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RESEARCH PAPER

Cis-isomerism and other chemical requirements of steroidal agonists and partial agonists acting at TRPM3 channels

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BACKGROUND AND PURPOSE

The transient receptor potential melastatin-3 (TRPM3) channel forms calcium-permeable, non-selective, cationic channels that are stimulated by pregnenolone sulphate (PregS). Here, we aimed to define chemical requirements of this acute steroid action and potentially reveal novel stimulators with physiological relevance.

EXPERIMENTAL APPROACH

We used TRPM3 channels over-expressed in HEK 293 cells, with intracellular calcium measurement and whole-cell patch-clamp recording techniques.

KEY RESULTS

The stimulation of TRPM3 channels was confined to PregS and closely related steroids and not mimicked by other major classes of steroids, including progesterone. Relatively potent stimulation of TRPM3-dependent calcium entry was observed. A sulphate group positioned at ring A was important for strong stimulation but more striking was the requirement for a *cis* (β) configuration of the side group, revealing previously unrecognized stereo-selectivity and supporting existence of a specific binding site. A *cis*-oriented side group on ring A was not the only feature necessary for high activity because loss of the double bond in ring B reduced potency and loss of the acetyl group at ring D reduced efficacy and potency. Weak steroid stimulators of TRPM3 channels inhibited effects of PregS, suggesting partial agonism. In silico screening of chemical libraries for non-steroid modulators of TRPM3 channels revealed the importance of the steroid backbone for stimulatory effects.

CONCLUSIONS AND IMPLICATIONS

Our data defined some of the chemical requirements for acute stimulation of TRPM3 channels by steroids, supporting the existence of a specific and unique steroid binding site. Epipregnanolone sulphate was identified as a novel TRPM3 channel stimulator.

Abbreviations

HEK 293 cells, human embryonic kidney cells; TRP, transient receptor potential

Introduction

TRPM3 is a Ca^{2+}-permeable member of the melastatin family of the transient receptor potential (TRP) cation channels (nomenclature follows Alexander *et al.*, 2009). It has been relatively little studied and its relevance to mammalian biology is poorly understood. Expression in kidney and brain was noted in initial studies but expression in a range of other tissues and cells has been detected subsequently

(Grimm *et al.*, 2003; Lee *et al.*, 2003; Wagner *et al.*, 2008). Constitutive channel activity has been detected (Grimm *et al.*, 2003) but there are also thought to be chemical activators of the channels. D-erythro-sphingosine was observed to stimulate human TRPM3 (Grimm *et al.*, 2005) but mouse TRPM3 channels were resistant (Wagner *et al.*, 2008). The most striking stimulation reported has been by pregnenolone sulphate (PregS), the sulphated form of pregnenolone (Preg), which derives from cholesterol and is the precursor for a wide range of steroid hormones. It causes rapid, large and apparently direct increases in TRPM3 channel activity (Wagner *et al.*, 2008). Similar results were observed for endogenous TRPM3 channels of pancreatic β-cells where there was functional coupling to insulin secretion (Wagner *et al.*, 2008). Unfortunately, the concentration of PregS required to stimulate TRPM3 channels was found to be relatively high, suggesting that the effect may only be of pharmacological relevance (e.g. relevant to the use of Preg and related steroids as non-prescribed dietary supplements). Nevertheless, PregS is a striking stimulator of TRPM3 and the data suggest that the TRPM3 channel is a previously unrecognized acute steroid sensor. In this study, our aim was to generate information on the chemical structure-activity relationships of TRPM3 channel stimulation by steroids with a view to enabling comparisons with other examples of ion channels that sense steroids and revealing key features and novel stimulators of TRPM3 channels that could potentially lead to a better understanding of the physiological or pathological relevance of the channels.

Methods

Cell culture

The cDNA for human TRPM3 channels (accession number AJ505026) was provided by C. Harteneck (Grimm *et al.*, 2003). For conditional overexpression in HEK293 cells, the T-Rex system (Invitrogen, Paisley, UK) was used. The human TRPM3 cDNA was stably incorporated into HEK293 cells under the control of a tetracycline-inducible promoter, such that addition of 1 μg/mL tetracycline (Tet+) induced robust expression of ectopic TRPM3 channels (Naylor *et al.*, 2008). As a control, cells not treated with tetracycline (Tet-) were used. Cells were maintained in Dulbecco's modified Eagle's medium-F12 + GLUTAMAX (Cat # 31331, Gibco, Paisley, UK) supplemented with 10% foetal calf serum, 100 units/mL penicillin/streptomycin (Sigma, St Louis, MO, USA) and selection antibiotics (10 μg/mL blasticidin and 400 μg/mL zeocin; Invitrogen) at 37°C in a 5 % CO_2 incubator. In a small number of experiments (Figure 3H only), we used mouse TRPM3α2 channel cDNA (accession number AJ544535) in pCAGGSM2-IRESGFP, which was a gift from SE Philipp (Wagner *et al.*, 2008). TRPM3α2 channels were co-transfected into HEK 293 cells with GFP using FuGENE 6 (Roche, Hertfordshire, UK) and functional studies were carried out 48 h after transfection.

Ca^{2+} measurement

For intracellular Ca^{2+} measurement, cells were plated at a confluence of 80–90% in clear-bottom poly-D lysine coated 96-well plates (Corning Inc., Corning, NY, USA) for 24 h prior to experiments. Immediately prior to recordings, cells were incubated for 1 h at 37°C in standard bath solution (SBS) containing 2 μM fura-2 acetoxymethyl ester (and 0.01% pluronic acid) and then washed with SBS once before adding the recording buffer (SBS with appropriate solvent). All experiments were performed at 23 ± 2°C. SBS comprised (in mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 8 and HEPES 10 in deionized water. Osmolality was adjusted to 290 mOsm/L using D-mannitol and pH was titrated to 7.4 using 4 M NaOH. Measurements were made on a 96-well fluorescence plate reader (FlexStation II^{384}, Molecular Devices, Sunnyvale, CA, USA). Fura-2 was excited by light of 340 and 380 nm and emitted light was collected at 510 nm. Wells of the 96-well plate were studied in a column format and loaded alternately for test and control conditions. Change (Δ) in intracellular calcium (Ca^{2+}_i) concentration is indicated as the ratio of fura-2 fluorescence (F) emission intensities for 340 and 380 nm (F ratio).

Electrophysiology

Borosilicate glass capillaries with an outside diameter of 1 mm and an inside diameter of 0.58 mm (Harvard Apparatus, Holliston, MA, USA) were used as the basis for pipettes. Pipettes were pulled to a tip diameter of approximately 1 μm using a PP-830 vertical 2-stage pipette-puller (Narishige, Tokyo, Japan). They were fire-polished using a tungsten carbide wire-heating element just prior to use. The final pipette resistance in solution was 3–5 MΩ. The pipettes were mounted on a CV203BU head-stage (Axon Instruments, Sunnyvale, CA, USA) connected to a three-way coarse manipulator and a micromanipulator (Newport 300P, Newport Corporation, Irvine, CA, USA). Electrodes comprised silver wires coated with chloride ions. Electrical signals were amplified and recorded using an Axopatch 200B amplifier and pCLAMP 8 software (Axon Instruments). Data were filtered at 1 kHz and sampled digitally at 2 kHz via a Digidata 1322A analogue to

digital converter (Axon Instruments). Recordings were made at least 5 min after break-through to the whole-cell configuration. Series resistances were <10 MΩ. The voltage-ramp protocol consisted of a step from a holding potential of 0 mV to –100 mV, followed by a 0.1 s ramp to +100 mV, before returning to 0 mV (repeated every 10 s). Analysis was performed off-line using Clampfit 8.2 (Axon Instruments) and Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA). The extracellular solution comprised (in mM): NaCl 130, KCl 5, CsCl 10, $MgCl_2$ 1.2, $CaCl_2$ 1.5, glucose 8 and HEPES 10, with pH titrated to 7.4 using 4 M NaOH. The osmolality of this solution was 295 mOsm/L. The patch-pipette solution comprised (in mM): Cs aspartate 80, CsCl 45, HEPES 10, BAPTA sodium 10, Na_2ATP 4; osmolality was adjusted to 290 mOsm/L using D-mannitol and the pH was titrated to 7.2 using 4 M CsOH. The pipette solution was filtered using a 0.2 µm membrane filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany), divided into aliquots of approximately 50 µL and stored at –20°C.

Rapid overlay of chemical structures

The rapid overlay of chemical structures (ROCS) methodology was used to identify chemical 'look-alikes' of the steroid PregS. ROCS is a three-dimensional method that employs Gaussian functions to align chemicals based on shape and/or chemical similarity (Tresadern *et al.*, 2009). The method is based on the assumption that two molecules have similar shapes if their volumes overlay well. ROCS was customized to incorporate specific chemical aspects including charge, hydrophobicity and hydrogen bonds (Tawa *et al.*, 2009). A structural similarity search using ROCS was applied to PregS using commercially available chemical libraries from Maybridge chemicals (Maybridge, Maybridge Chemicals, Cornwall, UK) and Key Organics (Cornwall, UK). These databases were generated with OMEGA software (OpenEye, Santa Fe, NM, USA). After completion of the ROCS run, the top 100 hits from each run were visualized using the OpenEye application VIDA software. The chemicals with the highest similarity to PregS were selected for functional assays.

Data analysis

Origin 7.5 software (OriginLab Corporation) was used for data analysis and presentation. Averaged data are expressed as mean ± standard error of mean. The amplitudes of signals were measured at the peak response. Control and test data were either compared using an independent Student's *t*-test (intracellular Ca^{2+} measurement) or paired Student's *t*-test (electrophysiology performed on the same cell). Probability (*P*) of less than 0.05 was considered statistically significant; n.s. (not significant) indicates $P > 0.05$. All intracellular Ca^{2+} measurement data are presented as N/n, where 'N' is the number of wells used in the 96-well plate and 'n' is the total number of independent experiments (i.e. on different 96-well plates). For patch-clamp recordings, the number of independent cell recordings is indicated by 'n'.

Materials

Steroids were purchased from Sigma or Steraloids and stock solutions were stored according to the suppliers' instructions. The following steroids were prepared as 10–100 mM stocks in 100% DMSO: PregS, progesterone, Preg, pregnenolone acetate (Preg-ac), dehydroepiandrosterone sulphate (DHEAS), 17β-oestradiol sulphate, corticosterone-21 sulphate, epipregnanolone sulphate (Epipregnas), pregnanolone sulphate (Pregnas), epiandrosterone sulphate (Epiandros) and androsterone sulphate (Andros). The following steroids were prepared as 10–50 mM stocks in 100% ethanol: 17β-oestradiol, dihydrotestosterone, aldosterone, cortisol, cholecalciferol and ergocalciferol. Dehydroepiandrosterone (DHEA) was prepared as a 50 mM stock in 100% methanol. Chemical structures of the relevant steroids are shown in Supplementary Figure S3.

Results

All data were from human TRPM3 channels except for the data of Figure 3H, which were generated from mouse TRPM3 channels.

Failure of progesterone and other steroid classes to stimulate TRPM3 channels

Human TRPM3 channel activity was measured by two independent techniques: an intracellular Ca^{2+} indicator to detect Ca^{2+} entry through Ca^{2+}-permeable TRPM3 channels (Figure 1A) and whole-cell patch-clamp to detect mixed cationic membrane current through TRPM3 channels (Figure 1B,C). In HEK 293 cells induced to express TRPM3 channels, strong and consistent responses to PregS were observed using either technique (Figure 1A–C). The current-voltage relationship of the PregS-induced current was outwardly rectifying and reversed polarity at approximately 0 mV (Figure 1B,C; and see Supplementary Figure S1). Responses were comparatively small or undetectable in cells that were not induced to express TRPM3

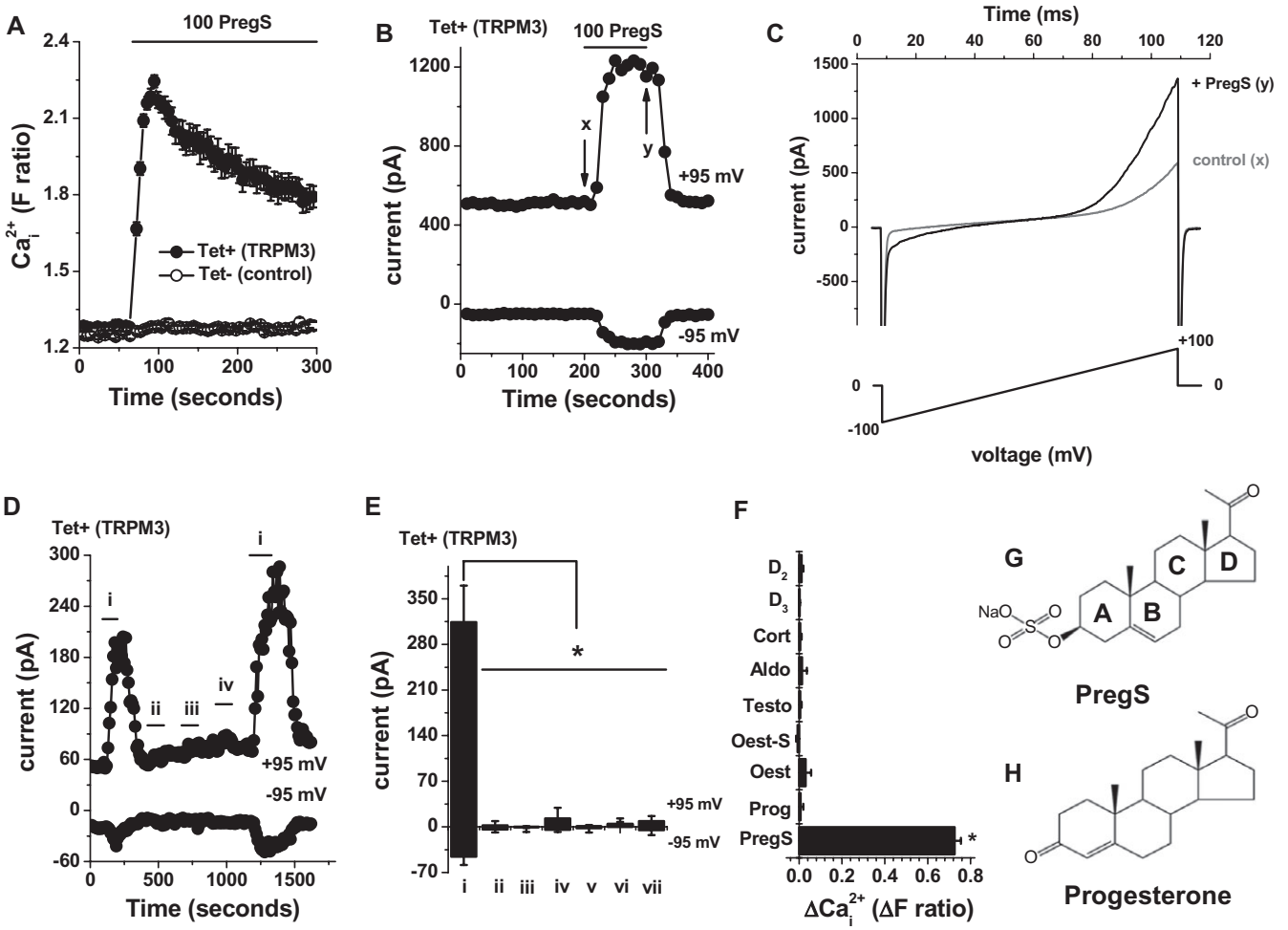

Figure 1

Selective stimulation of TRPM3 by pregnenolone sulphate (PregS). (A) Intracellular Ca^{2+} measurement showing the effect of acute application of 100 μM PregS in cells over-expressing TRPM3 channels (Tet+) and control (Tet-) cells. (B) Whole-cell voltage-clamp showing the induction of outward and inward currents after bath-application of 100 μM PregS. Data are representative of at least three independent recordings. To generate the current-voltage (I/V) relationships shown in (C), the amplitude of the current was measured at the time-points indicated by 'x' and 'y'. (C) The I/V relationship for the basal (control) and PregS-evoked (+PregS) current. The bottom panel shows the voltage-ramp protocol. (D) Example whole-cell voltage-clamp time-series plot showing the effect of bath-application of 50 μM each of PregS (i), progesterone (ii), 17β-oestradiol (iii) and dihydrotestosterone (iv) on outward and inward currents at +95 and –95 mV. The horizontal bars indicate the durations of compound application. (E) Mean data for experiments exemplified in (D) as well as separate experiments performed using 50 μM each of cortisol (v), cholecalciferol (vi) and ergocalciferol (vii). (F) Mean data from intracellular Ca^{2+} measurement showing the effects of PregS (5 μM), progesterone (Prog, 10 μM), 17β-oestradiol (Oest, 10 μM), 17β-oestradiol sulphate (Oest-S, 50 μM), dihydrotestosterone (Testo, 50 μM), aldosterone (Aldo, 50 μM), cortisol (Cort, 50 μM), cholecalciferol (D_3, 10 μM) and ergocalciferol (D_2, 10 μM). (G, H). The two-dimensional structures of PregS and progesterone. The four hydrocarbon rings (A, B, C and D) are labelled in PregS. N/*n* = 24/3 for (A), *n* = 4–5 for (E) and N/*n* = 24/3 for each steroid tested in (F). $^*P < 0.05$.

channels (Figure 1A, Supplementary Figure S2). The data suggest that TRPM3 channels were reliably stimulated by PregS in tetracycline-induced (TRPM3-expressing) HEK 293 cells and that background signals were small.

As a first step, other major classes of steroid were tested for their ability to stimulate TRPM3 channels in whole-cell patch-clamp and Ca^{2+}-measurement assays. In marked contrast to PregS, progesterone, 17β-oestradiol, 17β-oestradiol sulphate, dihydrotestosterone, aldosterone, cortisol, and vitamins D_2 and D_3 were ineffective at stimulating TRPM3 channels (Figure 1D–F). Chemical structures of the steroids are given in Supplementary Figure S3 but the structures of PregS and progesterone (the most closely related to PregS) are also shown in Figure 1G,H to highlight differences: an oxygen atom instead of a sulphate group at ring A, and a double bond in ring A rather than ring B. These differences prevent progesterone from stimulating

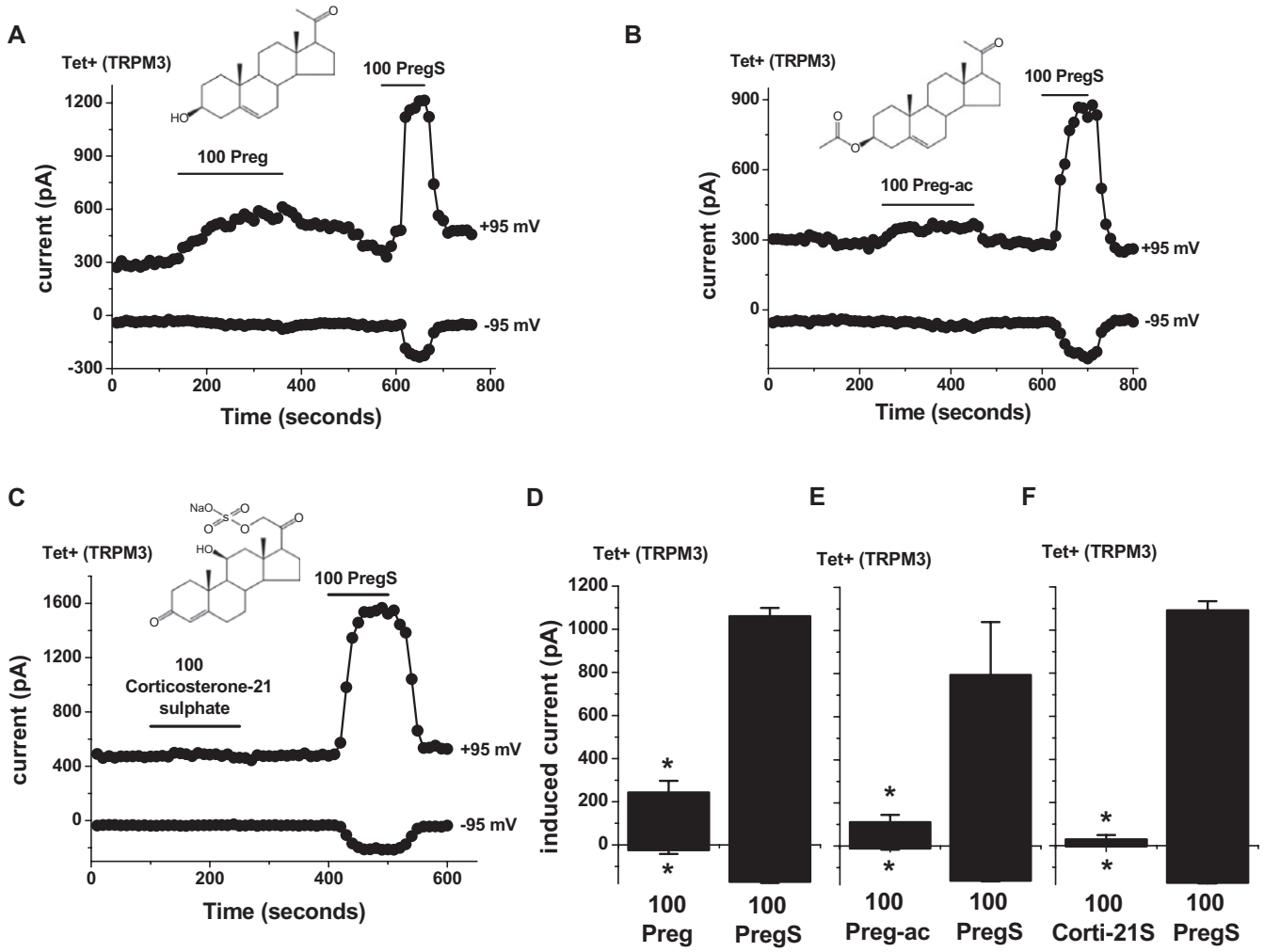

Figure 2

Significance of the ring A sulphate. Data were generated by whole-cell voltage-clamp in cells over-expressing TRPM3 channels (Tet+). (A, B and C) Time-series plots showing the effect of bath-application of 100 μM each of (A) pregnenolone (Preg), (B) pregnenolone acetate (Preg-ac) and (C) corticosterone-21 sulphate on the outward and inward currents. The responses elicited by 100 μM pregnenolone sulphate (PregS) were used as a positive control in all recordings. The horizontal bars indicate the duration of compound application. (D, E, F). Mean data for experiments illustrated in A, B and C, respectively. $n = 3$ for each steroid. *$P < 0.05$.

TRPM3 channels and suggest highly specific chemical requirements for stimulation of these cationic channels by steroids.

Importance of ring A sulphate for TRPM3 stimulation

Preg and Preg-ac are chemically the same as PregS except that the ring A sulphate is replaced by a hydroxyl or acetate group (Figure 2A and B). While both steroids were stimulators of TRPM3 channels, they had much weaker effects than PregS (Figure 2A,B,D,E). Corticosterone-21 sulphate is similar to progesterone but contains a sulphate group on ring D (Figure 2C). However, corticosterone-21 sulphate had no stimulatory effect on TRPM3 channels, suggesting that the ring position of the sulphate is important for activity (Figure 2C,F).

Ring B double bond affects potency but not maximum effect

Epipregnas is the same as PregS except that it lacks the double bond in ring B (Figure 3A). Importantly, Epipregnas was a strong stimulator of TRPM3 channels, evoking an ionic current that was similar to that evoked by PregS (Figure 3B–D). However, in Ca^{2+} measurement experiments, the potency and maximum effect of Epipregnas were less than those of PregS (Figure 3E).

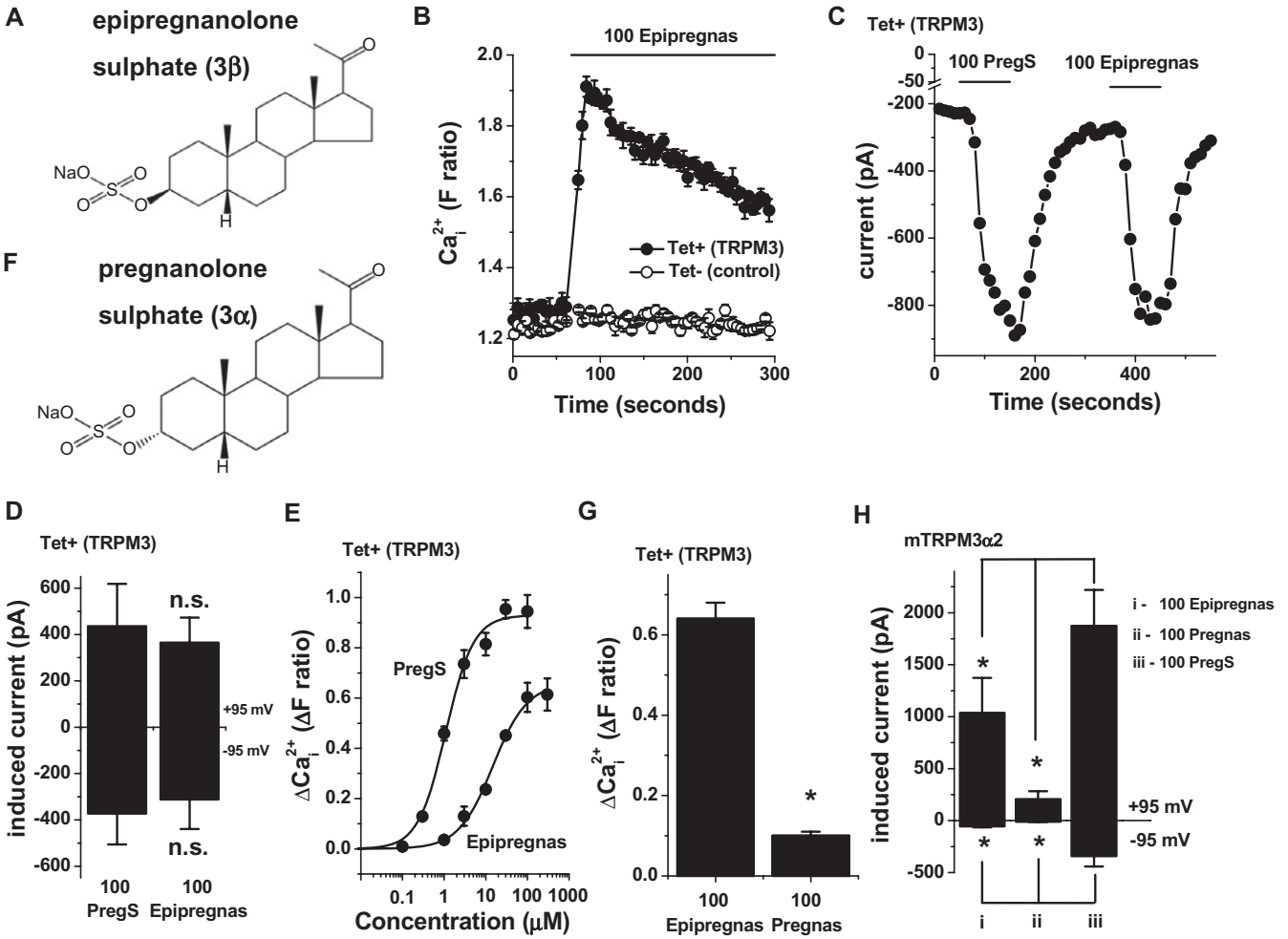

Figure 3

Stimulation by epipregnanolone sulphate. (A) Two-dimensional structure of epipregnanolone sulphate, indicating the conformation of the sulphate group in ring A. (B) Intracellular Ca^{2+} measurement data showing the effect of acute application of 100 μM epipregnanolone sulphate (Epipregnas) in cells over-expressing TRPM3 channels (Tet+) and control (Tet-) cells. (C) Whole-cell voltage-clamp recording showing the induction of inward currents at –95 mV upon bath-application of 100 μM pregnenolone sulphate (PregS) and then 100 μM Epipregnas. (D) Mean data from recordings made in the same cell comparing the currents elicited by 100 μM PregS and then 100 μM Epipregnas. (E) Concentration-response curves for the Ca^{2+} signals elicited by PregS and Epipregnas, giving approximate EC_{50} values of 1 and 14 μM respectively (N/*n* = 12/3). (F) The two-dimensional structure of pregnanolone sulphate (Pregnas). (G) Mean data from experiments performed in the same 96-well plate comparing the amplitude of the Ca^{2+} response elicited by 100 μM each of Epipregnas and Pregnas. (H) Mean whole-cell current data from HEK293 cells transfected with mouse TRPM3α2 channels comparing responses elicited by 100 μM Epipregnas, Pregnas or PregS. N/*n* = 12/3 for (B, G), *n* = 3 each for (D), and *n* = 7 each for (H). *$P < 0.05$.

Orientation of the ring A sulphate is critical

Pregnas is a stereo-isomer of Epipregnas, differing only in the orientation of the ring A sulphate group (Figure 3F). Pregnas was strikingly weaker at stimulating TRPM3 channels compared with Epipregnas (Figure 3G), showing critical dependence of TRPM3 channel stimulation on the *cis* (β) orientation of the sulphate group.

In separate experiments, we checked if mouse TRPM3α2 channels (Wagner *et al.*, 2008) respond similarly compared with human TRPM3 channels. In HEK 293 cells transfected with mouse TRPM3α2 channels, robust ionic currents were evoked by PregS or Epipregnas, whereas ionic currents evoked by Pregnas were much smaller and almost undetectable at negative voltages (Figure 3H). Therefore, mouse and human TRPM3 channels respond similarly.

The acetyl group at ring D is also important

DHEAS is the same as PregS, except that there is an oxygen instead of an acetyl group at ring D (Figure 4A). It therefore contains the *cis* (β) sulphate at ring A and the double bond in ring B, and so might be expected to be a full agonist at TRPM3 channels. However, previous study of mouse TRPM3

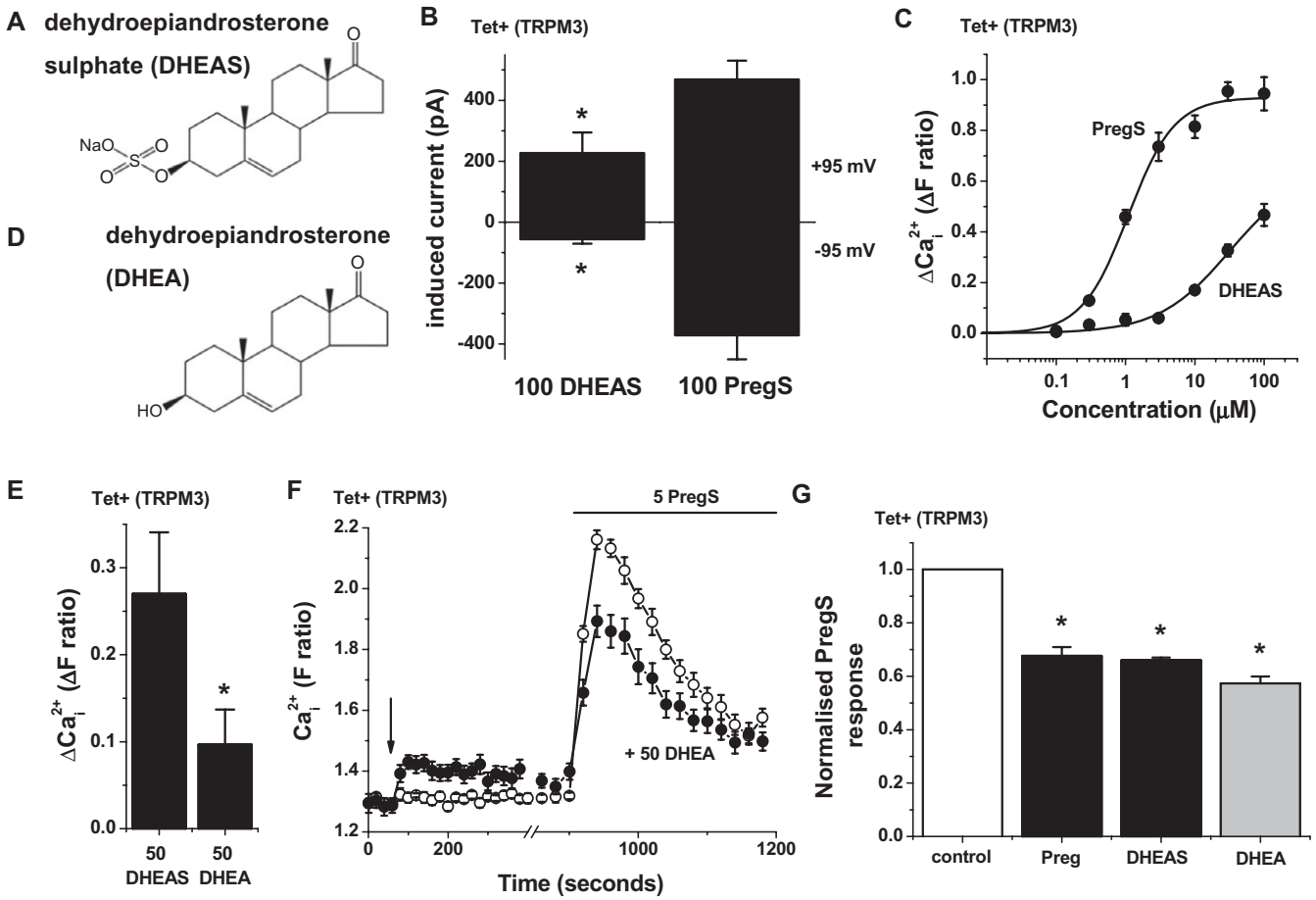

Figure 4

Partial agonism. Experiments were performed in cells over-expressing TRPM3 channels (Tet+). (A) Two-dimensional structure of dehydroepiandrosterone sulphate (DHEAS). (B) Mean data from whole-cell voltage-clamp performed in the same cell comparing the currents elicited by 100 μM each of DHEAS and pregnenolone sulphate (PregS). (C) Concentration-response curves for the Ca^{2+} signals elicited by PregS and DHEAS, giving approximate EC_{50} values of 1 and 33 μM respectively (N/*n* = 12/3). The PregS data are the same as those of Figure 3E. (D) Two-dimensional structure of dehydroepiandrosterone (DHEA). (E) Mean data comparing the Ca^{2+} responses elicited by 50 μM each of DHEAS and DHEA. (F) Indicated by the vertical arrow, acute application of 50 μM DHEA or solvent followed by 5 μM PregS indicated by the horizontal bar. (G) Mean data for the inhibitory effect of 50 μM each of pregnenolone (Preg), DHEAS and DHEA on the Ca^{2+} response elicited by 5 μM PregS. *n* = 3 for (B), N/*n* = 24/3 for (E) and (G). *$P < 0.05$.

channels found it to be only a weak agonist (Wagner *et al.*, 2008). Our experiments confirmed this result, showing DHEAS to be a weak stimulator of human TRPM3 channels compared with PregS (Figure 4B). The potency of DHEAS is also less than that of PregS (Figure 4C). As observed with PregS and Preg, DHEA was less effective than DHEAS (Figure 4D,E), further supporting the importance of the sulphate at ring A.

Partial agonists

Preg, DHEAS and DHEA are weak stimulators of TRPM3 channels, yet chemically related to PregS. We therefore hypothesized that they might be partial agonists at TRPM3 channels, that is, having the capability to inhibit effects of PregS. Indeed, this was the case because all of these steroids suppressed the effect of PregS (Figure 4F,G).

Cis (β) orientation of the ring A sulphate in the absence of the ring D acetyl group

Epiandros is the same as DHEAS except it lacks the double bond in ring B (Figure 5A). Andros is the same as Epiandros, except that the sulphate at ring A is in the *trans* (α) orientation (Figure 5B). Epiandros was an effective stimulator of TRPM3 channels with similar effect to DHEAS (Figure 5C,D). Andros, in contrast, had little or no stimulator effect (Figure 5E). These data further support the conclusion that stereo orientation of the ring A sulphate group is critical for stimulation of TRPM3 channels.

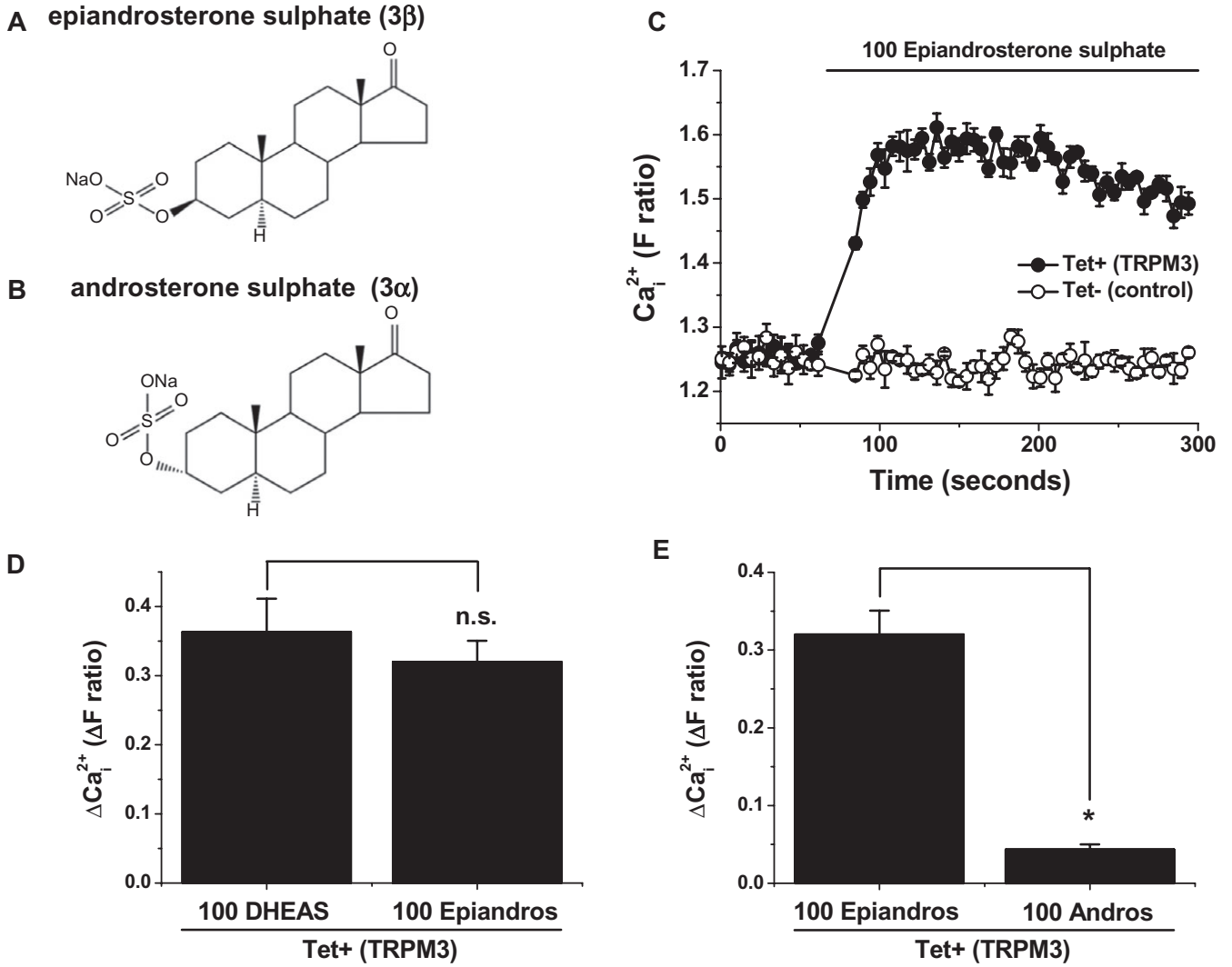

Figure 5

Importance of *cis* (β) orientation in ring A in the absence of an acetyl group at ring D. (A, B) Two-dimensional structures of epiandrosterone sulphate (Epiandros) and androsterone sulphate (Andros), indicating the conformation of the sulphate group in ring A. (C) Effect of acute application of 100 μM Epiandros in cells over-expressing TRPM3 channels (Tet+) and control (Tet-) cells. (D) Mean data comparing the amplitude of the Ca^{2+} responses elicited by acute application of 100 μM each of dehydroepiandrosterone sulphate and Epiandros. (E) Mean data comparing the amplitude of the Ca^{2+} responses elicited by acute application of 100 μM each of Epiandros and Andros. N/*n* = 12/3 for (D) and (E). **P* < 0.05.

Importance of the steroid backbone

To investigate if the steroid backbone is important in stimulation of TRPM3 channels, we performed *in silico* screens of chemical libraries to identify PregS look-alike compounds that lack the steroid backbone (Figure 6A). Thirty-two of the most promising structures were tested for stimulatory actions at TRPM3 channels in Ca^{2+} measurement experiments (Figure 6B). None gave significant stimulation of TRPM3 channels when used at 10 μM (Figure 6B). In cases where there was suggestion of an effect, the compounds were re-tested at the higher concentration of 100 μM but they again failed to give significant stimulation of TRPM3 channels (Figure 6C). However, 10 μM of one compound was found to inhibit TRPM3 channels stimulated by PregS (Figure 6D), suggesting that there was sufficient similarity with PregS (Figure 6E) for interaction at its putative binding site. Inability of the compounds to stimulate TRPM3 channels points to need for a specific steroid backbone in TRPM3 activation. Details of the chemicals are given in Supplementary Table S1.

Discussion and conclusions

The study shows that, like mouse TRPM3, human TRPM3 channels are strongly stimulated by PregS,

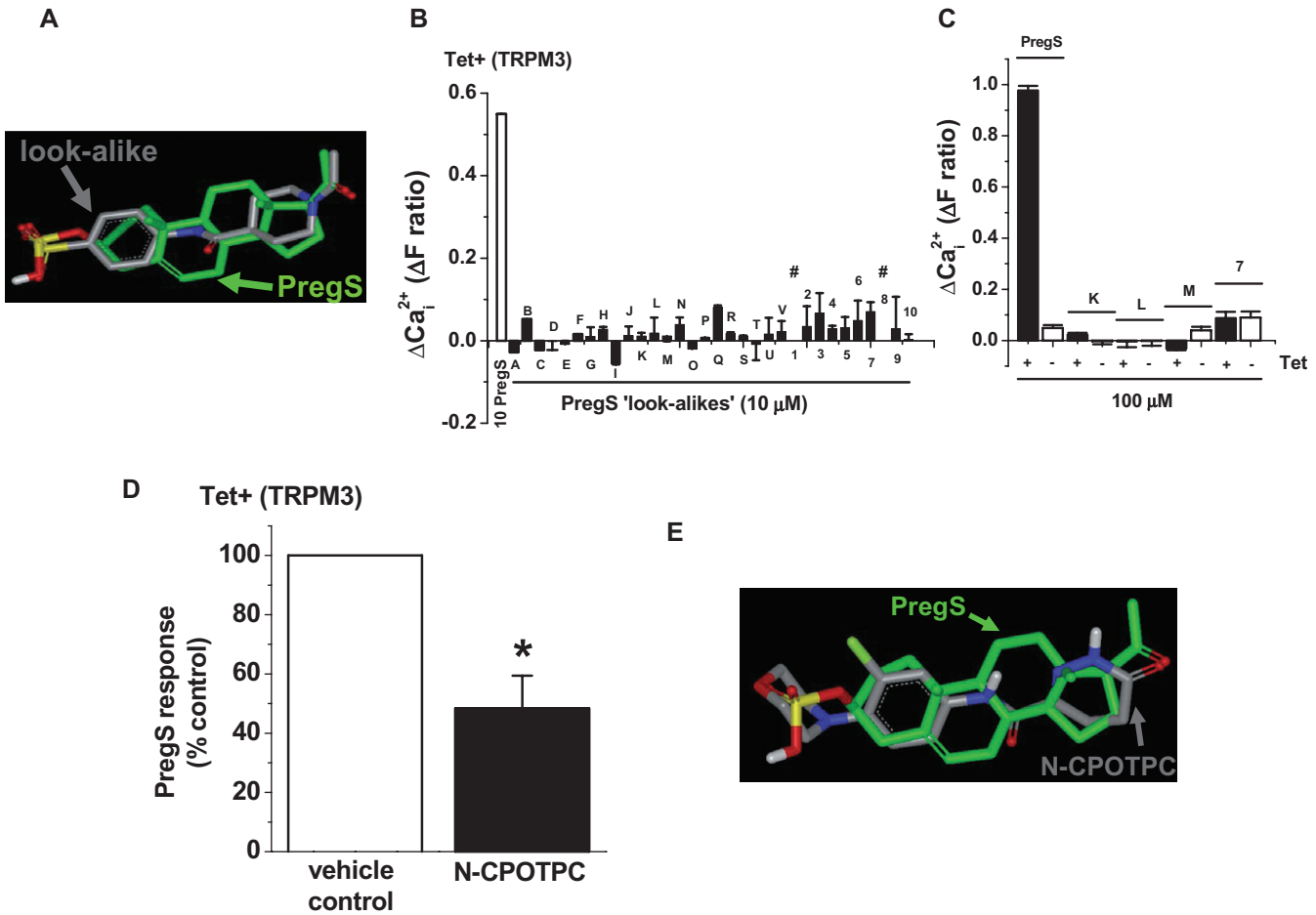

Figure 6

Chemicals lacking the steroid backbone. (A) Generated by rapid overlay of chemical structures (ROCS) methodology, an illustrative three-dimensional overlay of pregnenolone sulphate (PregS) with a look-alike chemical. (B) Data from intracellular Ca^{2+} measurement performed in cells over-expressing TRPM3 channels (Tet+) showing the amplitude of the signals elicited by acute application of 10 μM PregS and 32 selected chemical look-alikes (coded A–V and 1–10). The chemicals indicated by '#' produced fluorescent artefacts, so the data are not shown. (C) Data from intracellular Ca^{2+} measurement performed in cells over-expressing TRPM3 (Tet+) and control (Tet-) cells showing the amplitude of the signals elicited by acute application of 100 μM each of PregS and four chemical look-alikes (code-named K, L, M and 7). (D) Mean data for the inhibitory effect produced by 10 μM 'chemical 10' (N-(3-chloro-4-morpholinophenyl)-6-oxo-1, 4, 5, 6-tetrahydro-3-pyridazinecarboxamide; N-CPOTPC) on the Ca^{2+} response elicited by 1 μM PregS. (E) Overlap of N-CPOTPC with PregS. The data shown in (B) and (C) are an average of two wells of a 96-well plate per chemical; N/*n* = 12/3 for (D). **P* < 0.05. For details of the chemicals, see Supplementary Table S1.

only weakly stimulated by Preg, DHEAS or DHEA, and not stimulated by progesterone, oestradiol, testosterone or aldosterone. It adds that oestradiol sulphate, cortisol and vitamin D (cholecalciferol and ergocalciferol) do not stimulate TRPM3 channels. It shows that the sulphate of ring A is important for stimulation and that there is a striking requirement for the *cis* (β) configuration of this side group, revealing stereo-selectivity consistent with a specific binding site. Also revealed was partial agonism and importance of the double bond in ring B, the acetyl group at ring D and the general steroid backbone; the important but subtle differences in steroid structural requirements are shown in 3-D in Supplementary Figure S4. Consistent with these chemical requirements, Epipregnas was identified as a novel TRPM3 channel stimulator with slightly less efficacy and potency compared with PregS.

It has been suggested that the stimulation of TRPM3 channels by PregS has only pharmacological relevance based on electrophysiological studies of mouse TRPM3 channels (Wagner *et al.*, 2008). We note, however, that our investigation of human TRPM3 channels in Ca^{2+} measurement assays revealed greater potency, yielding a threshold near 0.1 μM rather than 1 μM, which brings it into the range of PregS concentrations detected in serum (Tagawa *et al.*, 2000). DHEAS also had considerably greater potency than indicated previously, bringing it into the range of physiological concentrations

(Tagawa *et al.*, 2000). The data were generated from a TRPM3 clone expressed ectopically in HEK 293 cells, possibly at levels that exceed those of native cells, but the data raise the possibility that physiological concentrations of PregS and DHEAS are sufficient to evoke functionally significant Ca^{2+}-entry through TRPM3 channels. The difference compared with the previous result (Wagner *et al.*, 2008) may reflect higher sensitivity of the Ca^{2+} measurement assays or differences in the human, compared with mouse TRPM3 channels. Although our study has identified a previously unrecognized TRPM3 channel stimulator with high efficacy (Epipregnas), it was not more effective or potent than PregS, and endogenous concentrations of Epipregnas are not thought to rise above 0.1 μM (Havlikova *et al.*, 2006; Hill *et al.*, 2007; Ocvirk *et al.*, 2009).

Steroids act through classical nuclear steroid receptors (Tsai and O'Malley, 1994) but rapid non-genomic effects are also well recognized (Losel and Wehling, 2003). Such acute actions of steroids on the NMDA and $GABA_A$ ligand-gated ion channels have received particular attention (Harrison *et al.*, 1987; Majewska, 1992; Poisbeau *et al.*, 1997; Park-Chung *et al.*, 1999; Mukai *et al.*, 2000; Charalampopoulos *et al.*, 2008; Schumacher *et al.*, 2008; Sedlacek *et al.*, 2008; Zheng, 2009). Like TRPM3 channels, NMDA channels show permeability to Ca^{2+} and Na^+, whereas $GABA_A$ channels are permeable to Cl^- and are inhibitory in the adult nervous system. As for the TRPM3 channels, PregS and DHEAS stimulate NMDA ion channels, whereas they inhibit $GABA_A$ channels (Demirgoren *et al.*, 1991; Wu *et al.*, 1991; Park-Chung *et al.*, 1999). Epipregnas, however, stimulates TRPM3 channels but inhibits NMDA and $GABA_A$ channels (Park-Chung *et al.*, 1997; 1999). There are other similarities and differences in the structure-activity relationships. Like TRPM3, $GABA_A$ channels are relatively resistant to modulation by Preg or DHEA (Harrison *et al.*, 1987; Demirgoren *et al.*, 1991; Park-Chung *et al.*, 1999; Wardell *et al.*, 2006) but, unlike TRPM3, $GABA_A$ channels are similarly affected by DHEAS and PregS, and $GABA_A$ and NMDA channels are sensitive to Pregnas or Andros (Park-Chung *et al.*, 1997; 1999; Kussius *et al.*, 2009). Therefore, the TRPM3 channel exhibits steroid structure-relationships that are distinct from those of the NMDA and $GABA_A$ channels, suggesting that TRPM3 channels may exist to sense a different neurosteroid profile.

Our data support the hypothesis that TRPM3 channels contain a specific steroid binding site that is quite well conserved between the human and mouse channels despite substantial sequence differences, particularly in the C-terminus (Oberwinkler *et al.*, 2005). Therefore, the membrane-spanning regions or the proximal N-terminus are likely to contain the steroid binding site or the amino acids involved in coupling TRPM3 channels to a steroid binding protein.

In summary, the study expands knowledge of steroid stimulation of TRPM3 channels and provides the first characterization of steroid-sensitivity of the human channel. Unexpectedly high potencies of PregS and DHEAS were detected in Ca^{2+} measurement assays, suggesting the potential physiological relevance of these agonists as stimulators of TRPM3-dependent Ca^{2+}-entry. Importance of *cis* (β) isomerism was revealed along with other highly specific chemical requirements around the steroid backbone; the data support the hypothesis that TRPM3 channels contain a specific steroid binding site. Evidence for partial agonism was obtained and a previously unrecognized TRPM3 channel stimulator (Epipregnas) was identified.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Current-voltage relationships (I-Vs) of steroid-induced currents. The I-V relationships of the currents induced upon bath-application of PregS (A), pregnenolone (Preg) (B), DHEAS (C) and epipregnanolone sulphate (Epipregnas) (D). Steroids were bath-applied at a final concentration of 100 μM. The I-Vs all show outward rectification at positive voltages and, in some cases, inward rectifi-

cation at negative voltages. In some experiments, the I-V tended towards a more linear relationship as observed previously when studying the same TRPM3 clone but using a different patch-clamp method and ionic solutions (Naylor *et al.*, 2008). However, in this study, we selected for experiments where the I-V showed strong outward rectification to enable easier comparison with a previous report on structure-activity relationships of steroid activation of TRPM3 (Wagner *et al.*, 2008).

Figure S2 PregS-evoked ionic currents were largely absent when TRPM3 over-expression was not induced. (a, b) Time-series plot from a whole-cell voltage-clamp experiment showing lack of effect of 100 μM PregS in control (Tet-) cells (A) and the I/V relationship (B). (C) Mean data for currents induced by 100 μM PregS in cells over-expressing TRPM3 (Tet+) and control (Tet-) cells (n = 3 for each).

Figure S3 Chemical structures of relevant steroids: sex steroids (A), corticosteroids (B) and secosteroids (C).

Figure S4 3D structures of strong and weak TRPM3 stimulators. Abbreviated names of steroids are the same as those used in the main paper.

Table S1 Details of the look-a-like chemicals.

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