronal and endothelial NO synthase, CSE activity is Ca^2 calmodulin dependent.[16] H_2S generated from L-cysteine by CSE exerts its biological action by sulfhydrating target proteins, a process that may augment guanylyl cyclase activity, thus, increasing intracellular cyclic guanosine monophosphate and relaxing smooth muscle.[17] However, in the current study relaxations to electrical field stimulation or GYY4137 were not changed by the soluble guanylyl cyclase inhibitor ODQ. This result, together with the fact that our protocol was performed in the presence of the NO synthase inhibitor L-NOARG, rules out the mediation of cyclic guanosine monophosphate/NO dependent mechanisms in H_2S induced relaxation. These results are in accord with those previously described for vascular smooth muscle, in which H_2S relaxations are produced in a guanylyl cyclase activation independent way that is unlike the mechanism responsible for the vasodilator action induced by NO and CO.[18]

H_2S inhibits superoxide anion formation via the adenylyl cyclase-PKA pathway in pig pulmonary arterial endothelial cells.[19] In our series the lack of effect of the PKA inhibitor KT5720 on electrical field stimulation or GYY4137 induced responses seems to rule out involvement of the PKA pathway in H_2S relaxations.

K_{ATP} channel activation mediates H_2S induced relaxation in vascular[3,4] and visceral[20] smooth muscle. In the pig bladder neck relaxations to GYY4137 were decreased in 80 mM KPSS precontracted strips. Increases in extracellular [K] inhibit K efflux via membrane K channels and the K_{ATP} channel inhibitor glibenclamide decreased electrical field stimulation or GYY4137 relaxations. Therefore, ionic conductance modifications via K_{ATP} channels are probably involved in H_2S relaxations. The lack of effect shown by large, intermediate or small K_{Ca} channel blockade initially rules out the involve-

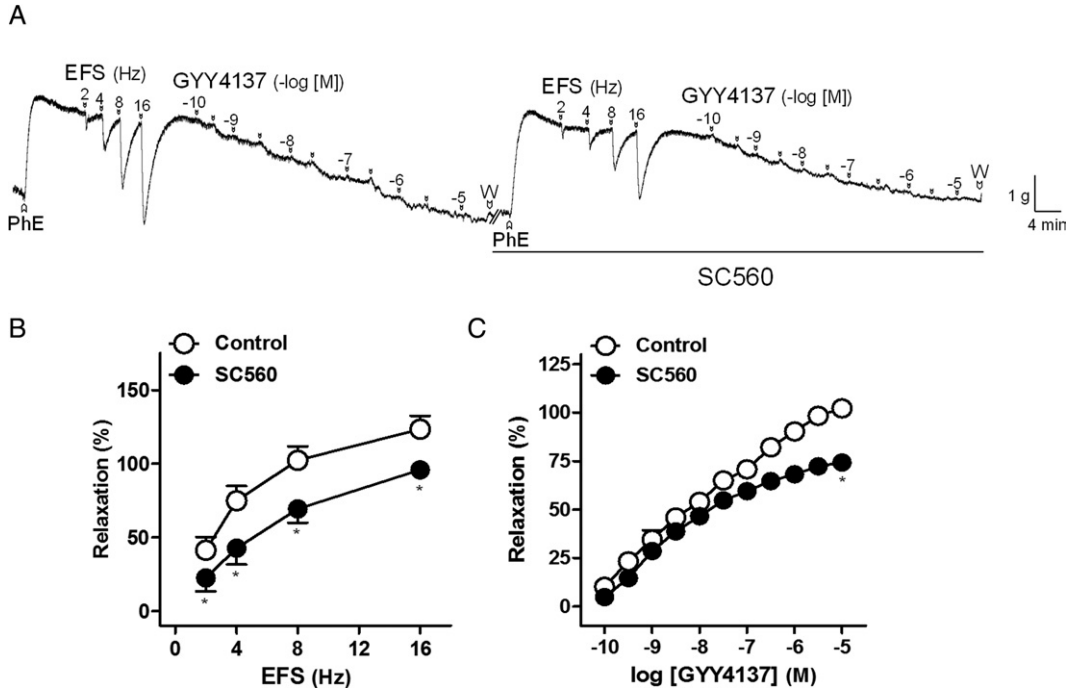

Figure 7. Isometric force recordings show relaxations evoked by 2 to 16 Hz electrical field stimulation (*EFS*) and 0.1 nM to 10 M GYY4137 in absence or presence of 1 M of selective COX-1 inhibitor SC560 on 1 M phenylephrine (*PhE*) precontracted pig bladder neck strips (*A*). *W*, wash. *g*, gm. Curves demonstrate frequency and concentration response relaxations to electrical field stimulation (*B*) and GYY4137 (*C*) in SC560 absence (*Control*) and presence. Results represent mean SEM of 6 preparations from total of 3 pigs. Asterisk indicates paired t test p 0.05 vs control.

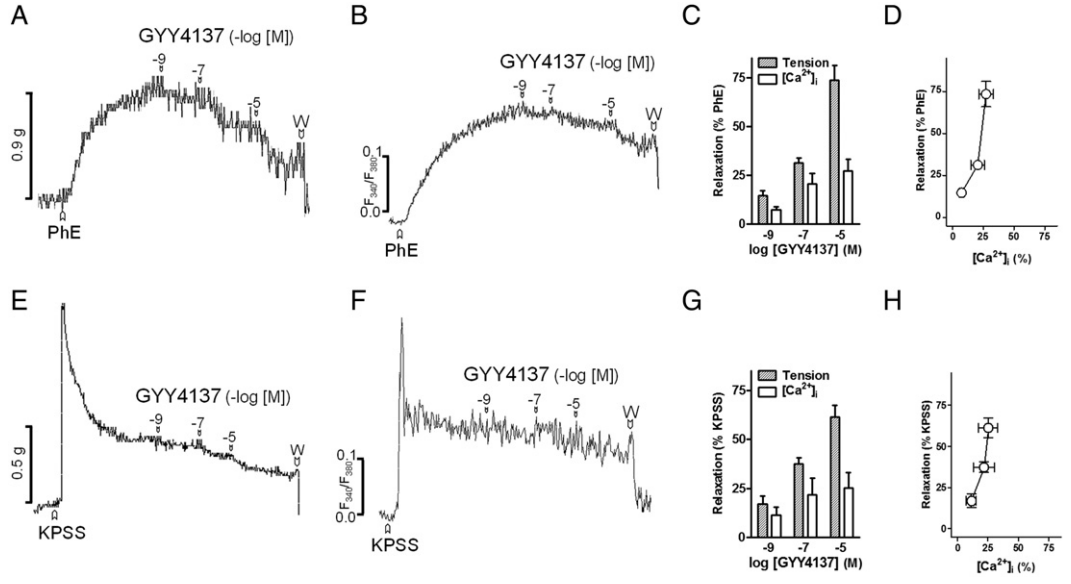

Figure 8. Simultaneous recordings of tension (*A* and *E*) and [Ca^2]$_i$, (*B* and *F*) reveal effects of cumulative doses of 1 nM, and 0.1 and 10 M of H_2S donor GYY4137 on 1 M phenylephrine (*PhE*) (*A* and *B*) or 80 mM KPSS (*E* and *F*) precontracted pig bladder neck strips. *g*, gm. Changes in tension and [Ca^2]$_i$ produced by GYY4137 on phenylephrine (*C*) and KPSS (*G*) induced contraction. Results are shown as mean SEM percent reversal of increases in tension and [Ca^2]$_i$ induced by phenylephrine or KPSS in each of 4 preparations from total of 4 pigs. Tension and [Ca^2]$_i$ relationships for GYY4137 of phenylephrine (*D*) and KPSS (*H*) precontracted bladder neck strips demonstrate Ca^2 desensitization mechanisms.

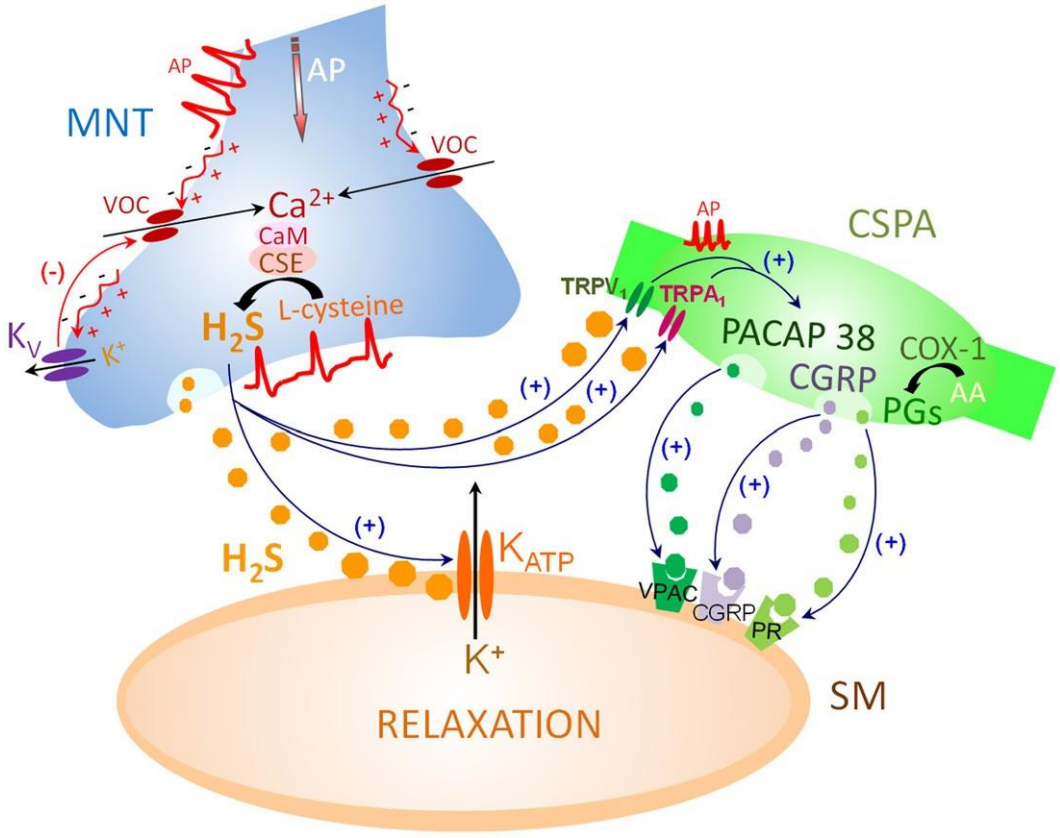

Figure 9. Proposed signaling of H_2S released from motor nerve terminal (*MNT*) in pig bladder neck. Arrival of action potentials (*AP*) at nerve ending evokes membrane depolarization and VOC channel activation with subsequent Ca^2 influx, which stimulates neural CSE via interaction with calmodulin (*CaM*), favoring H_2S synthesis from L-cysteine and release from nerves. H_2S diffuses across synaptic cleft, producing postjunctional K_{ATP} channel opening, membrane hyperpolarization and subsequent smooth muscle (*SM*) relaxation. H_2S promotes $TRPA_1$ and $TRPV_1$ channel activation from CSPAs, favoring release of $PACAP_{38}$, CGRP and prostaglandins (*PGs*) derived from arachidonic acid (*AA*) via COX-1 pathway. This produces smooth muscle relaxation by activating corresponding smooth muscle receptors. *PR*, progesterone receptor.

ment of these channels. In contrast, the potentiation of electrical field stimulation induced relaxation elicited by the K_v channel inhibitor 4-AP would be consistent with the presence of prejunctional K_v channels modulating the release of inhibitory neurotransmitters, as previously described.[7]

In vascular smooth muscle, in addition to K_{ATP} channel opening by H_2S, L-type VOC channels are subjected to direct modification by sulfhydryl reagents. This would support the notions that in the rat aorta H_2S induced vasorelaxation depends on extracellular Ca^2 entry,[21] and in rat cardiomyocytes H_2S and its donors are L-type Ca^2 channel inhibitors.[22] We investigated whether there might be GYY4137 inhibitory action on extracellular Ca^2 influx along with K_{ATP} channel activation. Results initially suggested the latter mechanism since GYY4137 produced bladder neck smooth muscle relaxation in preparations precontracted with K enriched solution, which promotes membrane depolarization with subsequent VOC channel activation. These relaxations were accompanied by simultaneous moderate decreases in smooth muscle $[Ca^2]_i$. These results agree with minor involvement of extracellular Ca^2 entry inhibition dependent mechanisms.

However, in our study most GYY4137 elicited relaxations were independent of decreases in $[Ca^2]_i$. Smooth muscle relaxation without a change in cytosolic $[Ca^2]$ is mediated through Ca^2 desensitization of contractile myofilaments. One of the most important Ca^2 desensitizing mechanisms is inhibition of the RhoA/Rho-kinase pathway, leading to MLCP activation.[23] MLCP independent Ca^2 desensitization mechanisms can also involve inhibitory phosphorylation of myosin light chain kinase by the activation of Ca^2 /calmodulin dependent protein kinase II.[24] Thus, in the bladder neck Ca^2 desensitization dependent mechanisms, probably via inhibition of one of these kinases, may be involved in GYY4137 induced relaxation. Our results agree with those in mouse gastric and colonic smooth mus-

cle, in which H_2S donors induce relaxation without significant changes in $[Ca^2]_i$ by Rho kinase dependent[25] and independent[26] regulation of MLCP activity.

H_2S donors produce rat detrusor contraction via the release of tachykinins, including substance P or neurokinin A, from CSPAs.[5] Sensory neuropeptides, such as $PACAP_{38}$[9] or CGRP,[10] are involved in bladder neck inhibitory neurotransmission. For this reason we investigated whether bladder neck H_2S induced relaxation could involve the release of sensory neuropeptides from CSPAs. The decrease in electrical field stimulation or GYY4137 relaxations caused by capsaicin, which produces functional blockade of CSPAs in the bladder neck,[10] and by the vanilloid receptor antagonist capsazepine would indicate that these responses are produced in part by inhibitory peptides released from CSPAs.

$TRPA_1$ is recognized as the main H_2S target in sensory neurons.[27] In our series the inhibition elicited by the $TRPA_1$ selective antagonist HC030031 on electrical field stimulation or GYY4137 responses suggests the involvement of these channels in H_2S relaxations. Moreover, the reduction in H_2S responses caused by blockading $TRPV_1$ with AMG9810 indicates that $TRPV_1$ channels are involved together with $TRPA_1$. Capsaicin inhibition of electrical field stimulation and GYY4137 relaxations was higher than that exerted by capsazepine, HC030031 and AMG9810. This suggests that other related ion channels on sensory neurons may be involved in H_2S relaxations, in addition to $TRPA_1$ and $TRPV_1$. The inhibition of electrical field stimulation and GYY4137 responses produced by blockading vasoactive intestinal peptide/PACAP and CGRP receptors suggests that H_2S promotes bladder neck smooth muscle relaxation via release of the sensory neuropeptides $PACAP_{38}$ and/or CGRP from CSPAs.

During the filling phase, the bladder shows local contractions and relaxations produced by basal myogenic mechanical activity, which may be increased by local release of COX derived prostanoids.[28] These spontaneous contractions can generate activity in afferent nerves, which may contribute to detrusor overactivity.[29] In rat detrusor $TRPV_1$ channel activation promotes the release of COX metabolites from sensory nerves, producing smooth muscle contraction.[30] In the bladder neck part of neurogenic relaxation is produced via prostanoid release from a COX-1 pathway.[11] For this reason we studied the possible involvement of a COX pathway in H_2S responses. In our study indomethacin, and SC 560, COX and COX-1 inhibitors reduced electrical field stimulation and GYY4137 elicited relaxations, respectively. This indicates that COX-1 derived prostanoids are involved in H_2S relaxations.

CONCLUSIONS

The current results suggest that H_2S produces smooth muscle relaxation of the pig bladder neck via K_{ATP} channel activation and $[Ca^2]_i$ desensitization dependent mechanisms with a minor role for extracellular Ca^2 influx inhibition. H_2S also promotes the release of inhibitory neuropeptides, such as $PACAP_{38}$ or CGRP, as well as COX-1 pathway derived prostanoids from CSPAs through the activation of $TRPA_1$, $TRPV_1$ and/or related ion channels in the sensory nerves (fig. 9).

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