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Etomidate and propylene glycol activate nociceptive TRP ion channels

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Abstract

Background: Etomidate is a preferred drug for the induction of general anesthesia in cardiovascular risk patients. As with propofol and other perioperatively used anesthetics, the application of aqueous etomidate formulations causes an intensive burning pain upon injection. Such algogenic properties of etomidate have been attributed to the solubilizer propylene glycol which represents 35% of the solution administered clinically. The aim of this study was to investigate the underlying molecular mechanisms which lead to injection pain of aqueous etomidate formulations.

Results: Activation of the nociceptive transient receptor potential (TRP) ion channels TRPA1 and TRPV1 was studied in a transfected HEK293t cell line by whole-cell voltage clamp recordings of induced inward ion currents. Calcium influx in sensory neurons of wild-type and *trp* knockout mice was ratiometrically measured by Fura2-AM staining. Stimulated calcitonin gene-related peptide release from mouse sciatic nerves was detected by enzyme immunoassay. Painfulness of different etomidate formulations was tested in a translational human pain model. Etomidate as well as propylene glycol proved to be effective agonists of TRPA1 and TRPV1 ion channels at clinically relevant concentrations. Etomidate consistently activated TRPA1, but there was also evidence for a contribution of TRPV1 in dependence of drug concentration ranges and species specificities. Distinct N-terminal cysteine and lysine residues seemed to mediate gating of TRPA1, although the electrophile scavenger N-acetyl-L-cysteine did not prevent its activation by etomidate. Propylene glycol-induced activation of TRPA1 and TRPV1 appeared independent of the concomitant high osmolarity. Intradermal injections of etomidate as well as propylene glycol evoked severe burning pain in the human pain model that was absent with emulsification of etomidate.

Conclusions: Data in our study provided evidence that pain upon injection of clinical aqueous etomidate formulations is not an unspecific effect of hyperosmolarity but rather due to a specific action mediated by activated nociceptive TRPA1 and TRPV1 ion channels in sensory neurons.

Keywords

Etomidate, propylene glycol, anesthetic, transient receptor potential ion channel, TRPA1, TRPV1, injection pain, nociception, sensory neuron, human pain model

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Background

The general anesthetic etomidate is one of the preferred drugs for the induction of anesthesia in cardiovascular risk patients as, in contrast to other substances such as propofol and thiopental, it causes only small changes in blood pressure and hardly exerts negative inotropic effects.[1] Due to its short context-sensitive half-life, etomidate is also used for sedation in short procedures such as cardioversion[2] and electroconvulsive therapy.[3]

However, etomidate exhibits some clinically relevant side effects. It can decrease release of cortisol and

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cortisone from the adrenal gland leading to a reduced stress response.[4] Etomidate can cause adrenal insufficiency even at a single dose whose sequelae have been discussed in recent years.[5–8] Long time administration can increase the morbidity and mortality of patients.[9] Especially application of etomidate to critically ill patients with sepsis has been under review, but the data concerning increased mortality or healthcare utilization remain inconclusive.[10]

Depending on the formulation and solvent, 4% to 90% of patients receiving etomidate intravenously suffer from burning pain during injection.[11–15] Moreover, etomidate preparations often cause venous sequelae such as thrombosis and thrombophlebitis in the injection vein.[11–15] Aqueous etomidate compounds containing propylene glycol (PG) as solubilizer (e.g. Hypnomidate®, JANSSEN-CILAG, Neuss, Germany) are known to exhibit these side effects, whereas a newer etomidate formulation based on a lipid emulsion as carrier (Etomidat-® Lipuro, B. Braun, Melsungen, Germany) induces nearly no pain or venous sequelae when injected.[13] The unphysiologically high osmolarity of PG-containing etomidate formulations (4965 mosmol/l) has been considered as an explanation of those unpleasant adverse effects. Although the disadvantage of injection pain can be eliminated symptomatically either by usage of the lipid-based etomidate formulations or by prior application of local anesthetics, the underlying molecular mechanisms remain poorly understood by which compound(s) in aqueous etomidate preparations and how nociceptive nerves are activated and generate pain.

Several groups have recently shown that local and general anesthetics directly activate the irritant sensor TRPA(Ankyrin)1 and the capsaicin receptor TRPV (Vanilloid)1 in primary nociceptive neurons, two members of the transient receptor potential (TRP) superfamily. Those ion channels have been identified as mediators for injection pain and pain-related behavior in rodents induced by propofol.[16–19] TRPA1 is involved in chemical nociception and can be activated by pungent ingredients of horseradish (allyl isothiocyanate (AITC)) and garlic (allicin) as well as by airway-irritating substances such as acrolein and isoflurane.[20–23] TRPV1 is a polymodal receptor and can be activated by capsaicin, the hot ingredient of chili peppers, noxious heat, low pH, and various other irritants.[24,25] Matta et al. have already shown that etomidate activates recombinant TRPA1 channels in cell line[17] but its relevance for etomidate-induced modulation of primary sensory neurons has not been analyzed.

The hypothesis of this study was whether etomidate could excite primary nociceptive neurons and specifically activate TRPA1 and/or other TRP channels, whether PG could interact with TRP channels, and whether both induce local pain in humans. Whole-cell patch-clamp recordings on HEK293t cells expressing recombinant TRP channels and calcium imaging experiments on cultured sensory neurons from wild type and knockout mice lacking *Trpa1* and/or *Trpv1* were performed. The ability of etomidate and PG to cause neurogenic inflammation was examined by measurements of release of the neuropeptide calcitonin gene-related peptide (CGRP) from isolated nerves of mice. Finally, the injection pain and flare response induced by different etomidate formulations and PG were explored in a human pain model.

Material and methods

Animals

Animal care and treatment were conducted according to the International Association for the Study of Pain guidelines,[26] and all procedures of this study were approved by the animal protection authorities (district government, Ansbach, Germany). Adult (8–12 weeks) female and male wild-type C57BL/6, *Trpv1*-knockout ($Trpv1^{-/-}$), *Trpa1*-knockout ($Trpa1^{-/-}$), and *Trpv1*/*Trpa1*-double-knockout mice ($Trpv1/Trpa1^{-/-}$) with C57BL/6 background were used. Original breeding pairs of $Trpv1^{-/+}$ and $Trav1^{-/+}$ mice were generous gifts from Dr. John Davis (formerly GSK, Harlow, UK)[27] and Dr. David Corey (Harvard University, Boston, MA)[28] and continuously backcrossed to C57BL/6. Double-knockout animals were generated in our animal facility by cross-matching knockouts of both strains. All animals were genotyped prior to experiments using previously reported primers.

Cell culture

Animals were killed in an atmosphere of rising CO_2 concentration. Dorsal root ganglion (DRG) cells from all spinal levels were excised, transferred into Dulbecco's modified Eagle's medium (DMEM) solution (GIBCO-Invitrogen, Germany) containing 50 μg/ml gentamicin (Sigma-Aldrich, Germany), and incubated in 1 mg/ml collagenase (Sigma type XI) and 0.1 mg/ml protease (Sigma) for 40 min at 37°C. The ganglia were then gently dissociated using a fire-polished silicone-coated Pasteur pipette, and neurons were plated onto borosilicate glass coverslips which had been coated with poly-D-lysine (0.2 mg/ml for 30 min, Sigma-Aldrich, Germany). Cells were cultured in serum-free TNB-100 basal medium supplemented with TNB 100 lipid-protein complex, 100 U/ml streptomycin, penicillin (all Biochrom, Germany), and mouse nerve growth factor (NGF, 100 ng/ml, Almone Labs, Israel) at 37°C under 5% CO_2

atmosphere. Calcium imaging experiments were performed within 20–30 h of dissociation.

Heterologous expression and mutagenesis

Human embryonic kidney (HEK) 293t cells were cultured in DMEM (GIBCO-Invitrogen, Germany), supplemented with 100 U/ml penicillin/streptomycin, 25 mM HEPES, 10% heat-inactivated fetal bovine serum (all GIBCO-Invitrogen), and 3 mM taurine (Sigma-Aldrich, Germany) at 37°C under 5% CO_2 atmosphere. Transient transfections were carried out either by calcium phosphate precipitation as described previously[29] or by the Nanofectin transfection method (PAA—The Cell Culture Company, Coelbe, Germany). HEK cells were plated in 35-mm culture dishes. After 6 to 10 h, 6.4 µl Nanofectin, 0.45 µg of the CD8-pih3m reporter plasmid, and 1.8 µg of the desired DNA were added to the cells and incubated for 12–14 h. Subsequently, cells were replated in 35-mm culture dishes and used for experiments within two days. HEK cells were transfected with TRPA1 from mouse (mTRPA1, pcDNA3-vector) or human (hTRPA1, pTRE2-vector) or with TRPV1-TRPV4, TRPM8 from rat (rTRPV1-TRPV4, rTRPM8, all pcDNA3-vector). Transfected cells were identified by immunobeads (CD-8 Dynabeads; Dynal Biotech, Norway). Mutagenesis of hTRPA1 (hTRPA1-C621S/C641S/C665S and hTRPA1-C621S/C641S/C665S/K710R) was performed with the Qiagen Plasmid Maxi Kit (Qiagen, Germany) using specifically designed mutagenic and selection primers. All constructs were confirmed by DNA sequencing. mTRPA1 was a generous gift from Dr Ardem Patapoutian (The Scripps Research Institute, La Jolla, CA, USA), hTRPA1 was a generous gift from Dr Paul A Heppenstall (EMBL, Monterotondo, Italy). All other cDNAs were generous gifts from Dr David Julius (University of California San Francisco, CA).

Ratiometric $[Ca^{2+}]_i$ measurements

Cells were stained by 5 µM fura-2 AM and 0.02% pluronic (both from Invitrogen, Carlsbad, CA, USA) for about 30 min. Following a 30-min washout period to allow for fura-2-AM ester hydrolysis, coverslips were mounted on an Olympus IX71 inverse microscope with a 10× objective. Fura-2 was excited at 340 and 380 nm with a Polychrome V monochromator (Till Photonics). Images were exposed for 200 µs and acquired at a rate of 1 Hz with a 12 bit CCD camera (Imago Sensicam QE, Till Photonics, Gräfelfing, Germany). Data were recorded and further analyzed using TILLvisION 4.0.1.3 software (Till Photonics, Gräfelfing, Germany). Background was subtracted before the calculation of ratios. Etomidate and AITC were applied for 30 s, and cells were exposed to capsaicin for 10 s. A 60-mM potassium stimulus (DRG cells) was applied as a control at the end of each experiment. The area under the curve of F340/380 nm ratios was quantified for regions of interest adapted to the neurons.

Patch-clamp recordings

Whole-cell voltage clamp recordings were acquired with an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, USA). Currents were filtered at 1 kHz and sampled at 5 kHz. All experiments were stored on a PC for off-line analysis using the pCLAMP 10 software (Axon Instruments/Molecular Devices). Current density was calculated by division of the evoked current by the measured cell capacitance. Microcal Origin 8.1 software (OriginLab Corp., USA) was used to perform curve fitting and to create figures. Patch pipettes fabricated from borosilicate glass tubes (TW150F-3; World Precision Instruments, Germany) were pulled to a resistance of 1.5 to 2.5 MΩ after heat polishing. Standard external solution contained 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 5 mM EGTA (adjusted with tetramethylammonium hydroxide to pH 7.4). Standard internal solution contained 140 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, and 5 mM EGTA (adjusted with potassium hydroxide to pH 7.4). All experiments were performed at room temperature, and cells were held at −60 mV. All solutions were applied with a polytetrafluorethylen glass multiple-barrel perfusion system.

Release of CGRP

Adult mice were sacrificed under rising CO_2 atmosphere, and sciatic nerves were exposed and excised from their origin in the lumbar plexus to the trifurcation into tibial, sural, and peroneal nerves. Isolated nerves were loosely tied around acrylic rods and placed in synthetic interstitial fluid (SIF), containing (in mM) 108 NaCl, 3.48 KCl, 3.5 $MgSO_4$, 26 $NaHCO_3$, 1.7 NaH_2PO_4, 1.5 $CaCl_2$, 9.6 sodium gluconate, 5.5 glucose and 7.6 sucrose, constantly gassed with carbogen (95% O_2. 5% CO_2) to pH 7.4, and positioned in a thermostatic shaking bath set to 32°C for a washout period of 30 min. Nerves were then consecutively passed through a series of incubation steps, each lasting for 5 min at 32°C. Basal CGRP release was determined in the first two incubation steps with test tubes containing SIF. The third step assessed stimulated CGRP release, while nerves were incubated with different test solutions. The last incubation period in SIF solution again allowed recovery of CGRP release levels. CGRP contents were determined using commercial enzyme immunoassays (Bertin Pharma, France) and

photometrical analysis using a microplate reader (Opsys MRTM, Dynex Technologies, USA).

Psychophysics

Studies on human volunteers were approved to fulfill the requirements of the Declaration of Helsinki by the local ethics committee of the Friedrich-Alexander University of Erlangen-Nuernberg, and experimental procedures were limited to four co-authors of the present study. Test persons were intradermally injected with different etomidate formulations and their respective carrier solutions in a double-blinded manner. The syringes were prepared by an uninvolved person, non-transparent tape was wrapped around the syringes to hide the solutions from view and avoid identification of lipid-based formulations; solutions were sterile filtered. Injections of 100 µl of each preparation were performed with 27-gauge needles in separate areas of both volar forearms. The following substances were tested: Etomidat-® Lipuro, Hypnomidate®, etomidate dissolved in Ringer's solution with methanol as solubilizer (pH adjusted to 7.4 with NaOH), Lipofundin®, PG 35% dissolved in Ringer's solution (pH adjusted to 7.4 with NaOH), and methanol in Ringer's solution (pH adjusted to 7.4). After injection of each formulation, pain was assessed on a numerical rating scale (0–10, 0 = no pain, 10 = maximum pain) every 15 s for a period of 10 min. To assess changes in superficial cutaneous blood flow following injection of etomidate formulation, laser Doppler imaging (LDI) was performed as described previously.[29] A rectangular region of the skin around the injection site was scanned by the laser Doppler imager (Moor, UK). Two scans were performed before each injection to define baseline skin perfusion, while the following scans were started immediately after injection of a formulation. Scans were performed at 0, 2.5, 5, 7.5, and 10 min after injection, each scan took 2 min. Area of superficial vasodilatation was analyzed with MDLI 3.0 software (Moore) and defined as pixels in which intensity exceeded the mean of basal values plus two standard deviations.

Chemicals

Etomidat-® Lipuro, Lipofundin® (both B. Braun Melsungen AG, Germany) and Hypnomidate® (JANSSEN-CILAG GmbH, Germany) were purchased from the hospital pharmacy of the University Clinics of Erlangen. Etomidate (Sigma-Aldrich, Germany), BCTC, HC-030031 (both Biotrend, Germany), AP-18, 2-aminophenyl borane (2-APB) (both Tocris Bioscience, UK) were dissolved in dimethyl sulfoxide to give stock solutions of 1–100 mM. AITC, capsaicin, menthol, and 4αPDD (all Sigma-Aldrich, Germany) were dissolved in ethanol to give stock solutions of 1 to 100 mM. PG and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich, Germany. All formulations, substances, and stock solutions were diluted with standard physiological buffers (pH 7.4) immediately before experimental use.

Statistical analysis

Calculations of statistical comparisons were performed with the Statistica 7.0 software package (StatSoft, USA) or with the Origin 8.2 software package (OriginLab Corporation, USA). Most data are presented as mean ± SEM. Calculated EC_{50} values are presented as mean and 95% confidence interval (CI). Statistical tests used are mentioned in the text or figure legends. Differences at p-values < 0.05 were considered statistically significant and marked with *.

Results

Etomidate activates and desensitizes mTRPA1

Matta et al.[17] previously reported that rat TRPA1 is activated by 100 µM etomidate. We first studied the activation characteristics of etomidate on mouse (m) TRPA1 transiently expressed in HEK293t cells. In whole-cell patch-clamp experiments, etomidate evoked inward currents at 100 µM and higher in a concentration-dependent manner with an EC_{50} of 375 µM (95% CI: 271–487 µM, n = 5–16 for each concentration, Figure 1(a) to (c)). In addition, 2500 µM etomidate was the highest concentration possible to test, as further increased concentrations led to loss of seal integrity. This etomidate concentration revealed declining currents during and off responses at the end of drug application (Figure 1(a)), suggesting that clinically used preparations of 8 mM etomidate could also exert a blocking effect on the channel. Three repeated applications of 1000 µM etomidate caused a significant desensitization of mTRPA1 with a mean inward current reduction between the first and the third etomidate application of 65% (95% CI: 43%–87%, n = 10, p = 0.0022, Figure 1(b)). Etomidate-induced currents on mTRPA1 were completely blocked by coapplication of the selective TRPA1-antagonist AP-18 (50 µM, remaining mean inward current: 3.2%, 95% CI: −3.9–10.3%, n = 5, p = 0.0004, paired t-test; Figure 1(d) and (e)).

Etomidate activates rTRPV1

Several thermosensitive TRP channels are expressed in sensory neurons which are presumably involved in heat, cold, and chemical nociception.[30] Therefore, we tested the activation of rat (r) TRPV1, TRPV2, TRPV3, TRPV4, and TRPM8 by etomidate in whole-cell patch-clamp experiments. In contrast to Matta et al.,[17]

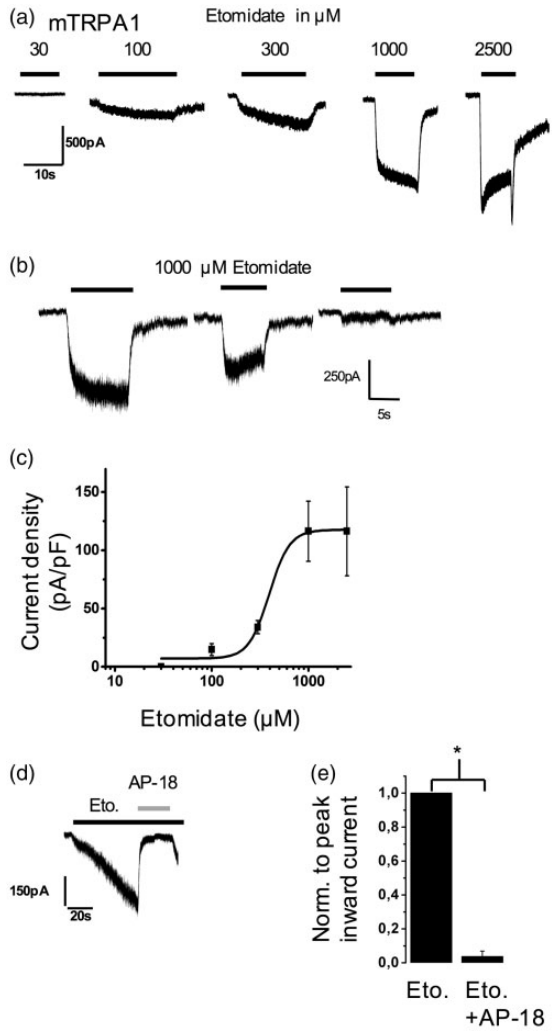

Figure 1. Etomidate activates and desensitizes mTRPA1. (a) Representative current traces of etomidate-evoked inward currents in HEK293t cells transiently expressing mTRPA1. To prevent desensitization only one concentration was tested on each cell. Cells were held at −60 mV, etomidate was applied until the current had reached a steady state. (b) Representative current traces evoked by three consecutive applications of 1000 µM etomidate, each applied at intervals of 2 min. (c) Concentration–response curve for etomidate-evoked inward currents in mTRPA1-expressing HEK293t cells; 6 to 10 cells were tested at each concentration. Data were fitted to the Hill equation. (d) Representative etomidate-evoked inward current blocked by the TRPA1 antagonist AP-18. 300 µM etomidate was applied until the current had reached a steady state, followed by a combined application of etomidate and 50 µM AP-18. (e) Normalized current amplitudes ± SEM measured in (d). Currents were normalized to the peak inward current during the steady state.
TRP: transient receptor potential.

we saw a strong activation of rTRPV1 by etomidate which, however, showed an inversely U-shaped dose–response curve; at 0.01 µM to 1 µM, etomidate evoked inward currents with increasing current amplitudes (current density: 0.01 µM: 9.1 ± 3 pA/pF, n = 6; 0.1 µM: 53 ± 16 pA/pF, n = 11; 1 µM: 245 ± 35 pA/pF, n = 29. Figure 2(a)). Higher concentrations of etomidate evoked decreasing current amplitudes (current density: 10 µM: 138 ± 47 pA/pF, n = 17; 100 µM: 104 ± 31 pA/pF, n = 13; 500 µM: 43 ± 10 pA/pF, n = 19; 2500 µM: 9 ± 1 pA/pF, n = 7; Figure 2(a)). Comparing the maximum effect of etomidate at 1 µM with that of capsaicin at 100 nM indicated that the prototypical TRPV1 agonist was not only more potent but also more efficient in the activation of inward currents through TRPV1 (Figure 2(a) and (c)).

Fitting the first three data points of the concentration–response curve to the Hill function revealed a half-maximal activation concentration of etomidate of approximately 240 nM for the activation of TRPV1 and an apparently linear decline of the current amplitude beyond 1 µM etomidate (Figure 2(b)). Xiao et al.[31] have shown that menthol exhibits an inversely U-shaped dose–response of mTRPA1. While low concentrations of menthol activate mTRPA1, higher concentrations block the receptor. We asked if the same principle could be an explanation for our findings with etomidate on TRPV1, which was confirmed by further experiments showing the concentration-dependent channel-blocking properties of etomidate on the capsaicin receptor. rTRPV1 was activated by 100 nM capsaicin until the current had reached a steady state, and then 100 µM etomidate was co-applied, causing a marked decrease in the capsaicin-evoked current. After switching back to capsaicin alone, the current increased again. The mean current reduction by 100 µM etomidate was 32% (95% CI: 20.2%–43.8%, n = 5, Figure 2(c)).

The selective TRPV1 antagonist BCTC (10 µM) was able to completely block the activation of TRPV1 induced by 1 µM etomidate (n = 5, data not shown). Unspecific effects did not occur, as non-transfected HEK293t cells showed no response to application of 1 µM etomidate (data not shown).

In additional experiments, we tested the activation of rTRPV2, rTRPV3, and rTRPV4 by 300 µM etomidate (each at least n = 4, Figure 2(d)). However, etomidate failed to activate any of these TRP channels. Matta et al.[17] described a block of rTRPM8 by 100 µM etomidate which we also detected in experiments using 1000 µM etomidate (Figure 2(d)). We observed a stabilization of the leak current which we interpreted as a block of tonically activated rTRPM8 channels. To verify that rTRPM8 does not show a similar bimodal activation curve-like rTRPV1, we also tested a lower concentration of 10 µM etomidate which did not activate rTRPM8 but induced the same leak-stabilizing effect as 1000 µM (data not shown).

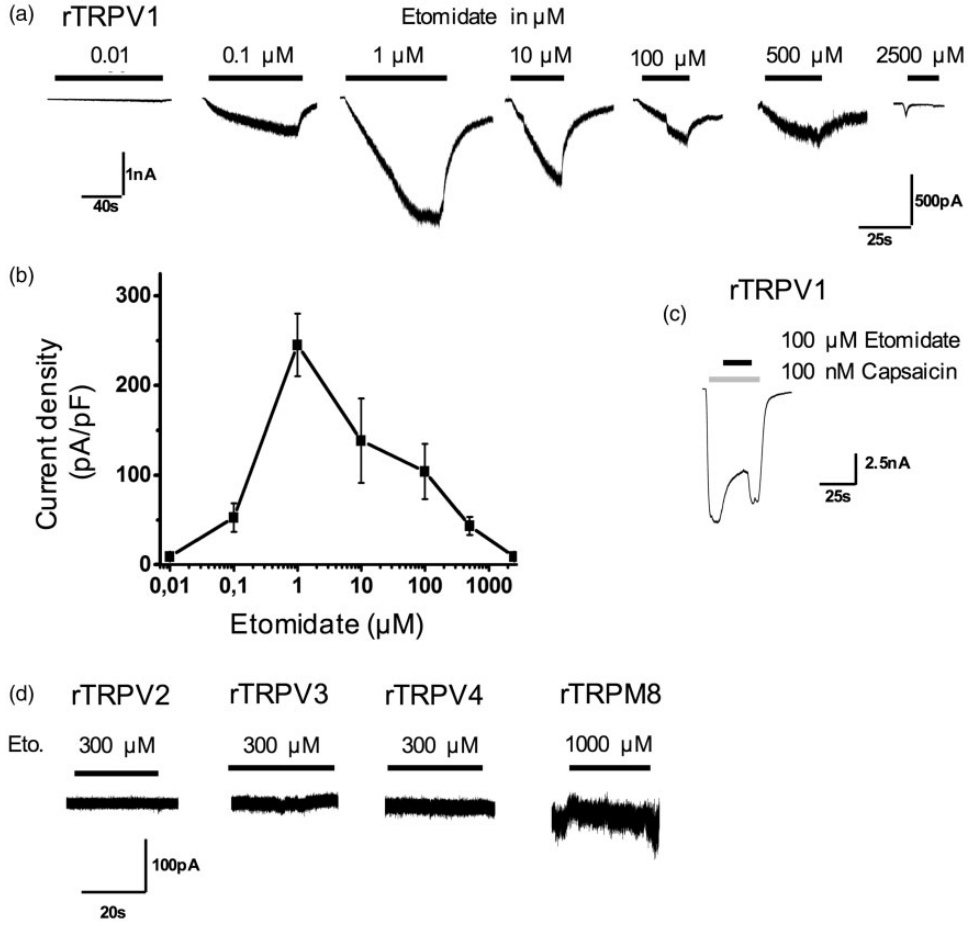

Figure 2. Etomidate activates rTRPV1. (a) Representative current traces evoked by 0.01–2500 µM etomidate in rTRPV1-expressing HEK293t cells. To prevent desensitization, only one concentration was tested on each cell. Cells were held at −60 mV, etomidate was applied for at least 20 s. Note the changed scales at 500 and 2500 µM etomidate. (b) Concentration–response curve for etomidate-evoked inward currents in rTRPV1-expressing HEK293t cells; between 6 and 29 cells were tested at each concentration. Note the bell-shaped curve progression suggesting channel block at etomidate concentrations > 1 µM. (c) Representative capsaicin-evoked inward current of a rTRPV1-expressing HEK293t cell blocked by 100 µM etomidate. Capsaicin (100 nM) was applied until the current had reached a steady state followed by a combined application of capsaicin and 100 µM etomidate. (d) Etomidate did not activate rTRPV2-, rTRPV3-, rTRPV4- or rTRPM8-expressing HEK293t cells. Cells were held at −60 mV, etomidate was applied for at least 20 s. Note the leak current-stabilizing effect of etomidate on rTRPM8. Channel expression was verified by a subsequent application of the TRPV2 and TRPV3 agonist 2-APB, the TRPV4 agonist 4αPDD and the TRPM8 agonist menthol (not shown); at least four cells were tested for each TRP channel.
TRP: transient receptor potential.

Etomidate-induced activation of TRPA1 shows species-dependent properties

As mentioned previously, Xiao et al.[31] described species-dependent activation of TRPA1 by menthol. We wondered if effects of etomidate on TRPA1 also show species-specific differences, and thus examined the activation of human (h) TRPA1 by etomidate. HEK293t cells expressing hTRPA1 produced small inward currents at an etomidate concentration of 30 µM (current density: 15 ± 5 pA/pF, n = 5, Figure 3(a)). 70 and 100 µM etomidate showed concentration-dependent increases in inward currents (current densities: 70 µM: 48 ± 8 pA/pF, n = 6; 100 µM: 131 ± 26 pA/pF, n = 17; Figure 3(a)). While 300 as well as 1000 µM etomidate did not evoke significantly greater inward currents than 100 µM (current densities: 300 µM: 136 ± 19 pA/pF, n = 8; 1000 µM: 128 ± 45 pA/pF, n = 5), 2500 µM of etomidate showed further increasing inward currents with a mean current density of 416 ± 90 pA/pF (n = 8, Figure 3(a)). In contrast to mTRPA1 calculation of a concentration–response curve of etomidate-induced inward currents on hTRPA1 revealed a bimodal course with two steep rises between 30 and 100 µM and between 300 and 2500 µM (Figure 3(b)). Concentrations from 10 to 1000 µM etomidate could be fitted to the Hill equation and an EC_{50} of 73 µM (95% CI: 73–81 µM) for the first rise of the curve was calculated. In general, etomidate seems to

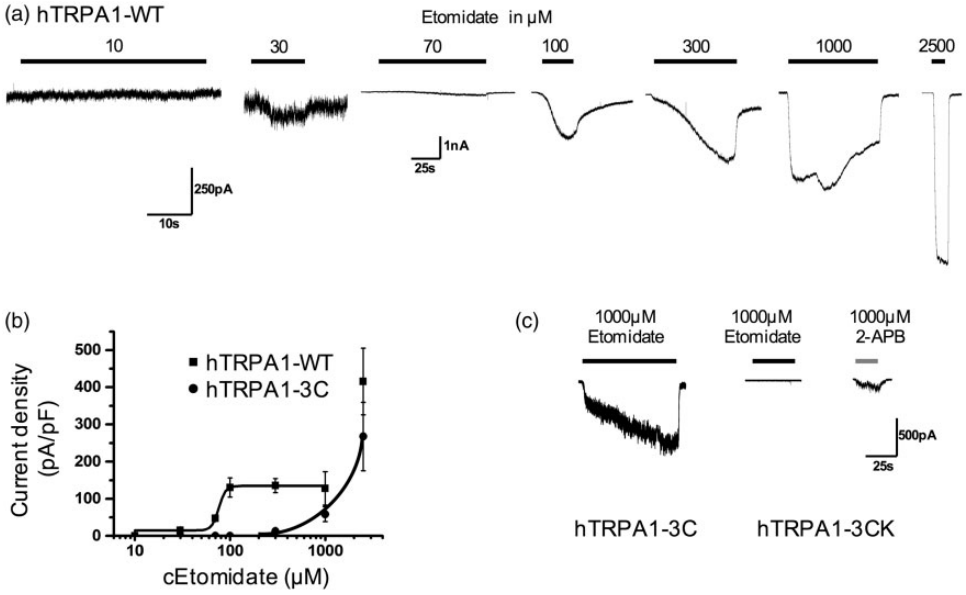

Figure 3. Activation of hTRPA1 by etomidate involves N-terminal cysteine and lysine residues. (a) Representative current traces of etomidate-evoked inward currents in HEK293t cells transiently expressing hTRPA1. To prevent desensitization, only one concentration was tested on each cell. Cells were held at −60 mV, etomidate was applied until the current had reached a steady state. (b) Concentration–response curve for etomidate-evoked inward currents in hTRPA1- and hTRPA1-C621S/C641S/C665S (3C)-expressing HEK293t cells; 6 to 10 cells were tested at each concentration. Data were fitted to the Hill equation. Note the plateau followed by a second slope at concentrations over 100 µM in the curve of hTRPA1. (c) Representative current traces of the hTRPA1 mutants C621S/C641S/C665S (3C) and C621S/C641S/C665S/K710R (3CK) evoked by 1000 µM etomidate. Cells were held at −60 mV, etomidate was applied for at least 25 s. In 3CK-expressing cells, channel expression was verified by a subsequent and effective application of the TRPA1 agonist 2-APB (not shown), while the functionality was tested using a single application of 2-APB 1000 µM per cell.
TRP: transient receptor potential; WT: wild type; 2-APB: 2-amino-phenyl borane.

have a greater potency in activating hTRPA1 than mTRPA1. One possible explanation for the observed results could be a second binding site in hTRPA1 with a lower affinity for etomidate but a stronger intrinsic effect.

Activation of hTRPA1 by etomidate involves N-terminal cysteine and lysine residues

Next, we were interested in the molecular mechanisms by which etomidate activates TRPA1. The inward currents evoked by low concentrations of etomidate (i.e. < 1000 µM) in TRPA1-expressing HEK293t cells resemble in general TRPA1-currents induced by reactive substances such as allylisothiocyanate (AITC) and acrolein. Two different groups have shown in the past that TRPA1 can be activated by these electrophilic substances through modification of distinct cysteine and lysine residues in the intracellular N-terminal ending of the channel protein.[32,33] We asked if such covalent modifications also apply to the effect of etomidate on hTRPA1. Initially, we tested the hTRPA1-mutant C621S/C641S/C665S (hTRPA1-3C) which was described by Hinman et al.[33] and which was shown to exhibit a reduced potency of AITC to evoke inward currents. As mentioned previously, 30 µM etomidate slightly activated hTRPA1, whereas the same concentration did not evoke any inward currents in hTRPA1-3C-expressing HEK293t cells. Inward currents induced by etomidate concentrations from 100 to 2500 µM displayed concentration-dependent increases in magnitude (current densities: 100 µM: 3 pA/pF, $n = 12$; 500 µM: 13 ± 4 pA/pF, $n = 5$; 1000 µM: 59 ± 20 pA/pF, $n = 5$; 2500 µM 268 ± 92 pA/pF, $n = 6$; Figure 3(b)). Similar to the data presented by Hinman et al.[33] on AITC-evoked activation of hTRPA1-3C, etomidate also showed a reduced potency and a resulting rightward-shift of the concentration–response curve in this mutant with an EC_{50} value of about 1390 µM compared to wild-type hTRPA1 (95% CI: 1040–1200 µM, Figure 3(b)). Moreover, Hinman et al. could also demonstrate that replacement of a lysine residue (K710) by arginine rendered hTRPA1-3C completely insensitive to AITC.[33] We tested this hTRPA1-3CK-mutant with etomidate and also found a complete loss of responsiveness (Figure 3(c)). Since the sensitivity to 2-APB, a non-electrophilic TRPA1 agonist not reacting with cysteines, was reported to be fully retained,[33] expression and functionality of the hTRPA1-3CK mutation were tested using 2-APB as control. In our hands, the quadruple

mutant-expressing HEK293t cells showed reproducible but reduced responses to 2-APB 1000 μM with current densities of 197 ± 168 pA/pF (n = 4; Figure 3 (c)), suggesting a partial loss of function of the hTRPA1-3CK mutant channel.

Covalent reactions with the TRPA1 protein can effectively be prevented by loading cells with the membrane permeable electrophile scavenger NAC.[19,34] However, pretreatment of hTRPA1-expressing HEK293t cells with NAC 5 mM did not prevent or reduce the activation of inward currents by etomidate 500 μM (n = 6, Supplemental Material Figure 2), suggesting TRPA1 activation by etomidate via a non-covalent protein modification.

Etomidate induces an increase in $[Ca^{2+}]_i$

Our results from whole-cell patch-clamp experiments on recombinant channels suggested that etomidate should activate sensory neurons expressing native mTRPA1 and/or mTRPV1. To corroborate this hypothesis, we first examined DRG neurons from wild-type C57BL/6 mice. As etomidate exerts its hypnotic actions via agonistic and sensitizing effects on gamma-Aminobutyric acid ($GABA_A$) receptor-channels and since these are also expressed in DRG neurons,[35] we conducted calcium imaging experiments in the presence of the $GABA_A$ antagonist picrotoxin (PTX, 100 μM). Under these conditions, 300 μM etomidate elicited an increase in $[Ca^{2+}]_i$ in AITC and capsaicin-sensitive neurons (Figure 4(a)). To verify a sufficient $GABA_A$ receptor block, 30 μM GABA was used as a control which had no effect, while PTX was present. Etomidate activated DRG neurons from wild-type mice in the presence of PTX with an EC_{50} of 762 μM (95% CI: 631–893 μM) and in absence of PTX with an EC_{50} of 743 μM (95% CI: 619–867 μM) (Figure 4(e), squares and triangles). To evaluate which receptors were involved in etomidate-induced $[Ca^{2+}]_i$ increase, we performed experiments with DRG neurons from wild-type mice and selective receptor antagonists. While application of PTX only caused a small reduction in etomidate-induced calcium increase, combined application of PTX and the TRPA1 antagonist AP-18 (15 μM) completely inhibited an increase in $[Ca^{2+}]_i$ upon etomidate application (analysis of variance, honest significant difference post hoc test, $p \leq 0.02$ each, Figure 4(b)). To further confirm this result, we tested DRG neurons from *Trpa1* knockout mice (*Trpa1*$^{-/-}$). In the presence of PTX 300 μM, etomidate did not evoke an increase in $[Ca^{2+}]_i$ in *Trpa1*$^{-/-}$ neurons (Figure 4(c)). Etomidate-evoked activation of *Trpa1*$^{-/-}$ neurons without PTX was minimal and provided a concentration–response curve with an EC_{50} of 41 μM (95% CI: 25–57 μM) (Figure 4(e), spheres). This corresponded well to the analysis of the relative contributions of TRPV1, TRPA1, and $GABA_A$ receptors to etomidate-evoked rises of $[Ca^{2+}]_i$. At a concentration of 100 μM etomidate, mainly cells also expressing $GABA_A$ receptors showed a Ca^{2+} increase (r = 0.913, p = 0.00, n = 239, Figure 4(d)), whereas the correlation to AITC (100 μM) and therefore to mTRPA1 was only weak (r = 0.277, p = 0.000004, n = 239, Figure 4(d), upper panels). The correlation to capsaicin and thus to mTRPV1 was negative (r = −0.096, p = 0.141, n = 235, not shown). At etomidate concentrations of 600 μM, the strong correlation to GABA responses (30 μM) was lost (r = 0.151, p = 0.000002, n = 934), while the correlation to AITC responses (100 μM) had notably increased (r = 0.569, p = 0.000000, n = 934, both Figure 4(d), lower panels). The correlation to mTRPV1 was weak again (r = 0.177, p = 0.000000, n = 309, not shown) seemingly independent of the concentration of the applied etomidate. Therefore, activation by lower μM concentrations of etomidate seems to be mediated by GABA receptors, whereas at higher μM concentrations, the increase in $[Ca^{2+}]_i$ seems to be mainly driven by mTRPA1.

While previous patch-clamp experiments indicated also a role for recombinant rat TRPV1 as a target of etomidate, the calcium imaging data from mouse DRG neurons did not confirm any contribution of the capsaicin receptor, suggesting major rat–mouse species differences for drug concentration ranges that were applicable on a cellular level, at least.

A well-established method for TRPV1 sensitization is preincubation with the protein kinase C (PKC) activator 4β-phorbol 12-myristate 13-acetate (PMA) which leads to phosphorylation of the serine residues 502 and 800 of TRPV1[36] and thus to enhanced agonist activity. We used DRG neurons from *Trpa1*$^{-/-}$ mice in the presence of 100 μM PTX and, after a first ineffective application of 300 μM etomidate, incubated them for 1 min with 100 nM PMA followed by a second etomidate application. But also after sensitization of mTRPV1, no activation of *Trpa1*$^{-/-}$ neurons could be observed (data not shown).

PG activates hTRPA1 and rTRPV1

It has been suggested that the injection pain from PG-containing solutions arises from the high osmolarity of those preparations and displays a rather unspecific effect.[15,37] Commercially available aqueous formulations of etomidate contain 35 volume-% PG which corresponds to a concentration of about 4770 mM. We were interested if PG had also specific effects on TRP channels. Therefore, we performed whole-cell patch-clamp recordings with non-transfected HEK293t cells (MOCK cells) and cells transiently expressing hTRPA1 or rTRPV1. Non-transfected HEK cells showed no response to 10% PG in standard external solution

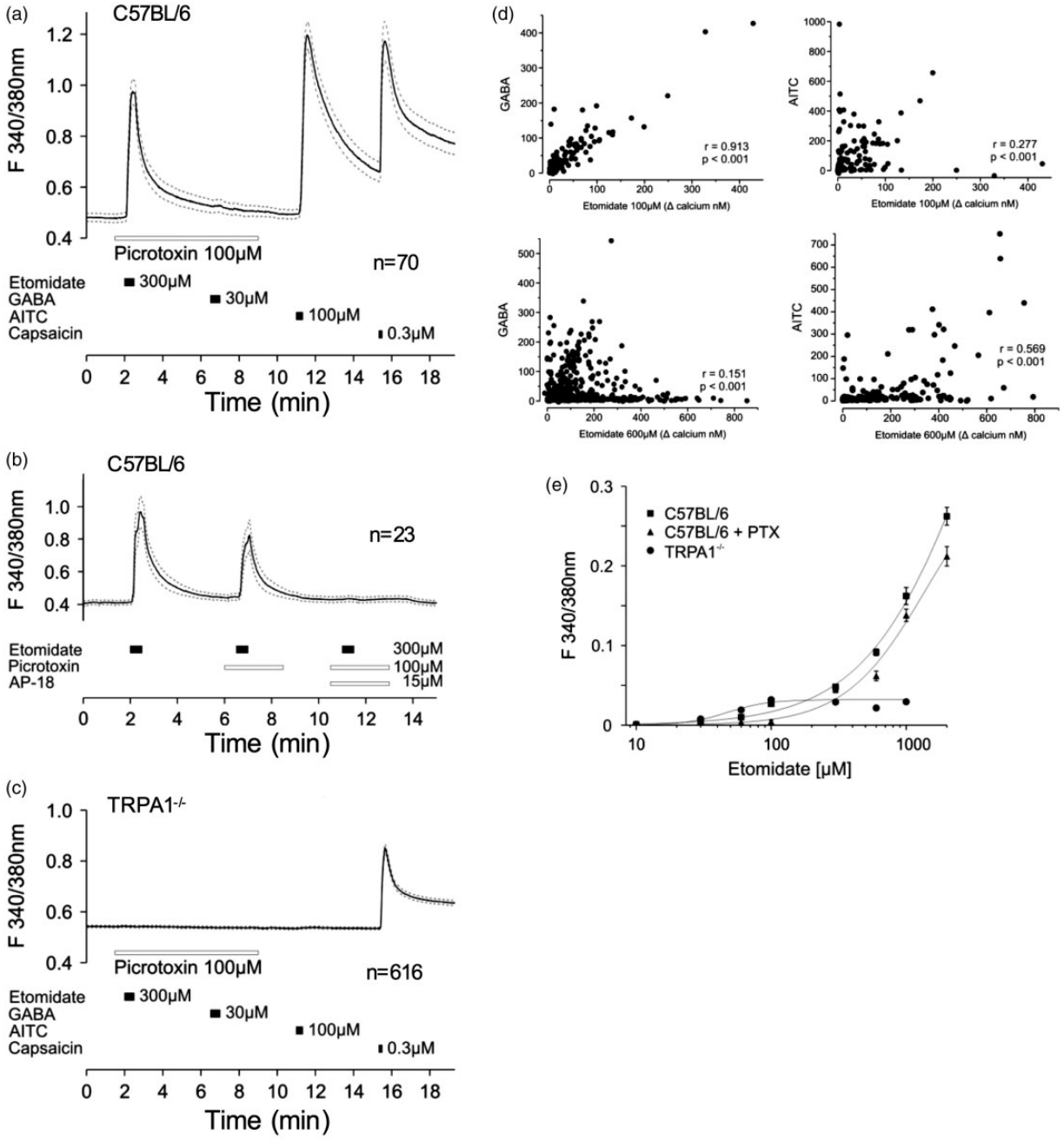

Figure 4. Etomidate induces an increase in $[Ca^{2+}]_i$ in sensory neurons. (a) Etomidate 300 µM lead to an increase in $[Ca^{2+}]_i$ in capsaicin- and AITC-sensitive DRG neurons from C57BL/6 mice during blockade of $GABA_A$-receptors by 100 µM PTX. Etomidate and GABA (30 µM) were applied for 30 s, AITC (100 µM) for 20 s, and capsaicin (0.3 µM) for 10 s. (b) Etomidate 300 µM induced an increase in $[Ca^{2+}]_i$ in DRG neurons from C57BL/6 mice. PTX 100 µM reduced etomidate-induced Ca^{2+} responses. Co-application of PTX and the TRPA1-antagonist AP-18 (15 µM) completely inhibited etomidate-induced Ca^{2+} increase. Etomidate was applied for 30 s, application of PTX either alone or in combination with AP-18 was started 30 s before and ended 120 s after etomidate. (c) Etomidate 300 µM did not lead to an increase in $[Ca^{2+}]_i$ in DRG neurons from $Trpa1^{-/-}$-mice during blockade of $GABA_A$ receptors by 100 µM PTX. Although neurons did not respond to 100 µM AITC applied as a control, 0.3 µM capsaicin evoked an immediate increase in $[Ca^{2+}]_i$ in a subpopulation of neurons. Etomidate was applied for 30 s, AITC for 20 s, and capsaicin for 10 s. (d) Etomidate (100 µM)-evoked Ca^{2+} increases in DRG neurons from C57BL/6 mice correlated better to responses evoked by 30 µM GABA than to responses evoked by 100 µM AITC. Note the inverse correlation at an etomidate concentration of 600 µM. Panels provide the product–momentum correlation coefficient *r*.
(e) Concentration–response curves for etomidate-evoked $[Ca^{2+}]_i$ increases in DRG neurons derived from C57BL/6 mice (with and without 100 µM PTX) and $Trpa1^{-/-}$ mice. Etomidate concentrations from 10 to 2000 µM were tested, the curves were fitted to the Hill equation. TRP: transient receptor potential; PTX: picrotoxin.

applied for 1 min (1366 mM, theoretical osmolarity 1687 mosmol/l, n = 5, Figure 5(a)). Cells showed a volume reduction indicating osmotic loss of intracellular water. After the end of PG application, cells grew again to their original volume. While cells expressing hTRPA1 did not show any activation by application of 1% PG (136 mM, theoretical osmolarity 457 mosmol/l, n = 13), 5% PG (683 mM, theoretical osmolarity 1004 mosmol/l),

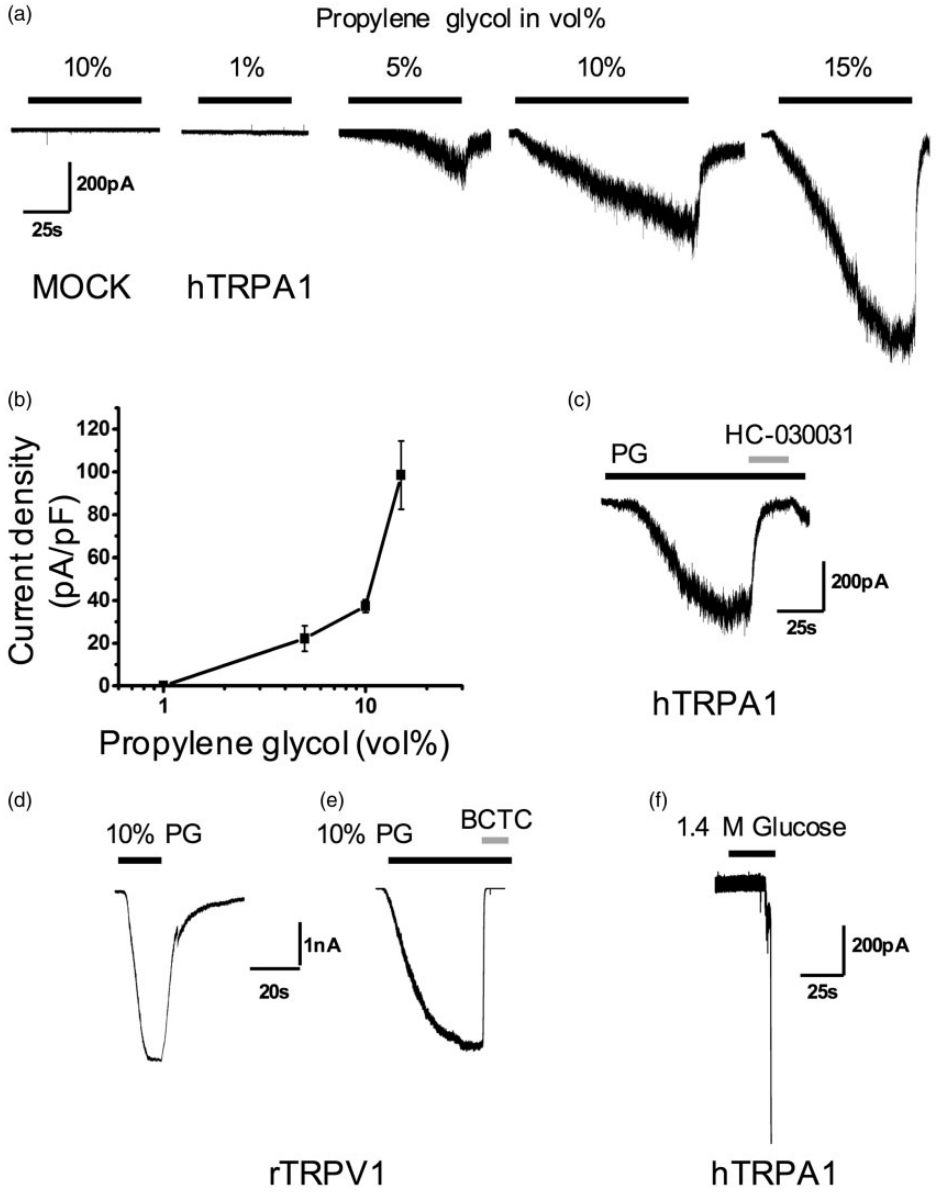

Figure 5. Propylene glycol (PG) activates hTRPA1 and rTRPV1. (a) Representative current traces of PG-evoked inward currents in non-transfected (MOCK) HEK293t cells or cells transiently expressing hTRPA1. To prevent desensitization, only one concentration was tested on each cell. Cells were held at −60 mV, PG was applied until the current had reached a steady state. (b) Concentration–response curve for PG-induced inward currents in hTRPA1-expressing HEK293t cells; 5 to 13 cells were tested at each concentration. Data were fitted to the Hill equation. (c) Representative PG-evoked inward current in an hTRPA1-expressing HEK293t cell blocked by the TRPA1 antagonist HC-030031. PG 10% was applied until the current had reached a steady state followed by a combined application of PG and 50 μM HC-030031. (d) Representative inward current of 10% PG in HEK293t cells expressing rTRPV1. Cells were held at −60 mV, PG was applied until the current had reached a steady state. (e) Representative PG-evoked inward current in an rTRPV1-expressing HEK293t cell blocked by the selective TRPV1 antagonist BCTC. PG 10% was applied until the current had reached a steady state followed by a combined application of PG and 10 μM BCTC. (f) Representative current in response to 1.4 M glucose (osmolarity corresponds approximately to the osmolarity of 10% PG). Note the loss of seal integrity during application of glucose.
TRP: transient receptor potential.

10% (1366 mM, theoretical osmolarity 1687 mosmol/l) and 15% PG (2050 mM, theoretical osmolarity 2371 mosmol/l) in a dose-dependent manner generated mean current densities of 22 ± 6 pA/pF ($n = 7$), 37 ± 3 pA/pF ($n = 13$) and 99 ± 16 pA/pF ($n = 5$), respectively (Figure 5(a)). From these data, an EC_{50} of approximately 11 volume-% (equating to a concentration of 1496 mM) was estimated (Figure 5(b)). Co-application of the selective TRPA1 antagonist HC-030031 (50 μM) reduced the mean inward currents induced by 10% PG to 9.2% (95% CI: 4–14.2%, $n = 5$, $p = 0.0021$, paired t-test, Figure 5(c)). Moreover, combination of PG (10%) and etomidate (300 μM) evoked an increase in inward currents by 2.9 ± 1.2-fold compared to preceding etomidate ($n = 8$, Supplemental Material Figure 1).

HEK293t cells expressing rTRPV1 also generated large inward currents upon application of 10% PG with a mean current density of 215 ± 26 pA/pF ($n = 12$; Figure 5(d)). This activation of TRPV1 was completely blocked by co-application of the TRPV1 antagonist BCTC (10 μM) (reduction of mean current to 0.5%, 95% CI: 0.1%– 0.9%, $n = 5$, $p = 0.0027$, paired t-test, Figure 5(e)).

It has recently been proposed that TRPV1 and TRPA1 could be activated by hyperosmotic stimuli.[38–40] To test if PG-mediated activation of both channels rather depends on a high osmolarity of the solution than on PG itself, we tested non-transfected, hTRPA1- and rTRPV1-expressing HEK293t cells for activation by hyperosmolar glucose solution. Neither hTRPA1 nor rTRPV1 (both $n = 5$) showed inward currents upon 1400 mM glucose (osmolarity similar 10% PG) in external solution (Figure 5(f)). In contrast to 10% PG, the seal always destabilized after 5 to 20 s of glucose application. During the time until destabilization of the seal, no reversible current could be detected. Application of 1400 mM glucose to untransfected HEK293t cells showed similar results as observed above with transfected HEK cells. In summary, we presume that the activation of hTRPA1 as well as rTRPV1 by PG is rather a specific effect of the substance than an effect of the high osmolarity of PG.

Etomidate and PG induce release of CGRP

Activation of peptidergic nociceptive nerve fibers leads to a calcium-dependent release of the proinflammatory neuropeptides CGRP and substance P (SP).[41] Measurement of CGRP release can serve as an index of nociceptor activity which represents a potentially painful input to the central nervous system. CGRP, SP, and other neuropeptides contribute to neurogenic inflammation, peripheral and central sensitization, and can initiate and aggravate (persisting) inflammatory processes.[41–43] After our cellular experiments, we asked if etomidate and PG would also activate nociceptive nerves in a native tissue preparation such as the isolated mouse sciatic nerve. The clinical formulations Hypnomidate® and Etomidat-® Lipuro do not contain any essential electrolytes and especially no calcium ions; therefore, we tested the effects of both formulations 10-fold diluted in SIF. Two preparations then contained an etomidate concentration of 0.82 mM. The 10-fold diluted Hypnomidate® solution additionally contained 3.5% of PG which acts as solubilizer for etomidate. Both 10-fold diluted Hypnomidate® and Etomidat-® Lipuro did not induce significant release of CGRP from sciatic nerves of C57BL/6 wild type mice (each $n = 8$; Figure 6(a)). We next created an SIF-based test solution with dimethyl sulfoxide as a solubilizer and an etomidate concentration of 0.8 mM which did not evoke any significant CGRP release either. Finally, an SIF-based solution with a concentration of 8 mM etomidate (nearly matching the concentration of the original clinical formulations) induced a massive and significant release of CGRP ($n = 8$, $p = 0.0117$; Figure 6(a)). Another SIF-based test solution containing 35% PG (corresponding to the PG concentration in Hypnomidate®) leads to an even greater release of CGRP from sciatic nerves ($n = 8$, $p = 0.0115$, all Wilcoxon matched pairs test, Figure 6(a)). Lipofundin®, which is the micellar carrier for etomidate in Etomidat-® Lipuro, had recently been tested and did not induce any CGRP release.[34] To find out which receptors mediate the etomidate-induced CGRP release, we performed equivalent experiments on sciatic nerves of knockout mice. *Trpa1*$^{-/-}$ mice showed a significant reduction in the response to etomidate (8 mM) by ~70% in comparison to C57BL/6 wild-type mice ($n = 8$, $p = 0.0209$; Figure 6(b)). *Trpv1*$^{-/-}$ mice even showed a reduction to ~11% ($n = 8$, $p = 0.0033$; Figure 6(b)), yet not significantly different from *Trpa1* knockouts. *Trpv1*/*Trpa1*$^{-/-}$ mice retained a small, still significant, CGRP release upon etomidate stimulation being, however, significantly reduced in comparison to wild-type mice ($n = 8$, $p = 0.0008$, all group comparisons Mann–Whitney U test; Figure 6(b)). Stimulation of the sciatic nerves with GABA (100 μM) caused no release of CGRP which had been already shown.[34] These findings support the conclusion that etomidate-evoked release of CGRP from isolated peripheral nerves is mediated by the activation of both TRPV1 and TRPA1, while peripheral GABA receptors,[35] if functionally expressed, do not play a role in this respect.

Etomidate formulations induce local pain upon intradermal injection in humans

To confirm the clinical relevance of our findings, we assessed the pain estimates evoked by different etomidate formulations in human volunteers. Four subjects received intradermal injections (just subepidermal) of either 50 μl of

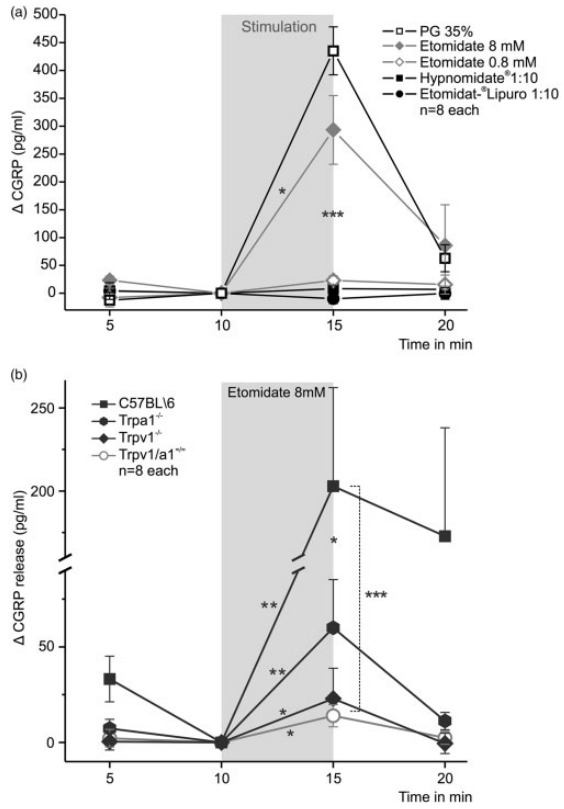

Figure 6. Etomidate and PG induce release of CGRP. (a) 10-fold diluted clinical formulations of Etomidat-® Lipuro and Hypnomidate® (both 0.8 mM etomidate) did not induce relevant CGRP release from isolated sciatic nerves of C57BL/6 mice. CGRP release was also not caused by 0.8 mM etomidate in SIF with dimethyl sulfoxide as solubilizer. A 10-fold higher etomidate concentration (8 mM) evoked significant release of CGRP as did a 35% PG solution in SIF. (b) Etomidat-induced CGRP release was significantly reduced in sciatic nerves from Trpa1$^{-/-}$-, Trpv1$^{-/-}$-, and Trpv1/Trpa1$^{-/-}$-knockout mice.
TRP: transient receptor potential; PG: propylene glycol; CGRP: calcitonin gene-related peptide.

Etomidat-® Lipuro, Lipofundin®, Hypnomidate®, 35 vol % PG in 0.9% saline, 8 mM etomidate in 0.9% saline with 8% methanol as a solubilizer and 8% methanol in 0.9% saline at different sites on both volar forearms in a double-blind fashion. In line with own clinical observations, Etomidat-® Lipuro and Lipofundin® did not cause considerable pain upon intradermal injection (Figure 7(a), top panel). However, application of Hypnomidate® and 35% PG evoked intense burning pain sensations immediately after injection in all volunteers with an average initial pain rating of 9 and 7.75 on the numerical rating scale (0 correlating to no pain and 10 to the maximal imaginable pain intensity) (Figure 7(a), middle panel). Pain upon injection of these solutions declined linearly and was rated only 4 and 2 on average after 2 and 3 min, respectively. Application of 8 mM etomidate (mimicking the clinically used concentration) with methanol as solubilizer caused the highest pain ratings, immediately after injection, the "maximal imaginable pain intensity" was shortly reached with an average rating of 9.75. Remarkably, this pain decayed very fast and was gone within 45 s. The control solution of 8% methanol in 0.9% saline caused an average rating of 4.75; however, the volunteers reported an unpleasant tingling sensation rather than a burning pain (Figure 7(a), bottom panel). Although the non-lipid solubilizers (PG and methanol) showed irritant effects of their own, our results indicate that etomidate itself has an algogenic potency that is strongly reduced in the micellar emulsion but not in the PG solution.

Etomidate formulations cause an axon reflex response in human skin

Particular C-fibers branch into widespread nerve fibers and terminals in the human skin. Activation of one of these terminals generates action potentials that are conducted orthodromically to the central nervous system, but they also invade the other branches antidromically. This initiates a release of neuropeptides such as CGRP and SP into the surrounding tissue which causes axon reflex vasodilatation, visible as a flare response and quantifiable by LDI of the area by an increased skin blood flow. As etomidate and PG caused CGRP release from the isolated peripheral nerve in vitro, we were interested if this could be reproduced in human skin in vivo. Fifty microliters of the clinical formulations of etomidate (Etomidat-® Lipuro, Hypnomidate®) and of their respective carrier solutions (Lipofundin®, 35% PG in aqueous solution) were injected intacutaneously. In both volunteers, Hypnomidate® and PG caused large flare areas clearly indicating an axon reflex vasodilatation caused by CGRP. Etomidat-® Lipuro and Lipofundin® only evoked local vasodilatation at the injection site. Representative LDI skin scans from one volunteer are shown in Figure 7(b), left panels depict skin perfusion before, right panels were taken 2 min after intradermal injection of a test substance. Figure 7(c) comprises quantitative analyses of the flare sizes (given as area in cm^2) developing over time based on the underlying data (Figure 7(b)).

These findings correlated well with the pain ratings that were much greater and sustained with both Hypnomidate® and PG than with the lipid emulsion of etomidate Etomidat-® Lipuro (Figure 7(a)).

Discussion

The TRP channels TRPV1 and TRPA1 are nowadays considered key players in acute and inflammatory states of pain and are also involved in neuropathic pain conditions.[44,45] Many local and general anesthetics interact with them and are able to sensitize, activate, or block them.[17,18] The results

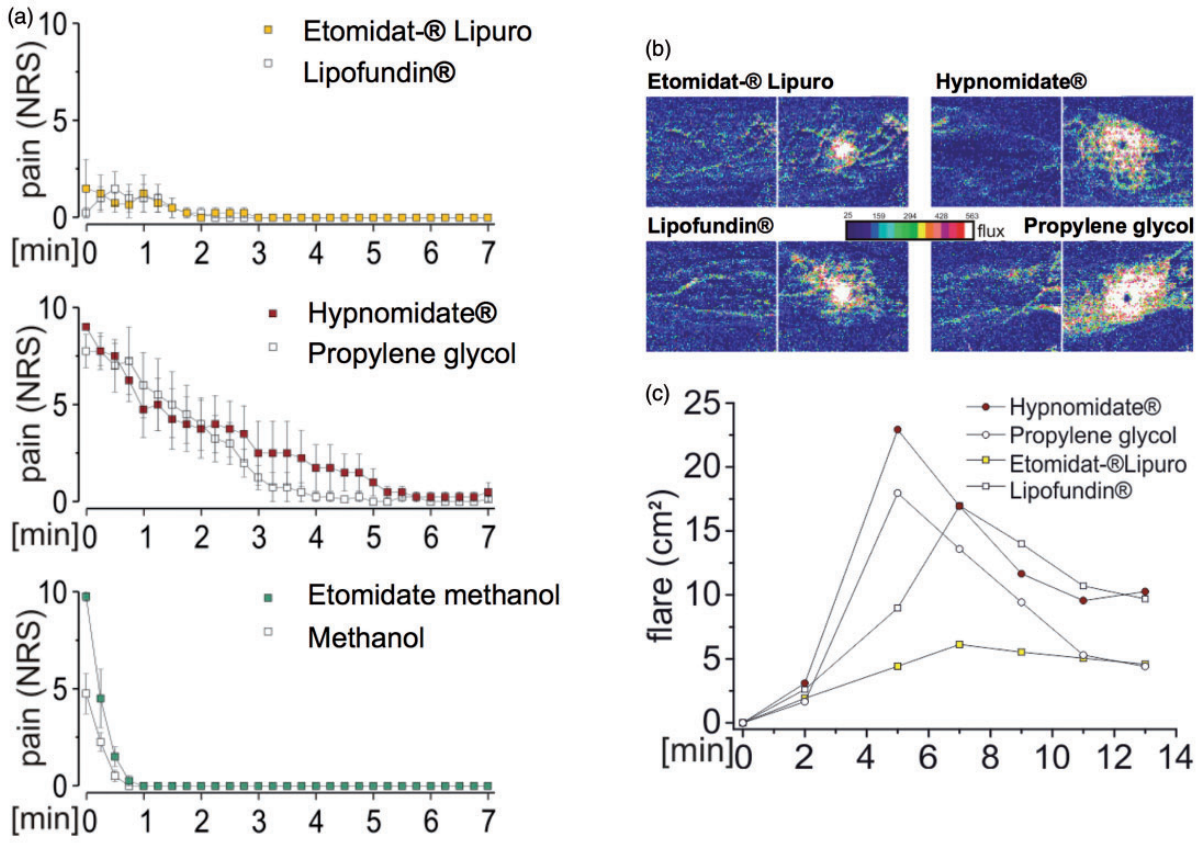

Figure 7. Etomidate formulations induce pain and axon reflex vasodilatation in human volunteers. (a) 100 µl Etomidat-® Lipuro (8.2 mM etomidate) or Lipofundin® injected intradermally in volar forearms of volunteers did not induce pain. 100 µl Hypnomidate® (8.2 mM etomidate) or 35% PG in Ringer's solution caused high values on the numerical rating scale (NRS) in all volunteers indicating severe pain. An 8.2 mM etomidate formulation with methanol as solubilizer caused highest values on the NRS; methanol alone in Ringer's solution produces significantly lower values on the NRS. (b) Measurements of regional skin perfusion by LDI 2 min after injection. Intradermal injections of 100 µl of Etomidat-® Lipuro, Lipofundin®, Hypnomidate® or 35% PG in Ringer's solution caused local vasodilatation. Hypnomidate® and 35% PG in Ringer's solution produced larger flare responses than lipid emulsion-based formulations after 2 min. Left-hand panels display baseline skin perfusion before injection. (c) Quantitative analyses of the flare sizes as area in cm^2 developing over time based on the data from (b), showing wider and extended flare reactions upon Hypnomidate® and PG than after Etomidat-® Lipuro. NRS: numerical rating scale.

of this study add etomidate and its widely used solubilizer PG to that list as activators of TRPV1 and TRPA1, inducing pain in clinically relevant concentrations.

Etomidate activates mouse and human TRPA1 and rat TRPV1

In patch-clamp experiments on HEK293t cells transiently expressing mouse or human TRPA1, etomidate shows concentration-dependent activating and desensitizing properties. The activation can be blocked by the TRPA1-specific antagonist AP-18 and does not occur in untransfected HEK-cells. While the activation of mTRPA1 shows a normally configured concentration–response curve with an EC_{50} of 375 µM, the activation of hTRPA1 differs from mTRPA1; hTRPA1 is already activated by 30 µM etomidate, followed by an activation plateau between concentrations of 100 and 1000 µM. Higher etomidate concentrations again show massively increasing membrane currents; thus, a bimodal concentration–response curve results with two increases and an EC_{50} of 73 µM for the first rise of the curve. Species differences in activation and blocking properties of TRPA1 agonists have previously been shown.[31,46] The observed difference in activation by etomidate could probably be explained by a second affinity center, only accessible in hTRPA1. It has previously been described that AITC and other electrophilic agonists such as methylglyoxal activate hTRPA1 via distinct cysteine and lysine residues.[32,33,47] Etomidate, although its molecular structure does not obviously seem electrophilic, showed a reduced potency on the hTRPA1 mutant C621S/C641S/C665S, analog to AITC, and the AITC-insensitive mutant hTRPA1-C621S/C641S/C665S/

K710R also appeared completely insensitive to etomidate. The rightward shifted concentration–response relationship of the hTRPA1-3C mutant seemed to follow the second rise of the wild-type curve at etomidate concentrations >300 μM which, thus, may correspond to the lysine K710 binding site of lower affinity. However, the lacking effect of NAC on the prevention of etomidate-induced hTRPA1 activation did not support an electrophilic interaction of the drug with cysteines and lysine within the TRPA1 channel protein. Thus, at high etomidate concentrations such as the clinically administered 8 mM other binding sites or indirect activation of TRPA1 seem to prevail.

TRPA1 is mainly expressed in the larger subset of TRPV1-positive nociceptive neurons; therefore, we tested TRPV1 for activation by etomidate in patch-clamp experiments. HEK293t cells overexpressing rTRPV1 showed inward currents starting at a concentration as low as 100 nM etomidate and with an EC_{50} of 240 nM, lying in the potency range of capsaicin acting on TRPV1.[48] Etomidate concentrations higher than 1 μM showed decreasing inward currents, and even higher concentrations also blocked capsaicin-induced inward currents. Therefore, the decreasing current amplitudes at higher concentrations are likely due to an additional, simultaneous channel-blocking effect of etomidate on TRPV1. In contrast to previous results showing a selective activation of TRPA1 but lack of effect on TRPV1 by etomidate (solely 100 μM),[17] our findings identify rTRPV1 as an additional molecular target of etomidate effectively activated at a lower concentration range with its maximum at 1 μM and blocked at concentrations above.

Etomidate activates $GABA_A$ and TRPA1 concentration dependently in mouse DRG neurons

In ratiometric calcium measurements, etomidate elicited an increase in $[Ca^{2+}]_i$ in AITC and capsaicin-sensitive DRG cells from wild-type C57BL/6 mice starting at a concentration of 30 μM and with an EC_{50} of about 760 μM. A fraction of this rise in $[Ca^{2+}]_i$ depended on the expression of $GABA_A$ receptors which depolarize sensory neurons. Therefore, experiments were performed under blockade of $GABA_A$ receptors by PTX. Without PTX an excellent correlation between the magnitudes of GABA and etomidate (at low concentrations) responses was found. However, no correlation between etomidate and capsaicin responses could be observed. Consistent with this, sensitization of TRPV1 by the PKC activator PMA failed to enable a TRPV1-dependent activation of mouse neurons by etomidate, signifying an apparent species variability between mouse and rat TRPV1 channels. On the other hand, the activation of the sensory neurons could be completely blocked by the TRPA1 antagonist AP-18, and etomidate (in presence of the $GABA_A$ blocker) was ineffective on DRG neurons of *Trpa1* knockout mice. Thus, it seems that at least in mouse DRGs, the activation of nociceptive neurons by etomidate is solely driven by $GABA_A$ receptors at low concentrations and by TRPA1 at higher concentrations.

PG activates hTRPA1 and rTRPV1

PG is a widely used vehicle for drugs that are scarcely water soluble, and it is found in oral, injectable, and topical formulations. PG is contained in cosmetics and is used to generate nicotine aerosol in electric cigarettes. Since the late 1970s, the Food and Drug Administration generally recognizes PG as safe.[49] It is known, however, that PG can produce airway irritation in rats upon inhalation[50] and it can cause venous sequelae such as pain, thrombophlebitis, and thrombosis upon injection. In general, all irritating effects of PG were attributed to the high osmolarity of PG-containing solutions.[51] With this study, we are able to show that PG, up to 10% at least, essentially acts via specific interactions with nociceptive receptor proteins of the TRP channel family. In whole-cell patch-clamp experiments, a reproducible and concentration-dependent activation of hTRPA1 and rTRPV1 could be observed. Inward currents in HEK293t cells expressing hTRPA1 or rTRPV1 depended on the expression of these proteins, and untransfected HEK cells showed no activation on application of 10 vol% PG. Furthermore, the activation of both proteins could be blocked by the specific TRP-antagonists HC-030031 and BCTC, respectively. Although it was reported that TRPA1 and TRPV1 could be activated by hypertonic solution,[39,40] in our study, PG-induced activation of hTRPA1 and rTRPV1 does not seem to depend on osmolarity, as control experiments with 1400 mM glucose (theoretical osmolarity 1721 mOsmol/l nearly matching the theoretical osmolarity 1687 mOsmol/l of 10% PG) showed no activation of hTRPA1 or rTRPV1 but led to a rather quick cell destabilization and loss of the seal which never occurred with 10% PG.

Clinical relevance

Many drugs used in the perioperative setting interact with the nociceptive system. Some of them such as propofol and etomidate do this quite obviously and produce burning pain upon intravenous injection.[13,14,17,34] Others such as the pungent volatile anesthetics isoflurane and desflurane cause laryngeal and bronchial irritation as well as neurogenic inflammation and can therefore induce laryngo- and bronchospasm especially in patients with sensitized airways in the context of asthma or in children.[18,23] In the past, the injection pain of etomidate-containing drug formulations was often attributed to the

vehicle PG. In contrast, we were able to show in cellular experiments that etomidate itself has algogenic properties and that these and the algogenic properties of PG are mediated by TRPA1 and TRPV1. Additional experiments demonstrating etomidate-induced release of CGRP from sciatic nerves of mice support the TRPA1/TRPV1-mediated activation of nociceptive neurons. Etomidate showed a steep concentration dependency inducing neuropeptide release only at a concentration of 8 mM as clinically employed, while 10-fold diluted solutions caused no significant release. PG showed a similar behavior with massive CGRP release at a concentration of 35% and no release at 3.5%. These findings were confirmed in translational, human experiments. A formulation of 8 mM etomidate with methanol as a solubilizer produced as high pain ratings as Hypnomidate® confirming the vehicle-independent algogenic effect of etomidate. Lipid-based Etomidat-® Lipuro with the same etomidate concentration caused no injection pain. Why? It was shown in the past that the injection pain of the lipophilic anesthetic propofol depends on its free concentration in the aqueous phase of the formulation, which is low in a lipid emulsion due to water–oil partitioning.[34,52,53] Therefore, it is most likely that the injection pain of the lipophilic etomidate also depends on the free concentration of the compound in the investigated formulations and is less, in fact absent, in the lipid emulsion. In formulations with organic solvents such as methanol, the free concentration of etomidate in the aqueous phase accords to the overall concentration of 8.2 mM, whereas in the investigated lipid-containing emulsion, the free concentration is only about 200 μM (personal correspondence with the manufacturer of Etomidat-® Lipuro by B. Braun Melsungen AG and own unpublished measurements), the vast majority of the drug being bound into lipid micelles. Considering the previously described steep concentration dependence for etomidate-induced CGRP release, it is likely that a free concentration of 200 μM etomidate is not high enough to activate TRPA1 and maybe TRPV1 to cause pain upon injection.

One inconsistency remains to be discussed. Etomidate activated recombinant rat TRPV1 but not native mouse TRPV1, while the effect of the clinically used etomidate concentration on intact isolated nerve was as much reduced in *Trpv1* as in *Trpa1* knockout mice. This may be explained by the difference in concentration ranges studied. In patch-clamp recordings, 2.5 mM etomidate was the highest concentration testable without losing seal integrity. Therefore, higher concentrations were also avoided on cultured DRG neurons in calcium imaging experiments to minimize unspecific drug effects. However, intact organ preparations usually demand higher (drug) concentration ranges than isolated cells, due to limited penetration through and dilution in complex tissues. In fact, the full clinical concentration of etomidate was required to evoke any and even massive CGRP release from intact nerves as well as a pain sensation in human subjects, depending on either TRPV1 or TRPA1. Both nociceptive ion channels are largely co-expressed and show intricate interactions. Activation of TRPV1 allows calcium ions to enter which, in turn, are known to activate TRPA1.[54] Vice versa, it has been shown that stimulated calcium influx through TRPA1 activates adenylyl cyclase and protein kinase A (PKA) which subsequently sensitizes TRPV1.[55] In addition, species differences between rat and mouse TRPV1 may account for the lesser sensitivity of the latter to etomidate and the need of much higher concentrations as clinically applied to be recruited. The structural variabilities between these TRP channels, including ligand binding sites, have recently been elaborated using cryo-electronmicroscopy.[56]

In conclusion, our study provided evidence that the general anesthetic etomidate in clinically relevant concentrations has algogenic properties mediated by direct and/or indirect activation of the nociceptive ion channels TRPA1 and TRPV1. Further, it demonstrated that also the solubilizing vehicle PG (35 volume-%) stimulates TRPA1 and TRPV1. Both etomidate and PG induced neurogenic inflammation by releasing neuropeptides from peripheral nerve fibers and elicited marked pain upon intradermal injection. These excitatory effects on nociceptive sensory neurons may underlie the burning pain sensation elicited by injection of aqueous etomidate formulations and contribute to neuronally mediated inflammation, both representing clinically relevant adverse effects during general anesthesia.

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Author contributions

FN, ME, and KK were involved in investigation and formal analysis; FN and ME have done visualization; resources were provided by BN, CN, and PWR; supervision was done by AL and PWR; the original draft was written by FN and ME; writing review and editing were done by PWR and KK; CN, PWR, and KK contributed to funding acquisition; AL, PWR, and

KK contributed to conceptualization and project administration.

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