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Elies, J, Johnson, E, Boyle, JP et al. (2 more authors) (2015) H2S does not regulate proliferation via T-type Ca2+ channels. Biochemical and Biophysical Research Communications, 461 (4). pp. 659-664. ISSN 0006-291X

https://doi.org/10.1016/j.bbrc.2015.04.087

H_2S does not regulate proliferation via T-type Ca^{2+} channels

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Running title: Hydrogen sulfide, proliferation and T-type Ca^{2+} channels

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ABSTRACT

T-type Ca^{2+} channels (Cav3.1, 3.2 and 3.3) strongly influence proliferation of various cell types, including vascular smooth muscle cells (VSMCs) and certain cancers. We have recently shown that the gasotransmitter carbon monoxide (CO) inhibits T-type Ca^{2+} channels and, in so doing, attenuates proliferation of VSMC. We have also shown that the T-type Ca^{2+} channel Cav3.2 is selectively inhibited by hydrogen sulfide (H_2S) whilst the other channel isoforms (Cav3.1 and Cav3.3) are unaffected. Here, we explored whether inhibition of Cav3.2 by H_2S could account for the anti-proliferative effects of this gasotransmitter. H_2S suppressed proliferation in HEK293 cells expressing Cav3.2, as predicted by our previous observations. However, H_2S was similarly effective in suppressing proliferation in wild type (non-transfected) HEK293 cells and those expressing the H_2S insensitive channel, Cav3.1. Further studies demonstrated that T-type Ca^{2+} channels in the smooth muscle cell line A7r5 and in human coronary VSMCs strongly influenced proliferation. In both cell types, H_2S caused a concentration-dependent inhibition of proliferation, yet by far the dominant T-type Ca^{2+} channel isoform was the H_2S-insensitive channel, Cav3.1. Our data indicate that inhibition of T-type Ca^{2+} channel-mediated proliferation by H_2S is independent of the channels' sensitivity to H_2S.

Key words:

proliferation; T-type calcium channel; gasotransmitter; vascular smooth muscle; hydrogen sulfide

1. INTRODUCTION

In recent years ion channels have emerged as a major family of target proteins for modulation by the gasotransmitters carbon monoxide (CO) and hydrogen sulfide (H_2S) [1-4]. Indeed, many beneficial and detrimental actions of these gases involve ion channel modulation [5-7]. One particularly important cellular process that involves ion channel activity and is also modulated by gasotransmitters is proliferation: in the vasculature, for example, vascular smooth muscle cells (VSMCs) can undergo phenotypic change, becoming non-contractile, proliferative cells to adapt to varying physiological and pathological situations [8-10]. This is important not only in developmental vasculogenesis and vascular repair but also in the development of cardiovascular diseases [8; 11; 12]. Progression of cancers is also dependent on profound cellular proliferation [13].

Interestingly, induction of heme oxygenase-1 (HO-1), which generates CO along with biliverdin and iron from the degradation of heme, is associated with proliferative vascular diseases [14; 15] and much evidence suggests that CO accounts for the known anti-proliferative effects of HO-1 in VSMCs [16-18]. HO-1 is also constitutively expressed in various types of cancer, where it may regulate proliferation and resistance to apoptosis, in part through formation of CO [19; 20]. By contrast, the effects of H_2S on proliferation appear to be cell-type specific; *In vitro* studies have shown that H_2S donors such as NaHS slow proliferation of VSMCs [21] yet can increase endothelial cell proliferation [22], and in some forms of cancer, such as colon cancer, H_2S promotes proliferation [23].

Ca^{2+} influx into cells is a requirement for proliferation as it regulates the activity of key transcription factors such as NFAT (nuclear factor of activated T-cells), via Ca^{2+}-dependent dephosphorylation by calcineurin [24]). The relative importance of different Ca^{2+} influx pathways contributing to proliferation are currently under investigation but there is compelling evidence for the involvement of voltage-gated T-type Ca^{2+} channels: in VSMCs, T-type Ca^{2+} channel expression increases during proliferation [25; 26], and they are required for VSMC proliferation both *in vitro* and in neointima formation observed following vascular injury [26-30]. In numerous forms of cancer high expression of T-type Ca^{2+} channels has been observed and, as in VSMCs, their expression supports proliferation [31]. These channels therefore represent an important therapeutic target for the treatment of both proliferative vascular diseases and cancer.

We have previously reported that CO is an effective inhibitor of all three isoforms of T-type Ca^{2+} channels (Cav3.1-3.3; [32]). Further evidence indicates that HO-1 induction suppresses VSMC

proliferation via CO-mediated inhibition of T-type Ca^{2+} channels [5]. More recently, we have demonstrated that H_2S can also inhibit T-type Ca^{2+} channels, but differs from CO in that it discriminates between subtypes; it is only effective in inhibiting Cav3.2, whilst Cav3.1 and Cav3.3 are unaffected by this gasotransmitter [33]. Given the known effects of H_2S on proliferation and the important involvement of T-type Ca^{2+} channels in this process, we have explored the possibility that H_2S, like CO, may regulate proliferation via inhibition of T-type Ca^{2+} channels.

2. METHODS

2.1 Cell culture

HEK293 cells: Wild type (WT; untransfected) HEK293 cells were cultured in minimum essential medium containing Earle's salts and L-glutamine, and supplemented with 10% (v/v) foetal bovine serum (FBS; Biosera, Ringmer UK), 1% (v/v) non-essential amino acids, 1% (v/v) antibiotic/antimycotic, and 0.1% (v/v) gentamicin. HEK293 cells stably expressing Cav3.1 and Cav3.2 T-type Ca^{2+} channels (a kind gift from Prof. E. Perez-Reyes; University of Virginia, Virginia USA), were cultured in WT HEK293 media, additionally supplemented with 1mg/ml G-418 to maintain selection pressure (All reagents from Gibco, Paisley UK; unless otherwise stated). HEK293/Cav3.2 cells were used at passages between P1 and P8, and WT HEK293 cells were used at passages between P1 and P6.

A7r5 cells (a smooth muscle cell line derived from rat thoracic aorta) were obtained from the European Collection of Cell Cultures (ECACC, Public Health England, Porton Down UK). They were grown in A7r5 complete media, consisting of Dulbecco's minimum essential medium containing 10% FBS (Biosera, Ringmer UK) and 1% glutamax (Gibco, Paisley UK).

Human coronary artery smooth muscle cells (hCASMCs) were obtained from ECACC (350-05a, Public Health England, Porton Down UK). They were grown in smooth muscle growth medium-2 supplemented with 5% FBS, growth factors (0.001% hEGF, 0.001% insulin, 0.002% hFGF-B) and gentamicin/amphotericin-B as described by manufacturers (Clonetics™ from Lonza, Germany). HCASMC were used at passages between P1 and P5.

All cell types were cultured in a humidified incubator at 37˚C (95% air: 5% CO_2) and passaged weekly.

2.2 Proliferation Assay

Cells were plated at $1x10^4$ / well and allowed to adhere for 6 hours in 24-well plates in complete growth media, then exposed to serum free medium (SFM) overnight. On day 0 of the assay, SFM was removed and replaced with 1ml of the relevant complete test media (vehicle or drug at the required concentration). To count cells, media was removed, cells were washed with 1ml of

Dulbecco's phosphate buffered saline (PBS) and 200µl of 0.05% trypsin-EDTA (Gibco, Paisley UK) was added (pre-warmed to 37˚C). Post-incubation, 800µl of complete media was added and the cell suspension centrifuged (600*g* for 6 minutes). Following removal of 950µl of media, 50µl of supernatant remained with the cell pellet, which was then re-suspended following addition of 50µl of 0.4% Trypan Blue (Thermo Scientific, Rockford USA) to exclude non-viable cells.
Media was retained from one well of each treatment, processed in the same manner as the cell samples, and any cells present were counted as an additional quantification of non-viable cells. Day 0 counts and media counts were performed using a hemocytometer. All other counts were performed using a TC10 Automated Cell Counter (Bio-Rad, Hemel Hempstead UK). Repeated counting from both test medium and trypsin suspension showed that no cells were lost in the counting procedure.

2.3 Real-Time Polymerase Chain Reaction (RT-PCR)

To determine mRNA expression levels of $Ca_v3.2$ and $Ca_v3.1$ channels, T75 flasks containing cells at 70-80% confluence were washed with PBS and cells dissociated using 0.5ml 0.05% trypsin-EDTA for 3 minutes (37˚C; 95% air: 5% CO_2). Enzyme activity was halted by adding 0.5ml ice-cold PBS. The cell suspension was then centrifuged (600*g* for 6 min) and RNA was generated from whole cell lysates using the Aurum Total RNA Mini Kit (Bio-Rad, Hemel Hempstead UK) following manufacturer's instructions. A cDNA template was generated from RNA samples using the iScript cDNA Synthesis Kit (Bio-Rad, Hemel Hempstead UK) following manufacturer's instructions (Reaction profile: 5 minutes at 25˚C, 30 minutes at 42˚C, 5 minutes at 85˚C, 5 minutes at 4˚C). Human Taqman probes (Applied Biosystems (ABI), UK) for $Ca_v3.1$ (CACNA1G), $Ca_v3.2$ (CACNA1H), and the endogenous housekeeper hypoxanthine phosphoribosyltransferase (HPRT1) were used with hCASMC. In all cases, 2µl of sample cDNA and 18µl of RT-PCR reaction mix (10µl Taqman Universal PCR Master Mix, 0.5µl Taqman probes (both from ABI), and 7.5µl RNase/DNase-free water (Gibco Cambridge UK)) was added to the required wells of a 96-well PCR plate (Applied Biosystems, Cambridge UK). RT-PCR was carried out using an ABI 7500 Real-Time PCR system (Reaction profile: 2 minutes at 50˚C, 10 minutes at 95˚C, 15 seconds at 95˚C for 60 cycles, 1 minute at 60˚C). Data were analysed using the 7500 software (ABI) and relative gene expression calculated using the $2^{-\Delta\Delta CT}$ method with HPRT1 as the endogenous control.

2.4 Electrophysiology

Ca^{2+} currents were recorded from A7r5 cells using the whole-cell configuration of the patch-clamp technique at room temperature (21-24ºC) as previously described [32] using an Axopatch 200A amplifier/Digidata 1300 interface controlled by Clampex 9.0 software (Molecular Devices, Sunnyvale, CA, USA). Offline analysis was performed using Clampfit 9.0. Pipettes (4–6 MΩ) were filled with (in mM): CsCl 120, $MgCl_2$ 2, EGTA 10, TEA-Cl 20, HEPES 10, Na-ATP 2, pH 7.2

(adjusted with CsOH). To optimise recording of T-type Ca^{2+} currents, cells were perfused with (in mM): NaCl 95, CsCl 5, $MgCl_2$ 0.6, $CaCl_2$ 15, TEA-Cl 20, HEPES 5, D-glucose 10, sucrose 30, pH 7.4 (adjusted with NaOH). Cells were voltage-clamped at -80mV and either repeatedly depolarized to -20mV (200ms, 0.1Hz) or to a series of test potentials ranging from -100mV to +60mV. All currents were low-pass filtered at 2kHz and digitised at 10kHz.

2.5 Data presentation and statistical analysis

Proliferation data are plotted example growth curves (with s.e.m., as each was performed in triplicate) and bar graphs representing normalised mean (with s.e.m.) proliferation on the final day of assessment, determined in at least 3 identical experiments. Statistical comparisons were made using ANOVA with Dunnett's post-hoc test.

RESULTS AND DISCUSSION

There is overwhelming evidence that H_2S is an important modulator of both physiological and pathological cardiovascular function. In addition to its tonic, physiological role as a regulator of blood pressure [34], it is also a major modifier of tissue remodelling resulting from cardiovascular diseases. Thus, for example, cardiac arteriolar hypertrophy and interstitial fibrosis observed in spontaneously hypertensive rats was prevented by daily administration of the H_2S donor NaHS [35]. Furthermore, neointima formation and VSMC proliferation following carotid artery balloon injury was suppressed following chronic NaHS administration [36]. Interestingly, this study also demonstrated that expression of cystathionine γ-lyase (CSE), the major vascular enzyme producing H_2S, was inhibited by balloon injury, a finding in agreement with its down-regulation in hypertension [37]. Since (a) the expression of T-type Ca^{2+} channels increases in VSMC proliferation [25; 26], (b) they are a prerequisite for VSMC proliferation and neointima formation following vascular injury [26-30] and (c) we have recently demonstrated that H_2S regulates the activity of the T-type Ca^{2+} channel Cav3.2 [33], the present study was conducted in order to investigate whether the inhibitory effects of H_2S on proliferation might be mediated via T-type Ca^{2+} channel inhibition. Our investigation was prompted not only by the importance of H_2S and T-type Ca^{2+} channels in VSMC proliferation, but also by the fact that CO, another gasotransmitter known to inhibit VSMC proliferation, appears to act in this way via inhibition of T-type Ca^{2+} channels [5].

Proliferation was firstly monitored in HEK293 cells over-expressing the H_2S-sensitive T-type Ca^{2+} channel Cav3.2 [33]. Over a 4 day period, the increase in cell number was reduced in a concentration-dependent manner by H_2S (applied as the donor NaHS; Fig. 1A), consistent with the known ability of H_2S to inhibit this class of T-type Ca^{2+} channel. As previously described [5], the rate of proliferation observed in Cav3.2-expressing cells in the absence of applied H_2S was

much greater than that observed in wild-type (WT; untransfected) HEK293 cells (Fig. 1B, plotted on the same Y axis scale as Fig. 1A for comparison). However, further reductions in this modest rate of proliferation were observed in WT cells in the presence of NaHS (Fig. 1B); these effects are more apparent when WT proliferation is plotted on a more restricted scale (Fig. 1C). Indeed, the degree of inhibition of proliferation caused by NaHS was not significantly different between WT and Cav3.2-expressing HEK 293 cells (Fig. 1D), as compared on day 4. This finding suggests that H_2S may not in fact inhibit proliferation specifically through inhibiting this class of T-type Ca^{2+} channel. To explore this possibility further, we examined proliferation in HEK293 cells stably expressing the H_2S insensitive T-type Ca^{2+} channel, Cav3.1 [33]. Our previous studies have indicated that current densities in the Cav3.1 and Cav3.2-expressing HEK293 cells are similar in magnitude (ca. 50-100pA/pF [32; 38]) and T-type currents are not detectable in WT cells (data not shown). Proliferation in these Cav3.1-expressing cells was rapid and monitored over a 3 day period. As shown in Fig. 2, H_2S also reduced proliferation in these cells in a concentration-dependent manner.

To explore any potential modulation of native T-type Ca^{2+} channels in VSMCs, and how this might impact on proliferation, we first explored its action in the rat aortic smooth muscle cell line, A7r5. We have previously shown that T-type (and not L-type) Ca^{2+} channels regulate proliferation in these cells [5] and, consistent with this, we found that the T-type Ca^{2+} channel inhibitor mibefradil inhibited proliferation in a concentration-dependent manner (Fig. 3A). Exposure of cells to NaHS similarly reduced proliferation in a concentration-dependent manner (Fig. 3B). Our recent work has suggested that A7r5 cells express both Cav3.1 and Cav3.2, but by far the predominant channel was Cav3.1, as determined via RT-PCR [5]. To examine directly whether T-type currents could be modulated by H_2S in A7r5 cells, we recorded whole-cell Ca^{2+} currents under conditions designed to optimise T-type Ca^{2+} channel resolution (see [5] and Methods). Under these conditions, NaHS was without effect on T-type currents, as exemplified by Fig. 3B, and quantified in Fig. 3C. We never observed significant modulation of currents (n = 5 cells at each concentration examined). These findings strongly suggest that the ability of H_2S to inhibit A7r5 proliferation does not occur via its ability to inhibit T-type Ca^{2+} channels expressed in these cells.

We also explored modulation of proliferation in human coronary artery smooth muscle cells (hCASMCs). Consistent with a role for T-type Ca^{2+} channels in proliferation, we found that Ni^{2+} caused a concentration dependent reduction in hCASMC proliferation, as monitored over 4 days (Fig. 4A). In three repeated experiments (not shown) Ni^{2+} only significantly ($P<0.01$) reduced proliferation at $\geq$ 100μM, suggesting the involvement of Cav3.1 rather than Cav3.2, since Cav3.2 is much more sensitive to Ni^{2+} [39]. In agreement with this suggestion, we next examined the relative expression of mRNA for the T-type Ca^{2+} channel isoforms, $Ca_v3.1$ and $Ca_v3.2$, using RT-

PCR. In three separate experiments, the $Ca_v3.1$ isoform was expressed at significantly higher levels than the $Ca_v3.2$ isoform, but both isoforms were detected (Fig. 4B; note different scales for each isoform). Despite the predominant expression of the H_2S insensitive channel Cav3.1, proliferation was reduced in a concentration-dependent manner by NaHS exposure (Fig. 4C). In three repeated experiments (not shown) the effects of NaHS were significant ($P<0.001$) at 100μM and 300μM. The data in both A7r5 and hCASMCs are consistent with the idea that H_2S suppresses proliferation independently of T-type Ca^{2+} channel modulation.

Our findings confirm and extend previous awareness that T-type Ca^{2+} channel activity promotes proliferation, as observed when over-expression of either Cav3.1 or Cav3.2 increases HEK293 cell proliferation (Figs 1 and 2), as we and others have shown previously [5; 40]. We also confirm that T-type Ca^{2+} channels modulate proliferation in A7r5 cells, and provide new data suggesting a similar role in hCASMCs (Fig. 4). At present, the mechanism by which T-type Ca^{2+} channels, specifically, can promote proliferation is not understood. In each cell type studied, we also demonstrate that H_2S inhibits proliferation, consistent with a previous report in rat aortic A10 smooth muscle [21]. Our results are also consistent with the observation that VSMCs isolated from $CSE^{-/-}$ mice show increased proliferation compared to wild type (WT) VSMCs, further indicating that H_2S has a 'breaking' effect on proliferation [41]. The major and unexpected finding of the present study, however, is that H_2S appears to suppress proliferation independent of any action on T-type Ca^{2+} channels. Thus, although the inhibition of proliferation in Cav3.2 expressing HEK293 cells by H_2S (Fig. 1) is consistent with its ability to inhibit this pro-proliferative channel, a similar degree of inhibition was also observed in WT cells which did not express Cav3.2. Furthermore, H_2S also inhibited proliferation of Cav3.1-expressing HEK293 cells (Fig. 2), despite the fact that this channel is insensitive to H_2S [33], and in A7r5 and hCASMCs, where the dominant T-type Ca^{2+} channel expressed is Cav3.1. It remains to be determined how H_2S suppresses proliferation, regardless of whether the proliferation is augmented by T-type Ca^{2+} channels.

The present data collectively suggest that, although CO can directly modulate VSMC proliferation via regulation of T-type Ca^{2+} channels, H_2S clearly differs in the means by which it exerts the same effect, despite its ability to inhibit at least one subtype of T-type Ca^{2+} channel. Thus, although ion channels represent a large and growing family of target proteins through which gasotransmitters exert their numerous, diverse biological activities, these agents clearly target additional signalling pathways with similar, important biological outcomes.

ACKNOWLEDGEMENTS

This work was supported by the British Heart Foundation.

FIGURE LEGENDS

Figure 1. NaHS inhibits proliferation in both Cav3.2-expressing HEK293 cells and wild type HEK293 cells. A. Line graph showing proliferation of Cav3.2-expressing HEK293 cells monitored over a 4-day period, in the absence (control, open circles) or presence of 1, 10 or 100μM NaHS as indicated. **B**. Line graph showing (on the same scale as (A))proliferation of wild type HEK293 cells monitored over a 4-day period, in the absence of drug (open circles), or during 1-100μM NaHS as indicated. **C**. Same data as plotted in (B) but with a magnified Y axis. **D**. Bar graph showing proliferative response of both WT HEK293 cells (bold bars) and Cav3.2-expressing HEK293 cells (open bars) on day 4 (mean ± s.e.m) in the absence and presence of NaHS, as indicated. Note normalised proliferation (compare to control in the absence of drug, n=3 for each cell type) is very similar for both cell types at each NaHS concentration. Statistical significance: * $p<0.05$; ** $P<0.01$.

Figure 2. NaHS inhibits proliferation in Cav3.1-expressing HEK293 cells. Upper: Line graph showing an example proliferation experiment using Cav3.1-expressing HEK293 cells monitored over a 3-day period, in the absence of drug (open circles), or in the presence of 30-300μM NaHS as indicated. Each point represents mean ± s.e.m of 3 repeats. Lower: Mean (with s.e.m.) normalised proliferation determined on day 3 in three experiments exemplified in upper graph. * $P<0.05$; ** $P<0.01$.

Figure 3. NaHS inhibits proliferation but does not modulate T-type Ca^{2+} currents in A7r5 cells. **A**. Upper: Line graph showing proliferation of A7r5 cells over a 3-day period in the absence (open circles) or presence of mibefradil. Each point represents mean ± s.e.m of 3 experiments. Lower: Mean (with s.e.m.) normalised proliferation determined on day 3 in three experiments exemplified in upper graph. *** $P<0.001$. **B**. Upper: as (A) but in the absence (open circles) or presence of NaHS. Lower: Mean (with s.e.m.) normalised proliferation determined on day 3 in three experiments exemplified in upper graph. ** $P<0.01$; *** $P<0.001$. **C.** Example, superimposed currents (identical in amplitude and time-course) evoked in a representative A7r5 cell before (Control) and during (NaHS) exposure to 100μM NaHS. The time-series graph taken from this cell plots successive current amplitudes (each shown by an open circle) evoked by repeated step depolarizations (-80mV to -20mV, 200ms duration, 0.2Hz). NaHS (100μM and 200μM) was applied via the perfusate for the periods indicated by the horizontal bars. **D.** Bar graph showing mean (with s.e.m., n= 5 cells in each case) effects of NaHS at 1-200μM.

Figure 4. NaHS inhibits proliferation in human coronary artery smooth muscle cells (hCASMCs). A. Line graph showing proliferation of hCASMC monitored over a 4-day period, in the absence (control, open circles) or in the presence of Ni^{2+}. **B**. Expression levels for Cav3.1

and Cav3.2 mRNA determined in hCASMCs. Channel expression is plotted as percentage of expression of the housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT1), taken from the same samples used to detect channel mRNA individual results from 3 separate experiments are shown. Note the difference in scales for each channel type. **C**. Line graph showing proliferation of hCASMCs monitored over a 4-day period, in the absence (open circles), or presence of NaHS as indicated.

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Figure 1

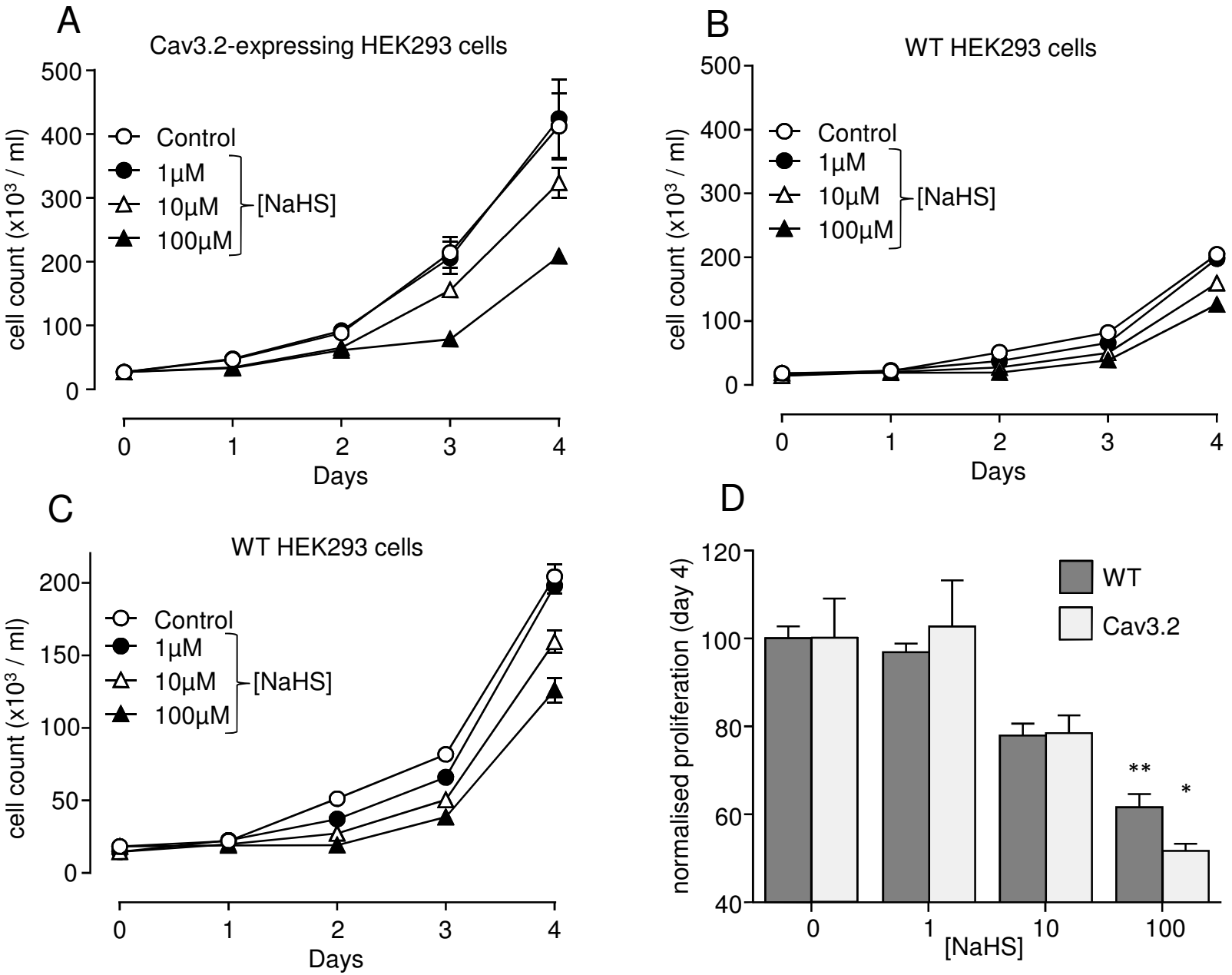

A
Cav3.2-expressing HEK293 cells
cell count (x10³ / ml)
500
400
300
200
100
0
Control
1μM
10μM
100μM
[NaHS]
0
1
2
3
4
Days
B
WT HEK293 cells
cell count (x10³ / ml)
500
400
300
200
100
0
Control
1μM
10μM
100μM
[NaHS]
0
1
2
3
4
Days
C
WT HEK293 cells
cell count (x10³ / ml)
200
150
100
50
0
Control
1μM
10μM
100μM
[NaHS]
0
1
2
3
4
Days
D
normalised proliferation (day 4)
120
100
80
60
40
WT
Cav3.2
**
*
0
1
10
100
[NaHS]

Figure 2

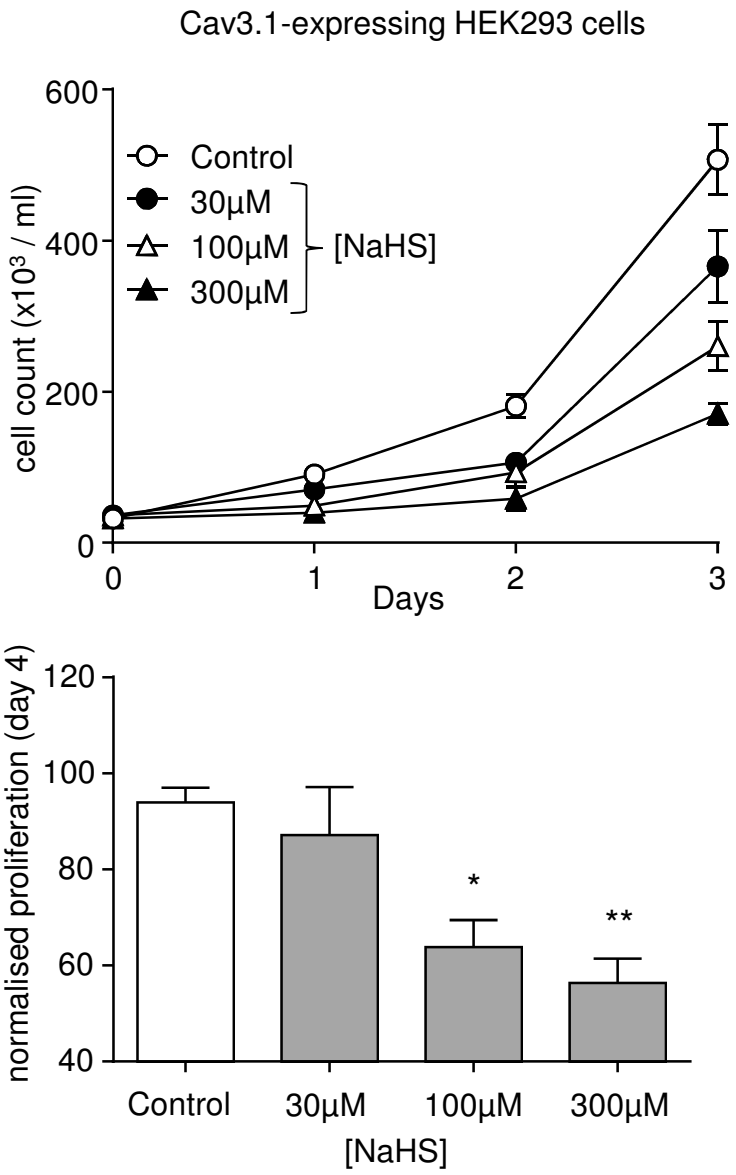

Cav3.1-expressing HEK293 cells
600
400
200
0
cell count (x10³ / ml)
Control
30μM
100μM
300μM
[NaHS]
0
1
2
3
Days
120
100
80
60
40
normalised proliferation (day 4)
*
**
Control
30μM
100μM
300μM
[NaHS]

Figure 3

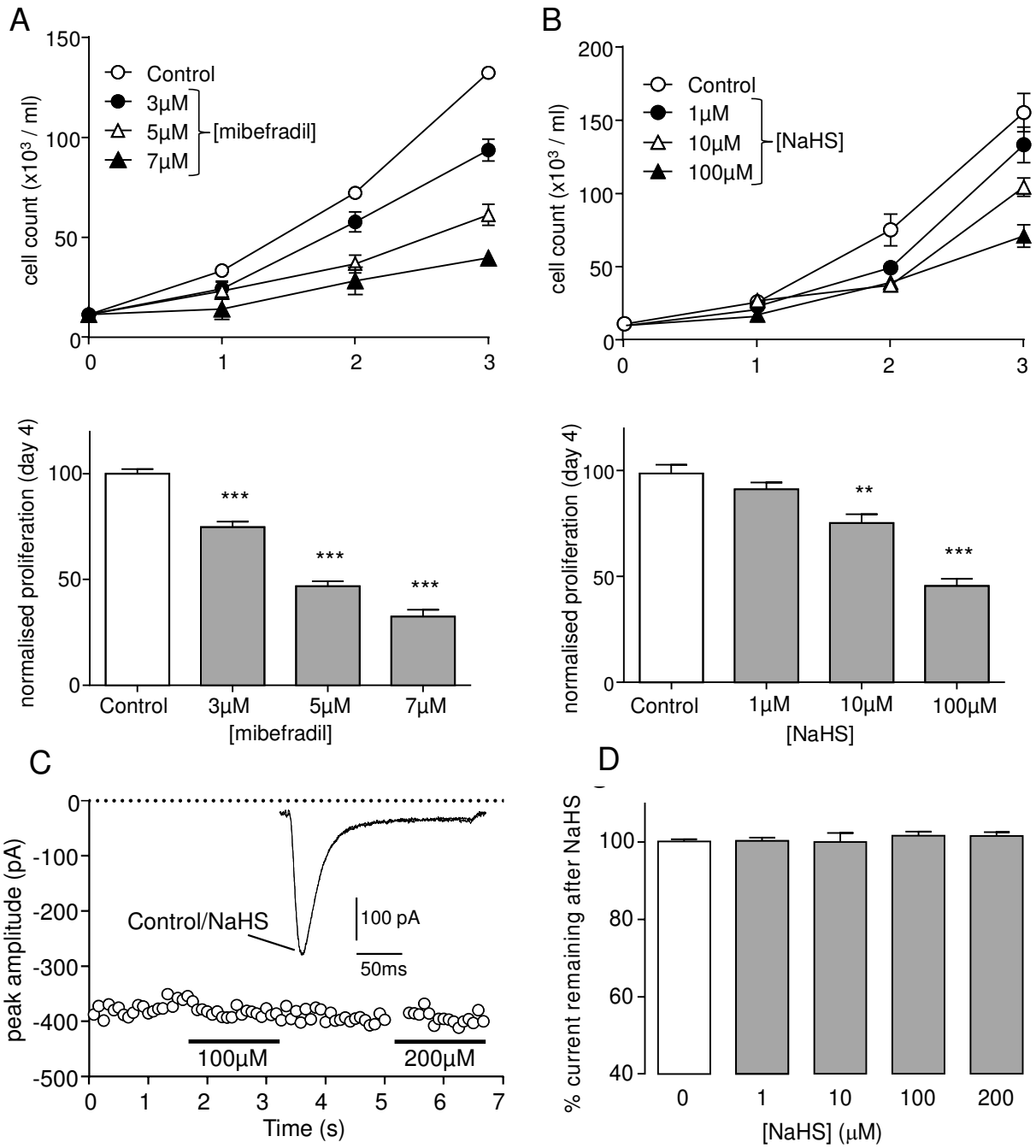
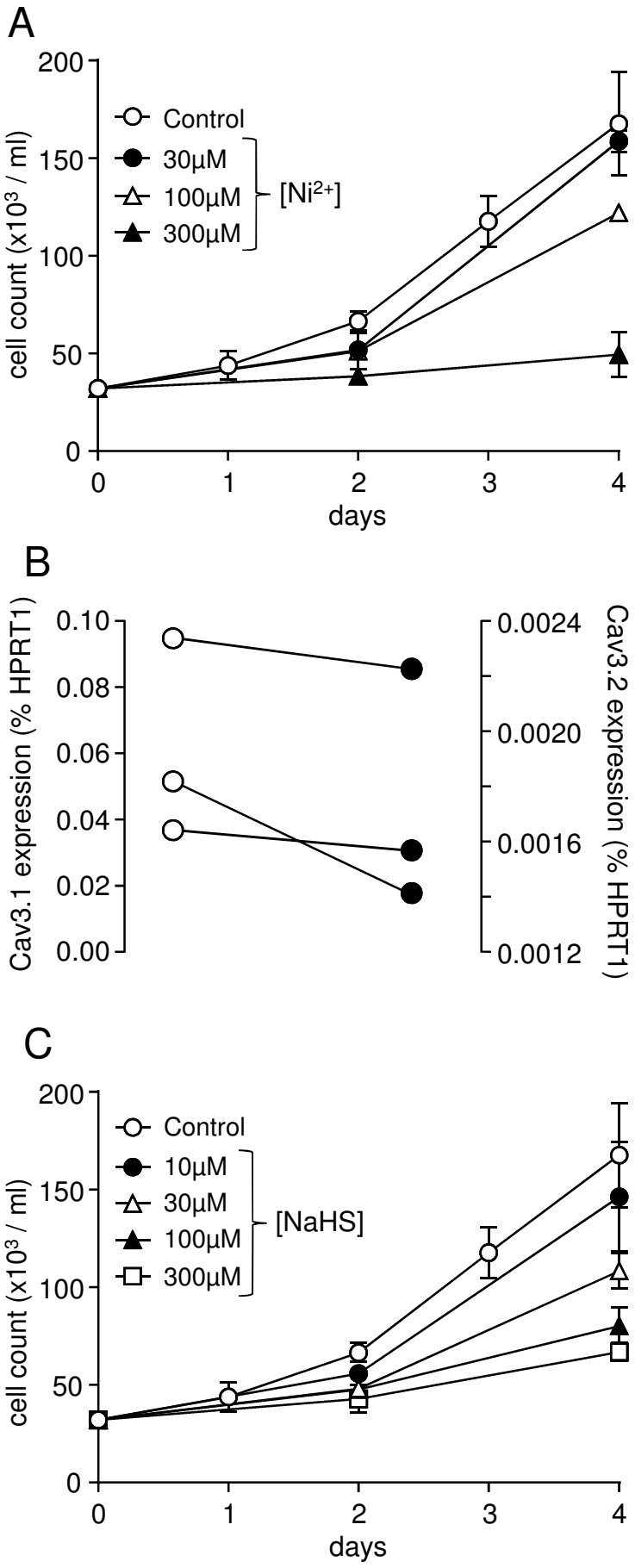

A
cell count (x10³ / ml)
Control
3μM
5μM
7μM
[mibefradil]
normalised proliferation (day 4)
Control
3μM
5μM
7μM
[mibefradil]
B
cell count (x10³ / ml)
Control
1μM
10μM
100μM
[NaHS]
normalised proliferation (day 4)
Control
1μM
10μM
100μM
[NaHS]
C
peak amplitude (pA)
Control/NaHS
100 pA
50ms
100μM
200μM
Time (s)
D
% current remaining after NaHS
[NaHS] (μM)

Figure 4


A
200
150
100
50
0
cell count (x10³ / ml)
Control
30μM
100μM
300μM
[Ni²⁺]
0
1
2
3
4
days
B
0.10
0.08
0.06
0.04
0.02
0.00
Cav3.1 expression (% HPRT1)
0.0024
0.0020
0.0016
0.0012
Cav3.2 expression (% HPRT1)
C
200
150
100
50
0
cell count (x10³ / ml)
Control
10μM
30μM
100μM
300μM
[NaHS]
0
1
2
3
4
days