Activation of K_{ATP} channels by H_2S in rat insulin-secreting cells and the underlying mechanisms

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H_2S is an important gasotransmitter, generated in mammalian cells from L-cysteine metabolism. As it stimulates K_{ATP} channels in vascular smooth muscle cells, H_2S may also function as an endogenous opener of K_{ATP} channels in INS-1E cells, an insulin-secreting cell line. In the present study, K_{ATP} channel currents in INS-1E cells were recorded using the whole-cell and single-channel recording configurations of the patch-clamp technique. K_{ATP} channels in INS-1E cells have a single-channel conductance of 78 pS. These channels were activated by diazoxide and inhibited by gliclazide. ATP (3 mM) in the pipette solution inhibited K_{ATP} channels in INS-1E cells. Significant amount of H_2S was produced from INS-1E cells in which the expression of cystathinonie gamma-lyase (CSE) was confirmed. After INS-1E cells were transfected with CSE-targeted short interfering RNA (CSE-siRNA) or treated with DL-propargylglycine (PPG; 1–5 mM) to inhibit CSE, endogenous production of H_2S was abolished. Increase in extracellular glucose concentration significantly decreased endogenous production of H_2S in INS-1E cells, and increased insulin secretion. After transfection of INS-1E cells with adenovirus containing the CSE gene (Ad-CSE) to overexpress CSE, high glucose-stimulated insulin secretion was virtually abolished. Basal K_{ATP} channel currents were significantly reduced after incubating INS-1E cells with a high glucose concentration (16 mM) or lowering endogenous H_2S level by CSE-siRNA transfection. Under these conditions, exogenously applied H_2S significantly increased whole-cell K_{ATP} channel currents at concentrations equal to or lower than 100 μM. H_2S (100 μM) markedly increased open probability by more than 2-fold of single K_{ATP} channels (inside-out recording) in native INS-1E cells ($n = 4$, $P < 0.05$). Single-channel conductance and ATP sensitivity of K_{ATP} channels were not changed by H_2S. In conclusion, endogenous H_2S production from INS-1E cells varies with *in vivo* conditions, which significantly affects insulin secretion from INS-1E cells. H_2S stimulates K_{ATP} channels in INS-1E cells, independent of activation of cytosolic second messengers, which may underlie H_2S-inhibited insulin secretion from these cells. Interaction among H_2S, glucose and the K_{ATP} channel may constitute an important and novel mechanism for the fine control of insulin secretion from pancreatic β-cells.

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Amounting evidence in recent years indicate that H_2S is an endogenous molecule of gas with important physiological functions (Wang, 2002). In the cardiovascular system, endogenous production of H_2S is mainly catalysed by cystathinonie gamma-lyase (CSE). Being a gasotransmitter, H_2S causes vasorelaxation at physiologically relevant concentrations (Zhao *et al.* 2001) and participates in the modulation of neuronal functions (Abe & Kimura, 1996). One of the mechanisms for cardiovascular actions of H_2S is activation of K_{ATP} channels. H_2S increased whole-cell K_{ATP} channel currents in rat aortic vascular smooth muscle cells (SMC) (Zhao *et al.* 2001). Similarly, in rat mesenteric artery SMCs H_2S at physiologically relevant concentrations stimulated K_{ATP} channels (Cheng *et al.* 2004). H_2S exerted a negative inotropic effect on heart. This effect was partially blocked by glibenclamide, a classical sulphonylurea K_{ATP} channel blocker (Geng *et al.* 2004). Although no electrophysiological recordings were performed on isolated cardiomyocytes, indication of involvement of K_{ATP} channels in cardiac effect of H_2S is noted.

K_{ATP} channels are sensitive to changes in intracellular ATP concentrations. Elevation of intracellular ATP level leads to closure of K_{ATP} channels in many metabolically

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active cells. In this way, the K_{ATP} channel is a coupling factor to link metabolic activity and membrane excitability. This feature is especially important for pancreatic β-cells. When circulating glucose level is elevated, glucose influx into pancreatic β-cells increases and so does ATP production. Consequential closure of K_{ATP} channels on plasma membrane depolarizes membrane and opens voltage-dependent calcium channels. The final eventuality of this chain reaction is increased insulin release due to increased intracellular free calcium. There is no doubt that K_{ATP} channels in β-cells are critical in regulation of glucose-induced insulin secretion (Cook *et al.* 1988; Ashcroft *et al.* 1989; Ashcroft & Gribble, 1998). However, beyond the regulatory role of glucose, via alteration of intracellular ATP level, on K_{ATP} channels, little is known about the existence of other endogenous regulators for K_{ATP} channels in pancreatic β-cells. By analogy to the stimulatory effect of H_2S on K_{ATP} channels in vascular SMCs, it is reasonable to believe that H_2S may be a novel K_{ATP} channel opener in pancreatic β-cells. To date, endogenous production of H_2S in the pancreas, effect of H_2S on insulin secretion, and interaction of H_2S with pancreatic K_{ATP} channels have not been determined.

In the present study, effects of H_2S on K_{ATP} channels in an insulin-secreting insulinoma cell line, INS-1E cells, were examined using the whole-cell and single-channel recording configurations of the patch-clamp technique. Endogenous levels of H_2S were either decreased by transfecting INS-1E cells with CSE-siRNA vector or dialysing the cells with a specific inhibitor for H_2S-generating enzyme, or increased by overexpressing CSE gene in INS-1E cells. Actual production of endogenous H_2S, expression level of H_2S-generating enzymes, and insulin secretion in INS-1E cells were determined. Our study characterized K_{ATP} channels in INS-1E cells, demonstrated the important regulatory role of H_2S on insulin secretion and pancreatic K_{ATP} channel activation for the first time, and revealed endogenous enzymatic production and metabolism of H_2S in insulin-secreting cells.

Methods

Cell culture

INS-1E cells derived from a rat insulinoma (kindly provided by Dr C. B. Wollheim, Geneva, Switzerland) were grown in a humidified (5% CO_2, 95% O_2) atmosphere for up to 2 days in Hepes-buffered RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol (Sigma), 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Sigma). For patch-clamp study, cultured INS-1E cells were placed in a Petri dish mounted on the stage of an inverted phase contrast microscope (Olympus IX70). For other biochemical and molecular biology assays, INS-1E cells were harvested and centrifuged at 500 g for 10 min after being rinsed twice with PBS solution.

Electrophysiological recording and analysis

Both whole-cell and single-channel recordings of K_{ATP} channel currents were performed as previously described (Cook & Hales, 1984; Zhao *et al.* 2001). Patch pipettes were pulled from borosilicate glass capillaries using a Flaming/Brown micropipette puller (Model P-87, Sutter Instruments). Pipette resistance was 8–12 MΩ for single-channel recordings and 1–5 MΩ for whole-cell experiments when filled with electrolyte solution. All electrophysiological recordings were performed at room temperature (20–24°C).

Whole-cell recordings were carried out with an Axopatch 200A patch clamp amplifier via a Digidata 1200 (Axon Instruments Inc.) interface, and analysed off-line using pCLAMP software (version 6.02; Axon Instruments Inc.). Test pulses of 600 ms were made with 10 mV increments from −150 to +50 mV. The holding potential was set at −20 mV. *I–V* relationships were constructed using stable current amplitude at the end of 600 ms test pulses. Pipettes were filled with a solution containing (mM): KCl 105, $MgCl_2$ 1.0, $CaCl_2$ 1.0, EGTA 10, and Hepes 10 (pH adjusted to 7.3 with KOH). Unless otherwise specified, ATP concentration of the pipette solution was 0.3 mM. The bath solution contained (mM): NaCl 102, KCl 40, $CaCl_2$ 1.0, $MgCl_2$ 1.2, glucose 4.5, and Hepes 10 (pH adjusted to 7.4 with NaOH). When glucose concentration was increased in some experiments, equimolar NaCl was removed to maintain osmolality of the bath solution. No leakage subtraction was performed to the original recordings and all cells with visible changes in leakage currents during the course of the study were excluded from further analysis.

For single-channel recording, inside-out configuration of the patch-clamp technique (Hamill *et al.* 1981) was used. Seal resistance was 10 GΩ. Single-channel currents were filtered at 1 kHz (eight-pole Bessel, −3 db) and recorded with 100 μs sampling interval in a gap-free model. Single-channel current records were displayed and analysed using pCLAMP 7.0 software (Axon Instruments Inc.). For each concentration of tested agents, at least 30 s of channel activity was directly recorded on computer hard disk. Open probability (NP_o), i.e. the fraction of time when the channels stay open within the total observation period with N representing the number of single channels in one patch (Wang & Wu, 1997) and single-channel conductance were determined from an all-point amplitude histogram using Fetchan and Pstat programs (Axon Instruments Inc.). The pipette solution contained (mM): KCl 140, $MgCl_2$ 1.2, EGTA 10 and Hepes 5 (pH adjusted to 7.2 with KOH). Inside-out patches were bathed in a solution

containing (mM): KCl 140, $MgCl_2$ 0.53, glucose 4.5, ATP 0.3, ADP 0.3 and Hepes 5 (pH adjusted to 7.4 with KOH).

Measurement of endogenous H_2S production

H_2S production rate was measured as previously described (Stipanuk & Beck, 1982) with modifications, which has been routinely used in our laboratory (Zhao *et al.* 2001, 2003; Cheng *et al.* 2004). Briefly, INS-1E cells cultured for 3–7 days were collected and homogenized in 50 mM ice-cold potassium phosphate buffer pH 6.8. The reaction mixture contained (mM): 100 potassium phosphate buffer pH 7.4, 10 L-cysteine, 2 pyridoxal 5′-phosphate, and 10% (w/v) homogenate. Cryovial test tubes (2 ml) were used as the centre wells, each containing 0.5 ml 1% zinc acetate as trapping solution and a filter paper 2 cm × 2.5 cm to increase air : liquid contacting surface. Reaction was performed in a 25 ml Erlenmeyer flask (Pyrex, USA). The flasks containing the reaction mixture and centre wells were flushed with N_2 before being sealed with a double layer of Parafilm. Reaction was initiated by transferring the flasks from ice to a 37°C shaking water bath. After incubating at 37°C for 90 min, 0.5 ml of 50% trichloroacetic acid was added to stop the reaction. The flasks were sealed again and incubated at 37°C for another 60 min to ensure a complete trapping of H_2S released from the mixture. Contents of the centre wells were then transferred to test tubes, each containing 3.5 ml of water. Subsequently, 0.5 ml of 20 mM *N*,*N*-dimethyl-*p*-phenylenediamine sulphate in 7.2 M HCl was added immediately followed by addition of 0.5 ml 30 mM $FeCl_3$ in 1.2 M HCl. Absorbance of the resulting solution at 670 nm was measured 20 min later with a spectrophotometer (Siegel, 1965). H_2S content was calculated against the calibration curve of standard H_2S solutions.

Measurement of insulin secretion from INS-1E cells

Native or transfected INS-1E cells were plated into 24-well plates at a density of 5×10^4 cells per well and tested 24–48 h later when cells reached about 80% confluence. Cells were maintained at 37°C for 2 h in glucose-free RPMI 1640 medium, washed and pre-incubated in glucose-free (0 mM) Krebs-Ringer-bicarbonate medium (pH 7.4) containing (mM): 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 10 Hepes and 0.1% BSA. After 30 min pre-incubation, cells were incubated for 30 min at 37°C in the presence of different glucose concentrations. At the end of each incubation period, the medium was collected and centrifuged for 10 min at 2500 *g* to remove cell debris. The supernatant was immediately stored at −20°C until insulin determination using the rat insulin ELISA kit (Mercodia AB, Sylveniusgatan, Uppsala, Sweden).

Western immunoblotting

Cultured cells were harvested and lysed in a lysis buffer (EDTA 0.5 M; Tris-Cl 1 M, pH 7.4; sucrose 0.3 M; antipain hydrochloride 1 μg ml^{-1}; benzamidine hydrochloride hydrate 1 M; leupeptin hemisulphate 1 μg ml^{-1}; 1,10-phenanthroline monohydrate 1 M; pepstatin A 1 μM; plenylmethylsulphonyl fluoride 0.1 mM, and iodoacetamide 1 mM). Extracts were separated by centrifugation at 14 000 g for 15 min at 4°C. SDS-PAGE and Western blot analysis were performed as previously described (Yang *et al.* 2004*a*). Briefly, equal amount of proteins were boiled in 1 × SDS sample buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) and resolved on a 10% SDS-PAGE gel, and transferred onto polyvinylidene chloride (PVDC) nitrocellulose membranes. Dilutions for the primary antibodies were 1 : 1000 for CSE, and 1 : 5000 for β-actin. HRP-conjugated secondary antibody was used at 1 : 5000. Immunoreactions were visualized by enhanced chemiluminescence (ECL) and exposed to X-ray film (Kodak Scientific Imaging film). Membranes were stripped by incubating in a buffer containing 100 mM β-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 6.8).

CSE-siRNA transfection of INS-1E cells

CSE-targeted 21 nucleotide siRNA was designed using a web based siRNA design program (http://www.ambion.com/techlib/misc/siRNA_finder_html) according to the AA-N19 rule (Brummelkamp *et al.* 2002; Lake & Castellot, 2003). The targeted sequence was localized at a position 192 bases downstream of the start codon of CSE (GenBank Accession No. NM001902). Forward (ggu uau uua ucc ugg gcu g dtdt) and reverse (cag ccc agg cua aau aac c dtdt) RNA strands with two 5′ deoxy-thymidine overhangs were commercially synthesized by Ambion (Austin, TX, USA). GADPH-targeted siRNA was also produced for optimizing transfection conditions. Negative control siRNA, a 21 nucleotide RNA duplex with no sequence homology with all known genes, was also purchased from Ambion. Transfection of siRNA into INS-1E cells was achieved using the siPORT lipid transfection agent from Ambion. Briefly, cells were plated overnight to form 60–70% confluent monolayers. CSE siRNA at 30 nM and the transfection reagent complex were added to cells in serum-free medium for 4 h. Fresh normal growth medium was then added and cells were incubated for another 20 h. As a control, negative siRNA was used to transfect INS-1E cells.

Construction of recombinant CSE adenovirus vector and infection of INS-1E cells

A PCR was performed to amplify opening read frame (ORF) of CSE (GenBank accession number AB052882) from reverse-transcribed rat vascular tissue using a set of primers: 5′-CGTCCCAGCATGCAGAAGAA-3′ and 5′-CAGTTATTCAGAAGGTCTGGCCC-3′. The amplified ORF of CSE was ligated into PCR2.1 vector (Invitrogen), and the *Kpn*I–*Xho*I restriction fragment of CSE was subcloned into the *Kpn*I–*Xho*I sites of the shuttle vector pAdShuttle-CMV (Qbiogene, Inc.), which contains cytomegalovirus promoter/enhancer element and simian virus 40 polyadenylation signals. Positive clone containing CSE ORF insert was sequenced to confirm the accuracy of the inserted CSE sequence. The resultant plasmid was linearized with *Pme*I and cotransformed with the adenovirus backbone vector pAdeasy-1 into *E. coli* BJ5183 cells by electroporation. Homologous recombinants containing CSE cDNA were detected by restriction endonuclease digestion and agarose gel electrophoresis. Recombinant CSE adenovirus vector (Ad-CSE) was then transformed into *E. coli* DH5α cells for large-scale amplification. The *Pac*I-digested E1-deleted replication-deficient Ad-CSE vector was then transfected into mammalian HEK-293 cells using calcium phosphate–DNA precipitates. The recombinant Ad-CSE was expanded, purified and titrated (He *et al.* 1998). The recombinant adenovirus encoding bacterial β-galactosidase (Ad-lacZ) derived from the same vector was used as a control. For adenoviral infection, subconfluent INS-1E cells were incubated with Ad-CSE or Ad-lacZ in serum-free media. After 4 h of incubation, media was removed, and cells were incubated in appropriate media for 48 h. The transfection efficiency of adenoviral vector in INS-1E cells was first determined by infecting cells with Ad-lacZ at various multiplicities of infection (MOI). The cells infected with Ad-lacZ were assayed for β-galactosidase expression by the *in situ* X-gal staining method (Hirooka & Sakai, 2004). At MOI $\geq$ 50, > 90% of cells showed nuclear staining for β-galactosidase. Subsequent experiments were performed at MOI of 50.

Real-time RT-PCR determination of the transcriptional level of CSE

Native untransfected cells, negative siRNA- or CSE siRNA-transfected cells were harvested from 100 mm culture dishes 24 h after transfection. Monolayers were rinsed twice with PBS, and total RNA was collected using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA). Contaminating DNA was avoided using the DNA-free kit (Ambion), and total RNA (2 μg) was reverse-transcribed into cDNA with AMV reverse transcriptase using random hexamer primers according to the manufacturer's protocol (Roche Applied Science, IN, USA). Controls containing no reverse transcriptase were used to safeguard for genomic DNA contamination in each sample.

Real-time PCR was performed in an iCycler iQ apparatus (Bio-Rad, Harcules, CA, USA) associated with the iCycler optical system software (version 3.1) using the SYBR Green PCR Master Mix. All PCRs were performed in a 20 μl volume using 96-well optical grade PCR plates and optical sealing tape. Negative controls for this experiment were samples without a template. Cycling conditions were 95°C for 90 s followed by 38 cycles of 95°C for 10 s and 60°C for 20 s. For quantification, the target gene was normalized to the internal standard gene β-actin. A standard curve was constructed with a series of dilutions of total RNA (Ambion) transcribed to cDNA using the protocol outlined above to confirm the same amplifying efficiency in PCR. A standard melting curve analysis was performed using a thermal cycling profile that began at 95°C for 1 min, decreased to 55°C for 1 min, and then ramped to 95°C in 1°C increments to confirm the absence of primer dimers. Product size was determined by running PCR products on a 1.8% agarose gel. Relative mRNA quantification was calculated by using the arithmatic formula '$2^{-\Delta\Delta CT}$', where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference cDNA (Yang *et al.* 2004*a*). Thus, this value yields the amount of the target normalized to an endogenous reference.

Chemicals and statistical analysis

H_2S-saturated solution (0.09 M) was freshly made by bubbling pure H_2S gas (Praxair; Mississauga, Canada) into Krebs' solution at 30°C for 40 min as previously described (Zhao *et al.* 2001, 2003; Cheng *et al.* 2004). At 37°C and pH 7.4, the concentration of H_2S in solution was relatively stable (Zhao *et al.* 2003). Data are expressed as mean ± S.E.M. Multiple comparisons were made with one-way ANOVA followed by a *post hoc* analysis (Tukey test). Statistical significance was set at $P < 0.05$.

Results

Endogenous H_2S production and insulin secretion from INS-1E cells

To investigate whether cultured INS-1E cells produced H_2S under different *in vivo* conditions, INS-1E cells were incubated with either 5 or 20 mM glucose for 24 h. Increase in extracellular glucose concentration from 5 to 20 mM significantly decreased endogenous production of H_2S in INS-1E cells by about 46% (Fig. 1*A*). Similar

inhibition of H_2S production in INS-1E cells was also observed with 16 mM glucose (not shown). To further examine whether this glucose-mediated endogenous H_2S production was regulated by specific enzymatic process, DL-propargylglycine (PPG), a selective inhibitor of CSE, was used. Lysed INS-1E cells were mixed with PPG to facilitate interaction of this inhibitor with cytosolically located CSE, and then H_2S production was assayed. It was found that PPG significantly inhibited H_2S production in INS-1E cells (Fig. 1*A*).

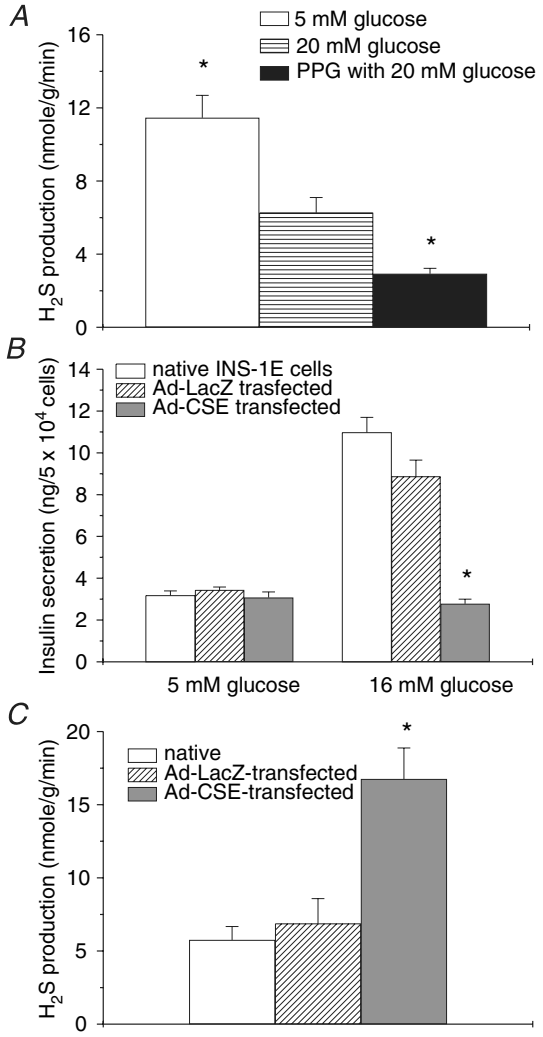


Figure 1. H_2S production and insulin secretion in INS-1E cells
A, glucose-mediated H_2S production in INS-1E cells. Endogenous production of H_2S was significantly decreased by high glucose concentration in the incubation medium. PPG (5 mM) treatment of INS-1E cells significantly reduced endogenous H_2S production. $^*P < 0.05$ *versus* other groups. $n = 4$ for each group. *B*, glucose-stimulated insulin secretion from INS-1E cells with Ad-CSE transfection to increase endogenous H_2S level. $^*P < 0.05$ *versus* native or Ad-LacZ-transfected INS-1E cells in the presence of 16 mM glucose. $n = 6$–8 for each group. *C*, production of H_2S in Ad-CSE-transfected INS-1E cells. Cells were cultured with 16 mM glucose in the medium. $^*P < 0.05$ *versus* other groups. $n = 4$–6 for each group.

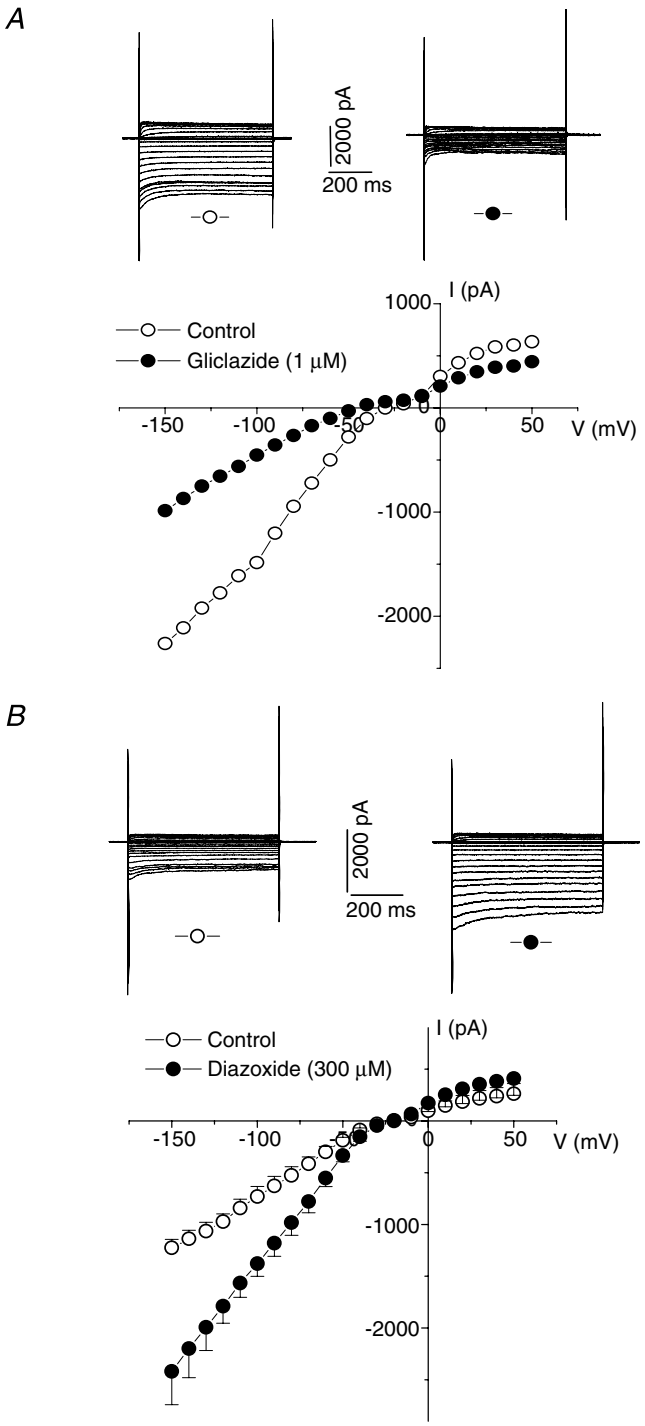


Figure 2. Pharmacological sensitivities of whole-cell K_{ATP} channels in INS-1E cells
Holding potential, −20 mV. *A*, inhibition of the whole-cell K_{ATP} channels by gliclazide in INS-1E cells. Representative K_{ATP} channel currents (top panel) and the mean *I–V* relationship of whole-cell K_{ATP} channels (bottom panel, $n = 5$) before and after the application of gliclazide at 1 μM. *B*, stimulation of whole-cell K_{ATP} channels by diazoxide in INS-1E cells. Representative K_{ATP} channel currents (top panel) and the mean *I–V* relationships of K_{ATP} channels (bottom panel, $n = 4$) before and after application of diazoxide at 0.3 mM.

High glucose affected insulin secretion from INS-1E cells. With an increased glucose concentration from 5 to 16 mM, insulin secretion from INS-1E cells became 3-fold greater (Fig. 1*B*). After transfection of INS-1E cells with Ad-CSE to overexpress CSE, insulin secretion at basal level (5 mM glucose) was not altered. However, high glucose-stimulated insulin secretion was virtually abolished (Fig. 1*B*). Significantly increased production of endogenous H_2S from Ad-CSE-transfected INS-1E cells is shown in Fig. 1*C*. Native INS-1E cells were also transfected with Ad-LacZ as a negative control. Neither basal nor high glucose-stimulated insulin secretion from INS-1E cells were altered by Ad-LacZ transfection (Fig. 1*B*).

Characterization of K_{ATP} channel in INS-1E cells

Gliclazide is a specific blocker of K_{ATP} channels in pancreatic β-cells (Trube *et al.* 1986; Ashcroft, 2000). Gliclazide (1 μM) decreased significantly whole-cell K_{ATP} currents in INS-1E cells from -1049.9 ± 115 to 522.8 ± 88 pA at -100 mV ($n = 5$, $P < 0.05$). Representative results of the effect of gliclazide on whole-cell K_{ATP} channels are shown in Fig. 2*A*. Diazoxide is a potent opener of K_{ATP} channels in pancreatic β-cells (Sturgess *et al.* 1988; D'hahan *et al.* 1999). Whole-cell K_{ATP} channels in INS-1E cells were significantly stimulated by diazoxide (Fig. 2*B*). Whole-cell K_{ATP} channels in INS-1E cells were also characterized by their sensitivity to intracellular ATP. K_{ATP} channel currents were 82.9 ± 8.6 pA pF^{-1} (–120 mV) with 0.3 mM ATP in the pipette solution ($n = 6$). When the ATP concentration was increased to 3 mM, K_{ATP} channel currents were reduced to 29.7 ± 5.6 pA pF^{-1} (–120 mV) ($n = 5$, $P < 0.05$ *versus* 0.3 mM ATP in the pipette solution).

With symmetrical K^+ (140 mM) in the pipette and bath solutions, K_{ATP} channels in INS-1E cells had a single-channel conductance of 78 ± 2.3 pS ($n = 5$) (Fig. 3*A*). These single-channel K_{ATP} currents appeared in rapid open–close transitions and in brief bursts. Open probability of single K_{ATP} channels at -60 mV was 0.08 ± 0.01 ($n = 6$). When gliclazide (1 μM) was added to the bath, activity of the single channel was greatly decreased with open probability reduced to 0.004 ± 0.001 ($n = 5$, $P < 0.05$) (Fig. 3*B*). When diazoxide (100 μM)

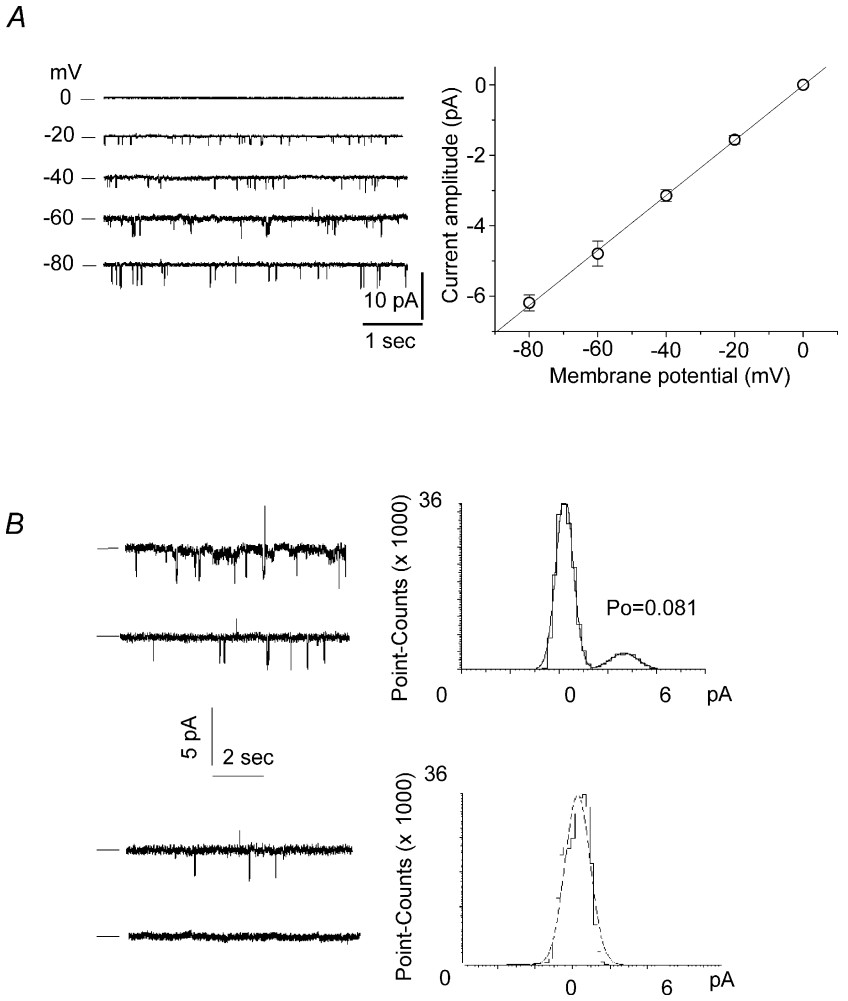
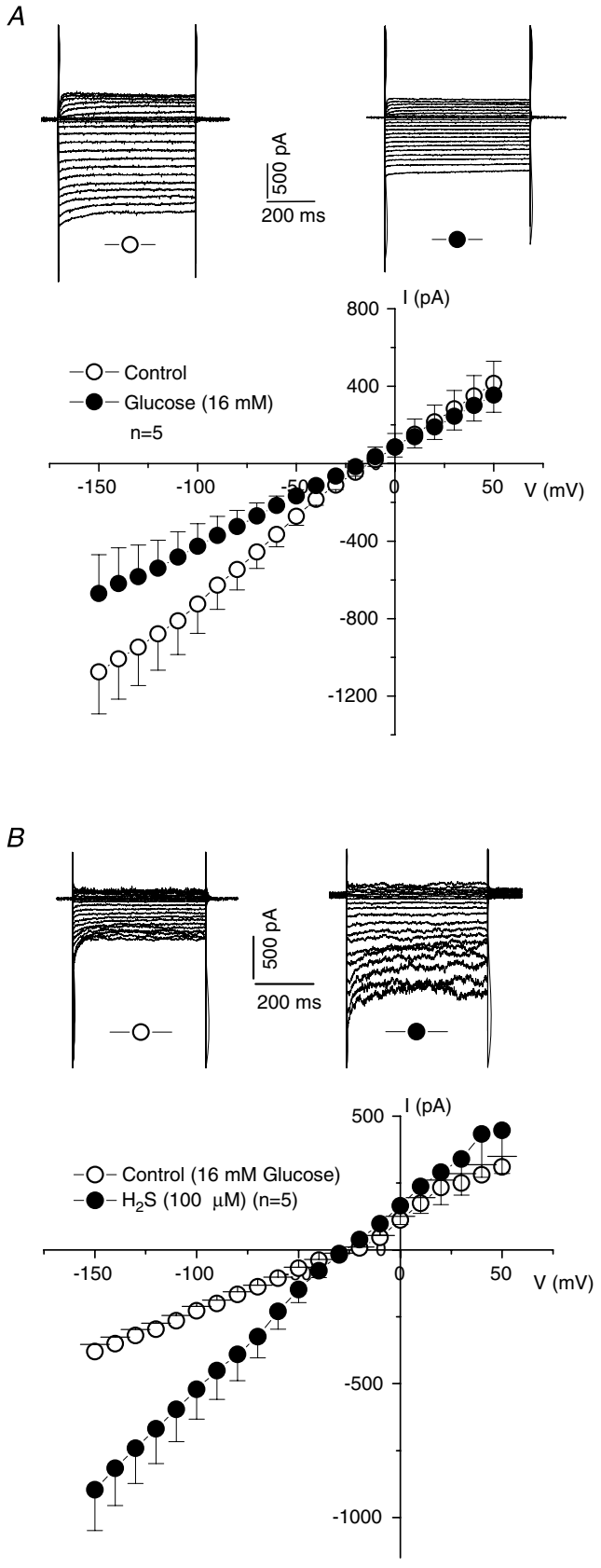


Figure 3. Single-channel characteristics of K_{ATP} channels recorded from inside-out membrane patches of INS-1E cells
A, representative single-channel K_{ATP} channel currents in one inside-out membrane patch (left panel) and the single-channel conductance of K_{ATP} channels (right panel, $n = 5$). *B,* open probabilities of single K_{ATP} channels in the absence (upper panel) and then presence (lower panel) of gliclazide (1 μM). Membrane potential, −60 mV.



was applied to inside-out patches, single-channel activity was greatly increased with open probability of single K_{ATP} channels changing from 0.09 ± 0.01 to 0.28 ± 0.03 ($n = 5$, $P < 0.05$). However, there was no significant change in amplitude of unitary inward current after application of diazoxide. Unitary current amplitudes of single-channel K_{ATP} currents were 4.68 ± 0.09 and 4.70 ± 0.06 pA (–60 mV) before and after application of diazoxide, respectively ($n = 5$, $P > 0.05$).

Effects of H_2S on K_{ATP} channels in INS-1E cells

K_{ATP} channels in INS-1E cells were also sensitive to glucose stimulation. After glucose concentration of the bath solution was changed from 5 mM to 16 mM, whole-cell K_{ATP} currents were significantly reduced (Fig. 4*A*). With 5 mM glucose in the bath solution, H_2S at 100 μM had no effect on whole-cell K_{ATP} currents in INS-1E cells ($n = 5$, $P > 0.05$). In the presence of 16 mM glucose, application of H_2S (100 μM) to INS-1E cells significantly increased K_{ATP} currents (Fig. 4*B*). Application of DL-dithothreitol (DTT) (3 mM) to INS-1E cells for 5 min did not significantly change K_{ATP} channel currents in INS-1E cells (79 ± 2.69 *versus* 86 ± 2.68 pA pF^{-1} at -100 mV, $n = 5$, $P > 0.05$). A lack of effect of DTT on K_{ATP} channels has also been reported previously in pancreatic β-cells (Islam *et al.* 1993; Krippeit-Drews *et al.* 1994). Since DTT is a reducing reagent, our result suggests that the stimulatory effect of H_2S on K_{ATP} channels in INS-1E cells is unlikely to be mediated by a general reducing effect.

To examine whether K_{ATP} channels in INS-1E cells were desensitized at resting conditions by high endogenous H_2S levels, we tried to lower endogenous H_2S level by pre-treating these cells with PPG. In the presence of PPG (5–10 mM) in the bath solution containing 5 mM glucose, H_2S at 100 μM did not alter the whole-cell K_{ATP} currents in INS-1E cells (812 ± 63 *versus* 796 ± 81 pA at -120 mV, $n = 5$, $P > 0.05$). In the next group of experiments, PPG (1 mM) was included in the pipette solution to dialyse cells. This manoeuver alone significantly reduced K_{ATP} channel currents by $40 \pm 9.6\%$ ($n = 5$,

Figure 4. Effects of glucose concentrations on basal K_{ATP} channel currents and interaction of H_2S with K_{ATP} channels in INS-1E cells

Holding potential, −20 mV. *A*, inhibitory effect of glucose on the whole-cell K_{ATP} channels in INS-1E cells. Representative K_{ATP} channel current traces (top panel) and the mean *I–V* relationships (bottom panel) of K_{ATP} channels with different glucose concentrations in the bath solutions. $n = 5$ for each group. $^*P < 0.05$. *B*, interaction of H_2S with whole-cell K_{ATP} channels in the presence of high glucose (16 mM) in the bath solution. Representative K_{ATP} channel current traces were shown in the top panel and the mean *I–V* relationships in the bottom panel of K_{ATP} channels in INS-1E cells before and after H_2S application. $n = 5$ for each group.

$P < 0.05$). More interestingly, including PPG in the pipette solution unmasked a dose-dependent stimulatory effect of exogenously applied H_2S on K_{ATP} channels (Fig. 5*A*). Even at a concentration of 10 μM, H_2S increased K_{ATP} channel currents by $69.4 \pm 5.7\%$ after PPG treatment ($n = 4$, $P < 0.05$, -120 mV) (Fig. 5*C*). Effects of H_2S on K_{ATP} channels were not different with either 1 or 5 mM PPG in the pipette solution (Fig. 5*A* and *B*), indicating that even at 1 mM PPG might already significantly inhibit CSE. CSE protein expression was confirmed in INS-1E cells with Western blot analysis (Fig. 6*A*). To further demonstrate that the preconditioning effect of intracellular PPG was related to reduced endogenous CSE activity, cultured INS-1E cells were transfected with CSE-siRNA to knock down endogenous expression of CSE gene. While negative-siRNA transfection did not alter translational or transcriptional expression levels of CSE, CSE-siRNA transfection reduced expression of the CSE gene by about 80% (Fig. 6*A* and *B*). In line with suppressed CSE gene expression, endogenous production of H_2S from CSE-siRNA-transfected INS-1E cells was significantly reduced in comparison with that of native INS-1E cells (Fig. 6*C*).

Whole-cell K_{ATP} channel current density was significantly smaller in CSE knock-down INS-1E cells

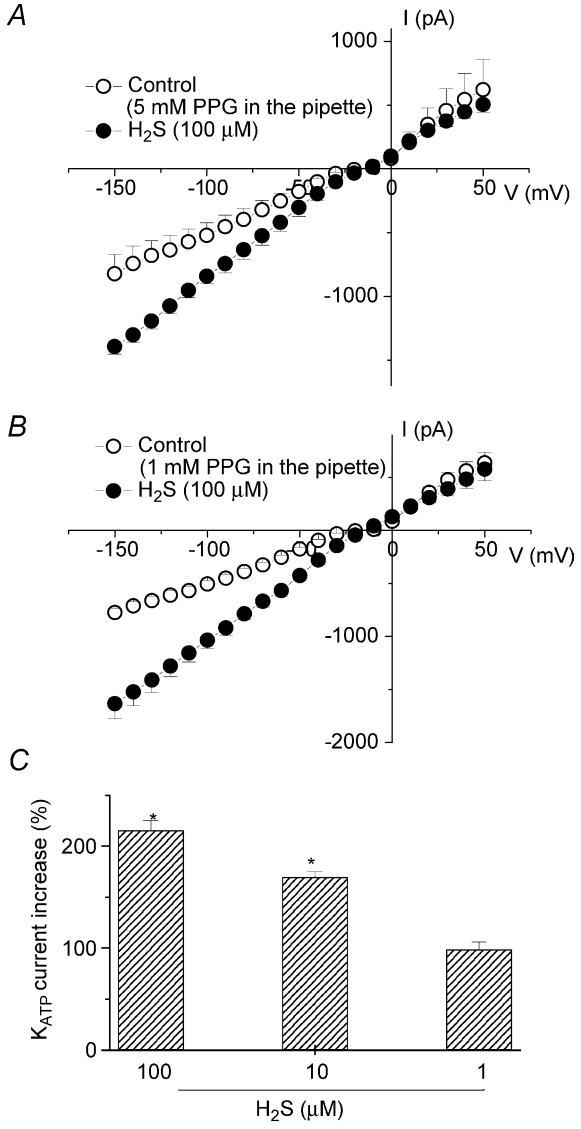


Figure 5. Stimulatory effect of H_2S on the whole-cell K_{ATP} channels of INS-1E cells in the presence of PPG in the pipette solution

A, bath application of H_2S at 100 μM increased inward K_{ATP} channel currents in INS-1E cells, which were dialysed with 5 mM PPG. $n = 4$. *B,* bath application of H_2S at 100 μM increased inward K_{ATP} channel currents in INS-1E cells, which were dialysed with 1 mM PPG. $n = 5$. *C*, the concentration-dependent stimulatory effects of H_2S on K_{ATP} channels with 1 mM PPG in the pipette solution. $n = 5$–6 for each group. $^*P < 0.05$ *versus* the recordings in the absence of H_2S.

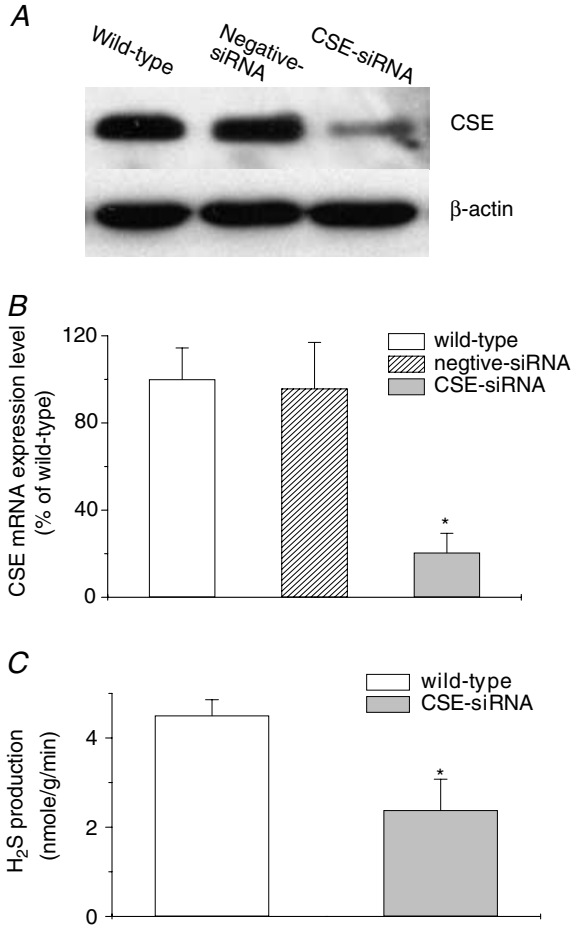


Figure 6. Expression of CSE gene and production of H_2S in cultured INS-1E cells

A, Western blot detection of CSE protein expression in INS-1E cells. *B*, real-time RT-PCR comparison of CSE expression levels in INS-1E cells with and without siRNA transfection. $n = 4$. $^*P < 0.01$. *C*, endogenous production of H_2S from INS-1E cells with or without transfection with CSE-siRNA. $n = 3$–5 for each group. $^*P < 0.05$ *versus* native INS-1E cells.

(26.8 ± 1.9 pA pF^{-1}, $n = 4$) than in native untransfected cells (46.4 ± 3.9 pA pF^{-1} at -120 mV, $n = 5$). Exogenous H_2S at 100 μM significantly increased whole-cell K_{ATP} channel currents in CSE-siRNA-transfected cells (Fig. 7). For example, an increase of $67.6 \pm 5.6\%$ in K_{ATP} channel currents was induced by H_2S at -120 mV ($n = 5$). Furthermore, it was found that PPG did not alter the function of single K_{ATP} channel in inside-out patches. Open probability of single K_{ATP} channels was 0.079 ± 0.004 after the application of PPG (1 mM) to the cytosolic side of inside-out patches, which was not different from that in the absence of PPG (0.083 ± 0.001 at -50 mV, $n = 4$, $P > 0.05$). This observation supports the notion that the inhibitory effect of PPG on K_{ATP} channels was mediated by CSE in the cytosolic milieu.

With the inside-out single-channel recording configuration, exposure of K_{ATP} channels in the excised membrane patch to endogenous H_2S was minimized. As such, desensitization of K_{ATP} channels in insulin-secreting cells by endogenous H_2S can be better confirmed. In this recording configuration, application of H_2S at low concentration (100 μM) significantly stimulated K_{ATP} channels (Fig. 8*A*). Open probability of single K_{ATP} channels was increased from 0.08 ± 0.002 to 0.26 ± 0.05 ($n = 4$, $P < 0.01$) and the closed time of single channels decreased from 189 ± 7 to 59 ± 6 ms ($n = 4$, $P < 0.01$) by 100 μM H_2S. However, H_2S did not change the open time of single channels, nor single-channel conductance of K_{ATP} channels. Effect of H_2S on single-channel activity at various membrane potentials was also examined. The linear current–voltage relation of single-channel currents was not changed by H_2S (100 μM) with a single-channel conductance of 78 ± 2.2 pS ($n = 6$) (Fig. 8*B*). To examine whether H_2S altered ATP sensitivity of K_{ATP} channels, ATP concentrations at the cytosolic side of inside-out patches were changed from 300 μM to 30 and 3 μM. Although open probability of single K_{ATP} channels was increased as ATP concentration decreased, H_2S increased open probability of K_{ATP} channels to the same degree with different ATP levels (Fig. 9). It appears that H_2S directly acts on K_{ATP} channel proteins, rather than altering ATP sensitivity of K_{ATP} channels.

Discussion

In recent years, the physiological importance of H_2S has gained increasingly recognition. Produced in many types of mammalian cells, H_2S modulates neuronal activities (Abe & Kimura, 1996), protects the heart from ischaemic damages (Geng *et al.* 2004), and participates in regulation of cellular apoptosis and proliferation (Yang *et al.* 2004*a*, *b*). One of the recognized mechanisms for cellular effects of H_2S is activation of K_{ATP} channels by this gasotransmitter, especially in vascular SMCs (Zhao *et al.* 2001, 2003; Cheng *et al.* 2004). K_{ATP} channels play a central role in regulating the function of insulin-secreting cells by coupling metabolic change with insulin secretion via changes in membrane potential (Cook *et al.* 1988; Ashcroft & Rorseman, 1989; Ashcroft & Gribble, 1998). However, little information is available on endogenous regulation of pancreatic K_{ATP} channels except the known effects of extracellular glucose and intracellular ATP. Interaction of H_2S with pancreatic K_{ATP} channels has not been investigated to date. It is reasoned that H_2S may also stimulate K_{ATP} channels in pancreatic β-cells as occurs in vascular smooth muscle cells. It should be aware that molecular composition of K_{ATP} channels is different among different cell types. For example, in pancreatic β-cells the K_{ATP} channel complex is composed of the pore-forming inwardly rectifying K^+ channel tetramer Kir6.2 and regulatory sulphonylurea receptors SUR1 (Saskura *et al.* 1995; Ashcroft, 1996; Gribble *et al.* 1997; Lorenz *et al.* 1998). In vascular smooth muscle cells,

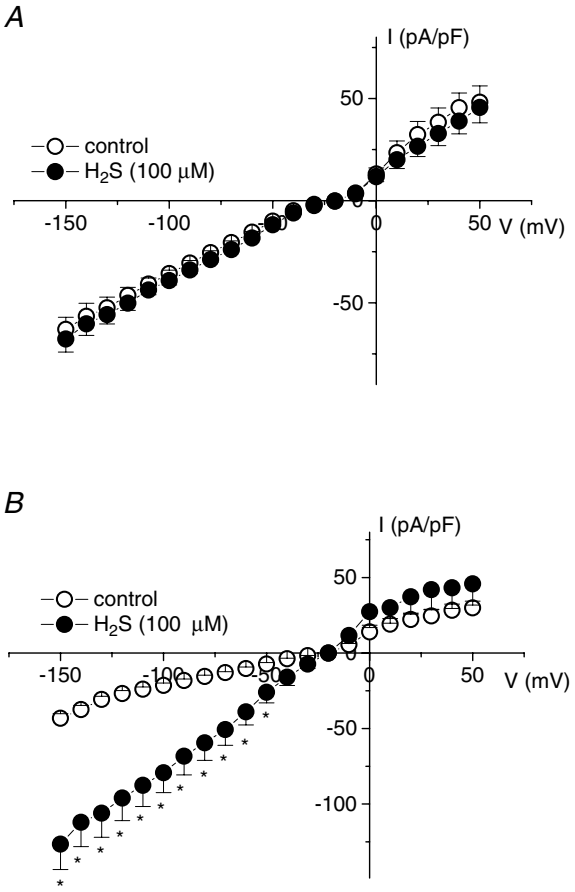
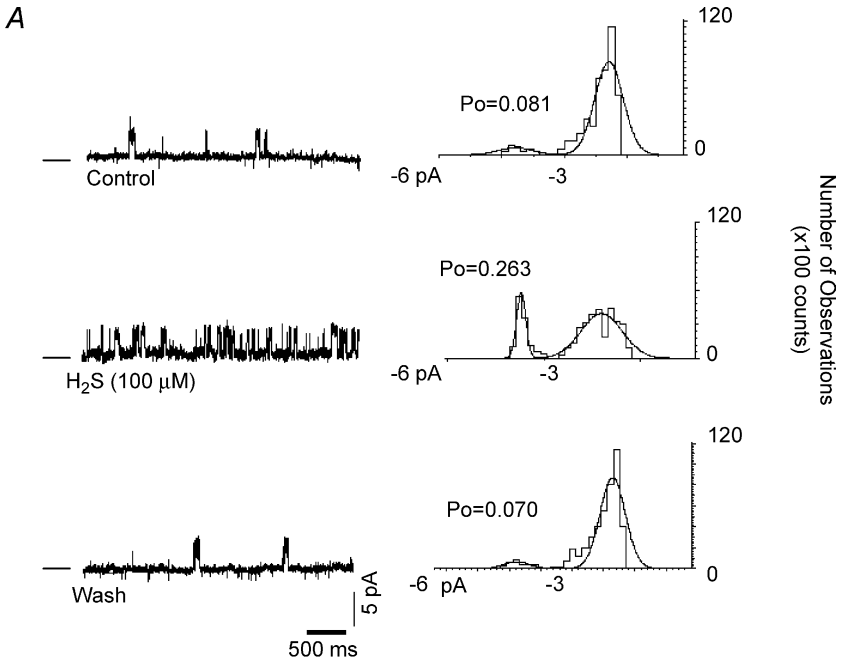


Figure 7. Effects of H_2S on the whole-cell K_{ATP} channels in CSE-siRNA-transfected INS-1E cells
A, H_2S (100 μM) had no effect on K_{ATP} channels in INS-1E cells transfected with negative siRNA. $n = 4$. *B*, H_2S (100 μM) significantly increased K_{ATP} channel currents in INS-1E cells transfected with CSE-siRNA. $n = 5$. $^*P < 0.01$.

however, K_{ATP} channel complex is combination of Kir 6.1 and SUR2B (Yokoshiki *et al.* 1998; William & Odle, 2003). In this context, interaction of H_2S with K_{ATP} channels in different cell types needs to be specifically investigated.

We recorded a 78 pS K_{ATP} channel in INS-1E cells. This single-channel conductance is typical of K_{ATP} channels reported in pancreatic β-cells (Ashcroft & Gribble, 1998). Other characteristics of K_{ATP} channels, including burst opening, ATP sensitivity, glucose sensitivity, voltage insensitivity and gliclazide sensitivity are all similar to those described in native pancreatic β-cells (Mukai *et al.* 1998). Moreover, 0.3 mM Mg-ATP was required for sustaining K_{ATP} channel currents in our study, a property shared by SUR1/KIR6.2 type of K_{ATP} channels (Inagaki *et al.* 1995; Gribble *et al.* 1997). Sulphonylureas such as gliclazide stimulate insulin secretion by closing K_{ATP} channels (Sturgess *et al.* 1985; Trube *et al.* 1986; Ashcroft, 2000). Potassium channel openers such as diazoxide inhibit insulin secretion by opening K_{ATP} channels (Trube *et al.* 1986; Dunne *et al.* 1987; Sturgess *et al.* 1988; Minami *et al.* 2003). In our study, diazoxide significantly increased, and gliclazide inhibited, K_{ATP} channel activity in INS-1E cells. K_{ATP} channels in INS-1E cells were also inhibited by high glucose concentration in the bath solution (16 mM) or ATP (3 mM) in the pipette solution. These features are hallmarks of K_{ATP} channels in insulin-secreting cells (Cook & Hales, 1984; Ashcroft & Rorseman, 1989; Ashcroft & Gribble, 1998). Insulin secretion and K_{ATP} channel



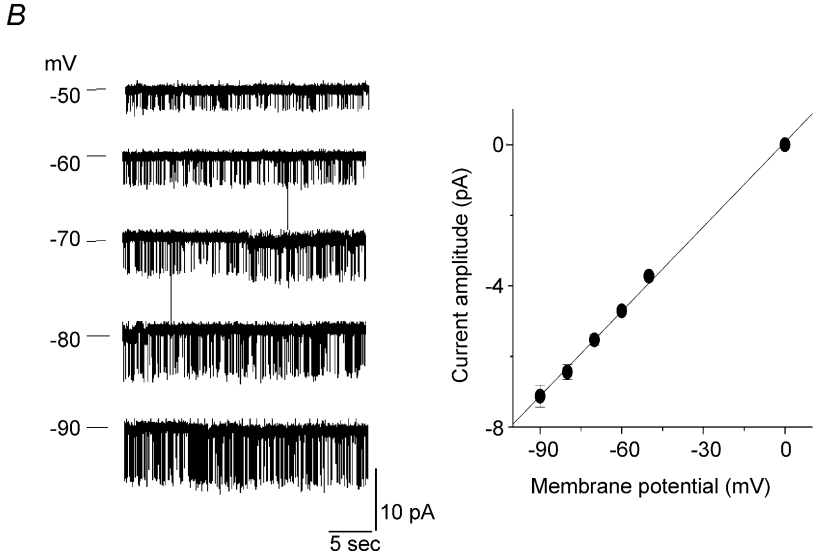


Figure 8. Activation of single K_{ATP} channels in INS-1E cell by H_2S, recorded in inside-out configuration with symmetrical 140 mM KCl solutions
A, changes in open probability of single K_{ATP} channels induced by H_2S. The close states of the channel are indicated by a bar beside each record. Membrane potential, +60 mV. Changes in opening probability (P_o) are shown in the right panel. *B*, the effect of H_2S on the single-channel conductance of K_{ATP} channels in INS-1E cells. Representative original records of K_{ATP} channels in the presence of H_2S (100 μM) are shown in the left panel. The *I–V* relationship of K_{ATP} channels under these conditions is shown in the right panel. $n = 6$ for each data point.

functionality in this way respond to changes in glucose levels. These pharmacological and biophysical properties indicate that K_{ATP} channels in INS-1E cells share the same characteristics with those in pancreatic β-cells (Ashcroft & Gribble, 1998; Mukai *et al.* 1998).

INS-1E cells are derived from an insulinoma pancreatic β-cell line (Janjic *et al.* 1999; Merglen *et al.* 2004). These cells exhibit stable differentiated β-cell phenotype, and secrete insulin in response to glucose and non-nutrient secretagogues via the stimulation of K_{ATP} channels and a minor amplifying pathway (Merglen *et al.* 2004). Significant amounts of H_2S were produced by INS-1E cells. We have achieved a partial knockdown of the CSE gene in INS-1E cells using the CSE-siRNA technique. Since this partial knockdown significantly reduced the production of H_2S, it is believed that CSE is the main enzyme for the production of H_2S in INS-1E cells. More importantly, our study demonstrated that this endogenous H_2S production in INS-1E cells was mediated by a variance in glucose concentrations, thus providing physiological regulatory mechanisms for H_2S production. Functional correlation of H_2S levels in INS-1E cells is realized by insulin secretion from these cells. By reducing endogenous H_2S production in INS-1E cells, high glucose also stimulated insulin secretion. Furthermore, over-expression of the CSE gene in INS-1E cells via Ad-CSE infection significantly increased endogenous H_2S production, thus inhibiting high glucose-stimulated insulin secretion.

In the present study, we demonstrated for the first time that H_2S activated K_{ATP} channels in INS-1E cells. Without manipulating the endogenous H_2S level, exogenous H_2S at concentrations equal to or lower than 100 μM had no effect on whole-cell K_{ATP} channels in INS-1E cells. This phenomenon might be explained as K_{ATP} channels in INS-1E cells were desensitized to endogenous H_2S at resting conditions. When INS-1E cells were incubated with a high concentration of glucose (16–20 mM), these cells became sensitive to exogenous H_2S that significantly increased K_{ATP} channel activity at 100 μM. This sensitizing effect of high glucose concentration on K_{ATP} channels could be linked to a high glucose-induced decrease in endogenous H_2S production. This hypothesis was further verified by directly inhibiting CSE activity in INS-1E cells. When PPG (1–5 mM) was used to dialyse INS-1E cells to inhibit CSE and endogenous production of H_2S, whole-cell K_{ATP} channel currents were greatly increased by exogenously applied H_2S in a concentration-dependent manner. Another line of evidence supporting conditioning of K_{ATP} channels in INS-1E cells by endogenous H_2S was derived from single-channel recording studies. Exogenous H_2S significantly increased open probability of single K_{ATP} channels in inside-out membrane patches of INS-1E cells. With this cell-free recording configuration, substrates and enzymes for endogenous H_2S production are eliminated. Therefore, it is highly possible that K_{ATP} channels in insulin-secreting cells have been desensitized by a high level of endogenous H_2S at resting conditions. Removal or reduction of endogenous H_2S would re-sensitize K_{ATP} channels so that these channels regain their sensitive response to H_2S. We propose that the endogenous H_2S level has one switch for turning on or off K_{ATP} channels in insulin-secreting cells, whereas the glucose level regulates endogenous H_2S production. Under physiological conditions with low extracellular glucose (~5 mM), endogenous H_2S level is high, which would keep K_{ATP} channels mostly in their open state, thus hyperpolarizing the membrane of insulin-secreting cells. This will result in low activity of voltage-dependent calcium channels and low secretion of insulin from insulin-secreting cells. When the glucose concentration of plasma is elevated, endogenous H_2S production in insulin-secreting cells is

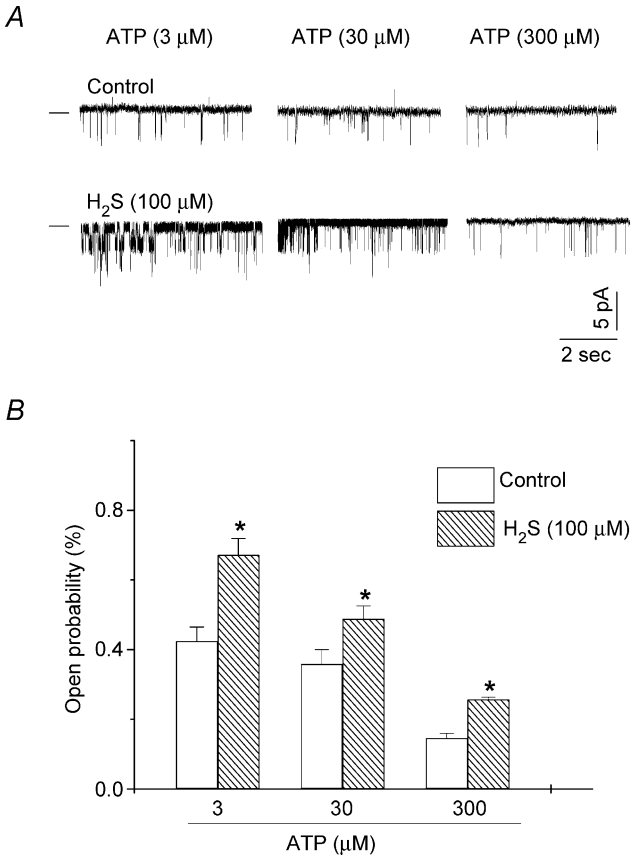


Figure 9. The stimulatory effect of H_2S on K_{ATP} channels in INS-1E cells was not related to ATP sensitivity of these channels
Single K_{ATP} channel currents in inside-out membrane patches ($n = 4$) were recorded with symmetrical 140 mM KCl solutions. *A*, representative single K_{ATP} channel currents in inside-out membrane patches before and after H_2S (100 μM) of the bath solution at −60 mV membrane potential with different concentrations of ATP. Bar beside each trace indicates the closed state of the single channel. *B*, increasing concentrations of ATP in the cytosolic side of the membrane patches reduced open probability of single K_{ATP} channels, but did not alter the relative stimulatory effect of H_2S. $^*P < 0.05$ *versus* control.

decreased. Consequent closure of K_{ATP} channels leads to increased insulin secretion. In our previous studies, it has been shown that the vasorelaxant effect of H_2S was not mediated by any known second messengers, including cGMP, cAMP and PKC pathways (Zhao *et al.* 2001, 2003; Zhao & Wang, 2002). In the present study, we showed that H_2S directly activates K_{ATP} channels in cell-free inside-out membrane patches. We also showed that ATP sensitivity of K_{ATP} channels was not changed by H_2S. Taken together, these observations suggest that the interaction of H_2S and K_{ATP} channels is not mediated by cytosolic second messengers. As a reducing agent, H_2S may reduce selective cysteine residues of K_{ATP} channel protein, altering its functional status. However, application of a classical reducing agent (DTT) to INS-1E cells did not replicate the excitatory effect of H_2S on K_{ATP} channels, suggesting that other mechanisms should be sought to explain the interaction of H_2S with the K_{ATP} channel complex in a tissue-/cell type-specific manner.

In summary, K_{ATP} channels in insulin-secreting INS-1E cells share many common features with their counterparts in native pancreatic β-cells. H_2S increased the activity of these K_{ATP} channels by increasing single-channel open probability, but not single-channel conductance. An endogenous high level of H_2S in INS-1E cells sets up the tune for K_{ATP} channel activity and thus insulin-secreting level at resting conditions. An increase in extracellular glucose concentration lowers endogenous H_2S level. This will have two effects. Firstly, activity of K_{ATP} channels in INS-1E cells will be significantly reduced so that insulin secretion will be increased. Secondly, K_{ATP} channels in INS-1E cells will be partially closed and re-sensitized to H_2S. Subsequent changes in the endogenous H_2S level would exert a much greater effect on the functional status of K_{ATP} channels under these conditions. Interaction among H_2S, glucose, and K_{ATP} channels in insulin-secreting cells may constitute an important and novel mechanism for the fine control of insulin secretion from pancreatic β-cells, which is initially triggered by changes in glucose concentration, under physiological and different pathophysiological conditions.

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