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Apomorphine Is a Bimodal Modulator of TRPA1 Channels

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ABSTRACT

Apomorphine is a non-narcotic derivative of morphine, which acts as a dopamine agonist and is clinically used to treat "off-states" in patients suffering from Parkinson's disease. Adverse effects of apomorphine treatment include severe emesis and nausea, and ulceration and pain at the injection site. We wanted to test whether sensory transient receptor potential (TRP) channels are a molecular target for apomorphine. Here, we show that rTRPV1, rTRPV2, rTRPV3, and mTRPV4, as well as hTRPM8, and rTRPM3, which are expressed in dorsal root ganglion neurons, are insensitive toward apomorphine treatment. This also applied to the cellular redox sensor hTRPM2. On the contrary, human TRPA1 could be concentration-dependently modulated by apomorphine. Whereas the addition of apomorphine in the low micromolar range produced an irreversible activation of the channel, application of higher concentrations caused a reversible voltage-dependent inhibition of heterologously expressed TRPA1 channels, resulting from a reduction of single-channel open times. In addition, we provide evidence that apomorphine also acts on endogenous TRPA1 in cultured dorsal root ganglion neurons from rats and in the enterochromaffin model cell line QGP-1, from which serotonin is released upon activation of TRPA1. Our study shows that human TRPA1 is a target for apomorphine, suggesting that an activation of TRPA1 might contribute to adverse side effects such as nausea and painful injections, which can occur during treatment with apomorphine.

Introduction

Transient receptor potential A1 (TRPA1) is activated by a variety of proalgesic agents (McNamara et al., 2007), environmental irritants (Bautista et al., 2006), and pungent dietary compounds such as mustard oil, allyl isothiocyanate (AITC), cinnamon (cinnamaldehyde), and garlic (allicin) (Bandell et al., 2004; Bautista et al., 2005). The mechanisms of TRPA1 activation are diverse, ranging from covalent modification by electrophilic substances (Hinman et al., 2006; Macpherson et al., 2007) to activation by physical stimuli such as cold and mechanical forces (Karashima et al., 2009; Vilceanu and Stucky, 2010). In addition to its predominant expression in sensory neurons of the dorsal root and trigeminal ganglia, where TRPA1 contributes to the sensation of chemical pain, recent evidence also suggests its presence in a variety of non-neuronal cells, including skin cells (Atoyan et al., 2009; Oehler et al., 2012) and cells within the respiratory tract (Nassini et al., 2012) and the gastrointestinal tract (Stokes et al., 2006). In enterochromaffin cells (ECs), for example, a possible contribution of TRPA1 to the regulation of gastrointestinal motility might base on a release of serotonin upon channel activation (Doihara et al., 2009c; Nozawa et al., 2009).

Apomorphine, a aporphine derivative, approved in human and veterinary medicine. It is currently used for the treatment of "off-states" (episodes of poor motor function as far as complete immobility) in late stage Parkinson's disease and is subcutaneously administered. Moreover, recent studies suggest a dopamine receptor–independent beneficial effect of apomorphine in a mouse model of Alzheimer's disease (Himeno et al., 2011). Other medical indications include treatment of erectile dysfunction (Dula et al., 2001). In animals, apomorphine is used to induce severe vomiting after ingestion of poisonous substances. Treatment with apomorphine is commonly accompanied by adverse effects including nausea, vomiting, and hypotension, as well as local reactions at the injection site such as erythema, pain, and itching.

Chemically, apomorphine is derived from morphine, but lacks its effect on opioid receptors. Apomorphine is structurally similar to dopamine and acts as an agonist on central dopamine receptors (e.g., in the striatum), which explains its therapeutic effect in Parkinson's disease. The emetic effect of apomorphine is thought to result from a direct stimulation of dopaminergic receptors in the chemoreceptor trigger zone (CTZ).

Here, we demonstrate a bimodal modulation of TRPA1 channels by apomorphine with an activation of channels in the low micromolar range and an initial potentiation followed by a block at high apomorphine concentrations. Apomorphine not only acts on heterologously expressed TRPA1 channels, but also triggers HC-030031–sensitive rises in intracellular calcium in a subpopulation of dorsal root ganglion (DRG) neurons that are also activated by AITC. Furthermore, we show that serotonin is released from enterochromaffin QGP-1 cells upon stimulation of TRPA1 by apomorphine. Taken together, our study shows that TRPA1 is a target for

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ABBREVIATIONS: AITC, allyl isothiocyanate; CTZ, chemoreceptor trigger zone; DRG, dorsal root ganglion; EC, enterochromaffin cell; HBS, HEPES-buffered solution; TRP, transient receptor potential.

apomorphine, suggesting that activation of TRPA1 might contribute to some of the side effects that occur during apomorphine therapy.

Materials and Methods

Cell Culture and Reagents. We used a HEK293 cell line that was stably transfected with human TRPA1 ($HEK293_{TRPA1}$) as well as a parental HEK293 cell line for control experiments (Hill and Schaefer, 2007). To generate stable cell lines expressing the respective TRP channels, CFP-tagged rat TRPV1, YFP-tagged rat TRPV2, YFP-tagged rat TRPV3, YFP-tagged mouse TRPV4, and human TRPM2 were stably transfected in HEK293 cells as previously described (Urban et al., 2012). The stable myc-tagged rat TRPM3 cell line was a kind gift from S. Philipp (Universitaet des Saarlandes, Homburg, Germany), and generation of the HEK_{TRPM3} cell line has been described elsewhere (Fruhwald et al., 2012). HEK293 cells were grown in Earle's minimum essential medium (Biochrom Berlin, Germany) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, supplemented with 1 mg/ml geneticin (G418) (Invitrogen Corp., Carlsbad, CA) except for the parental HEK293 cell line. For transient transfection with human TRPM8-CFP, cells were seeded in 35-mm culture dishes and transfected at 80% confluency with 2 μg plasmid DNA and 4 μl FuGENE HD reagent (Roche Applied Science, Penzberg, Germany). QGP-1 cells were a kind gift from B. Wiedenmann (Charite, Berlin, Germany) and were cultured in RPMI 1640 medium (PAA Laboratories, Pasching, Austria) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. HC-030031 was obtained from Tocris Bioscience (Bristol, UK). *R*-(-)-apomorphine ((*R*)-5,6,6*a*,7-tetrahydro-6-methyl-4*h*-dibenzo[*de*,*g*]quinoline-10,11-diol) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), if not stated otherwise. Apomorphine was dissolved in aqueous solution immediately before the start of experiments.

Preparation of DRG Neurons. Wistar rats (mixed sexes) aged 8 weeks were used in the study. Animals were killed under CO_2 and decapitated to obtain cell cultures of DRG neurons. After isolation of thoracic and lumbar DRG neurons, cells were plated onto 25-mm glass coverslips, coated with poly(L-lysine) (25 μg/ml) (Sarstedt, Nümbrecht, Germany) and kept in Dulbecco's modified Eagle's medium (Biochrom) supplemented with 30 mM glucose, 2.5 mM L-glutamine, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 μg/ml gentamicin, 20% fetal bovine serum, 100 ng/ml nerve growth factor, 1% ITS liquid media supplement, containing 1.0 mg/ml recombinant human insulin, 0.55 mg/ml human transferrin, and 0.5 μg/ml sodium selenite, (Sigma-Aldrich). Primary cultures of rat DRG neurons were maintained for up to 2 days in a humidified atmosphere (37°C, 5% CO_2) before experiments.

Analysis of $[Ca^{2+}]_i$. For single-cell $[Ca^{2+}]_i$ analysis of QGP-1 and HEK293 cells expressing the respective TRP channels, cells were seeded

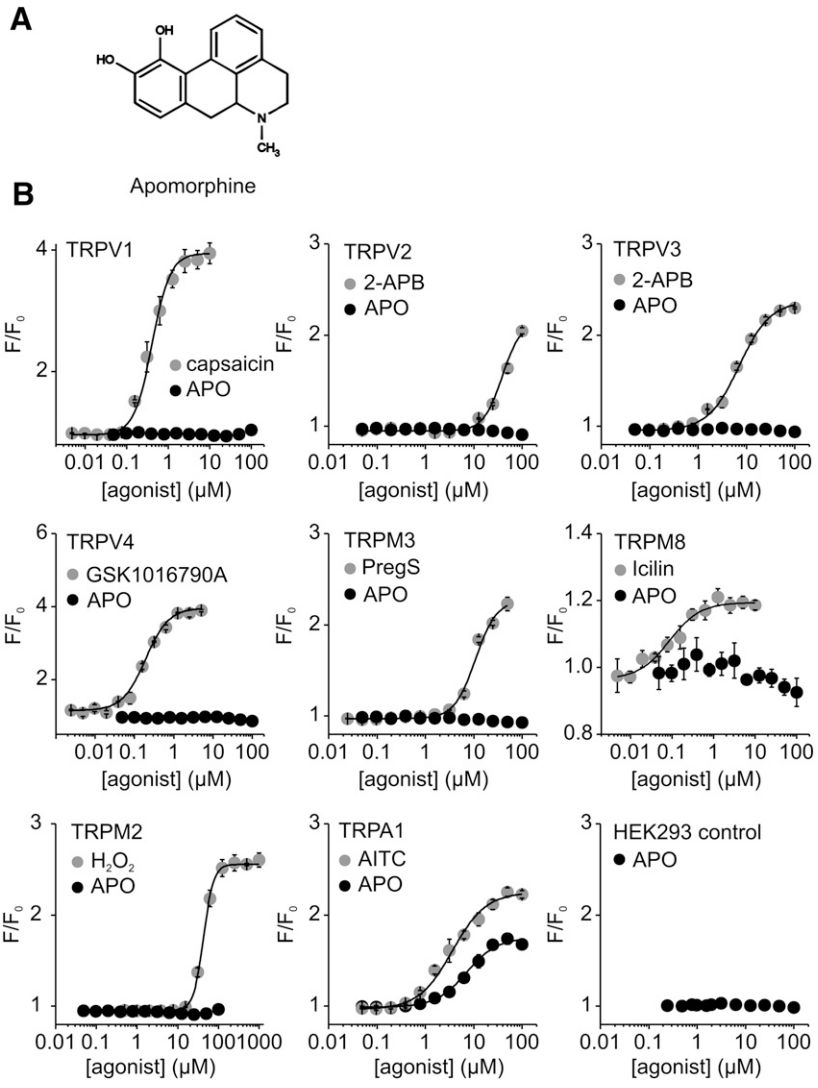

Fig. 1. Apomorphine (APO) selectively activates TRPA1 but no other sensory TRP channel. (A) Structure of apomorphine. (B) Concentration response curves for several sensory TRP channels were determined using Fluo-4–loaded HEK293 cells heterologously expressing TRPV1-4, TRPM3, TRPM8, TRPM2, or TRPA1. Parental HEK293 cells serve as the control. Data represent the means ± S.D. together with the best fit to a three-parameter Hill equation. Each cell line was stimulated with apomorphine (up to 100 μM, black symbols) and, except for parental HEK293 cells, with the respective activator for the TRP channel as described in the figure (gray symbols). An EC_{50} of 7.1 μM could be calculated for TRPA1 activation by apomorphine (versus 3.6 μM for AITC-induced activation).

on 25-mm glass coverslips and allowed to attach for 24 h. At about 60% confluency, cells were incubated with 2 μM Fura-2/acetoxymethyl ester (Fura-2/AM; Molecular Probes, Eugene, OR) in a HEPES-buffered solution (HBS) containing 10 mM HEPES, 134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4 at 37°C for 30 min, rinsed, and mounted in a bath chamber for monochromator-assisted (TILL Photonics, Graefelfing, Germany) digital epifluorescence videomicroscopy, built around an inverted microscope (Zeiss Axiovert 100; Carl Zeiss, Jena, Germany). The fluorescence of Fura-2 was sequentially excited at 340, 358, and 380 nm through the imaging objective (Fluar 10×/0.5; Carl Zeiss, Jena, Germany). Emitted light was filtered through a 512-nm long-pass filter and recorded with a 12-bit cooled charge-coupled device camera (IMAGO; TILL Photonics, Graefelfing, Germany). The calcium concentration was defined as previously described (Lenz et al., 2002).

To generate concentration response curves, cells were loaded with 4 μM Fluo-4/AM (Invitrogen Corp, Carlsbad, CA) and seeded into pigmented clear-bottom 384-well microwell plates (10,000 cells/well; Corning Inc, Corning, NY). Activation of the respective channel was followed by measuring increases in the fluorescence intensity of Fluo-4. Measurements were carried out using a custom-made fluorescence plate imaging device as previously described (Nörenberg et al., 2012).

Catalase was purchased from Sigma-Aldrich. To remove thymol, catalase was centrifuged at 13.000 × *g* for 5 min, washed, and resuspended in the same volume of 50 mM phosphate buffer. Before experiments, 500–2500 U/ml catalase was mixed with 500 μM hydrogen peroxide or 20 μM apomorphine.

Electrophysiology. Patch clamp experiments were performed in the whole-cell or inside-out configurations using a Multiclamp 700B amplifier together with a Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA) under the control of pCLAMP 10 software. Coverslips with HEK_{TRPA1} or QGP-1 cells were transferred to a continuously perfused recording chamber (500 μl volume) and mounted on the stage of an inverted microscope. Patch pipettes were fabricated from borosilicate glass with a typical resistance of 3–5 MΩ. Whole-cell series resistances were compensated by 75%. The extracellular solution contained 140 mM NaCl, 5 mM CsCl, 2 mM $MgCl_2$, and 10 mM HEPES (pH 7.4 adjusted with NaOH). The pipette solution contained 140 mM CsCl, 4 mM $MgCl_2$, 10 mM HEPES, and 10 mM EGTA (pH 7.4 adjusted with CsOH). All experiments were performed at room temperature. Whole-cell currents were filtered at 3 kHz (4-pole Bessel filter) and sampled continuously at 5 kHz. Voltage ramps from −80 mV to +80 mV or −60 mV to +60 mV (500-ms duration) were applied every second. For inside-out recordings, standard whole-cell extracellular solution was used in the patch pipette, and the bath solution contained 140 mM KCl, 25 mM NaCl, 10 mM HEPES, 2 mM $MgCl_2$, and 5 mM EGTA (pH 7.2 adjusted with KOH). Single-channel currents were filtered at 3 kHz and sampled with 10 kHz.

5-Hydroxytryptamine Release Experiments. The method was performed as described previously (Nozawa et al., 2009). In brief, QGP-1 cells were seeded in 24-well plates at 2 × 10^5 cells/1 ml RPMI supplemented with 10% FCS and cultured for 72 h. The medium was removed and the cells were washed with HBS containing 2 μM fluoxetine and 0.1% bovine serum albumin. The HBS was replaced with 0.25 ml HBS containing different stimulants (300 μM AITC, 300 μM AITC with 100 μM HC-030031, 300 μM apomorphine, 300 μM apomorphine with 100 μM HC-030031) and cells were incubated for 1 h at 37°C. The supernatants were collected, centrifuged, and stored at −80°C until 5-hydroxytryptamine (5-HT) measurements were performed using an enzyme immunoassay kit (Beckman Coulter, VillePinte, France). Dimethylsulfoxide served as control.

Results

Apomorphine Induces TRPA1-Dependent Calcium Influx in $HEK293_{TRPA1}$ Cells. We investigated the effect of apomorphine on different TRP channels, utilizing Fluo-4–based calcium measurements (Fig. 1). To obtain concentration response curves, experiments were carried out in a 384-well format. Neither the warm/heat receptors rTRPV1, rTRPV2, rTRPV3, and mTRPV4, nor the warm receptor rTRPM3 or the cold and menthol receptor hTRPM8 were activated by apomorphine at concentrations of up to 100 μM. In addition, also the redox-sensitive TRPM2 channel was insensitive to apomorphine treatment. The same applied to the parental HEK293 control cell line. Functional expression of the TRP channels was confirmed by establishing concentration response curves utilizing their respective activators (TRPV1, capsaicin; TRPV2 and TRPV3, 2-APB; TRPV4, GSK 1016790A; TRPM3, pregnenolone sulfate; TRPM8, icilin; TRPA1, AITC; TRPM2, hydrogen peroxide). HEK293 cells stably expressing hTRPA1 ($HEK293_{TRPA1}$), however, showed a robust increase in intracellular calcium upon apomorphine addition. Measurements of concentration response curves gave an EC_{50} of 7.1 μM for the apomorphine-induced activation compared with 3.6 μM for AITC-induced activation of TRPA1. We further confirmed the activation of TRPA1 by apomorphine in Fura-2–based radiometric single-cell calcium

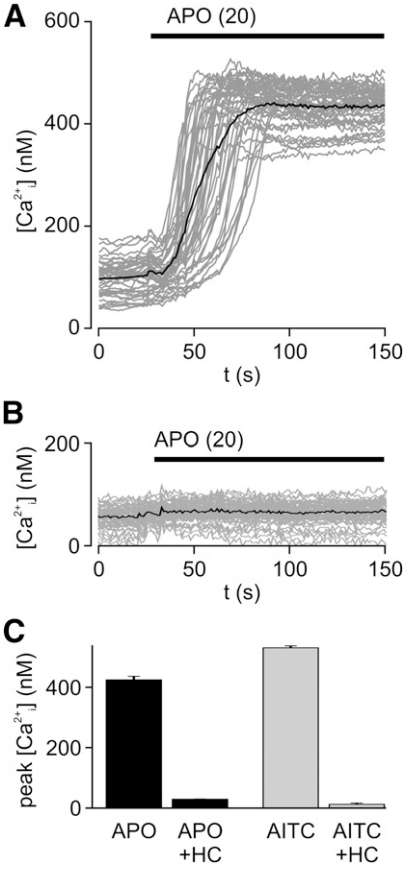

Fig. 2. Apomorphine (APO) induces an elevation of $[Ca^{2+}]_i$ in TRPA1-expressing HEK293 cells. (A) Representative single-cell time-lapse analysis of $[Ca^{2+}]_i$ of Fura-2–loaded $HEK293_{TRPA1}$ cells stimulated with apomorphine (20 μM) revealed strong responses of single cells (gray lines) and the mean response (black line). (B) Preincubation of $HEK293_{TRPA1}$ cells with HC-030031 (HC; 100 μM) completely prevented the apomorphine-induced calcium rise. (C) Statistical analysis of several experiments such as in (A) and (B) (data represent mean values ± S.D.). As a reference, responses of HEK_{TRPA1} cells to AITC treatment (20 μM) are included in the figure (gray bars).

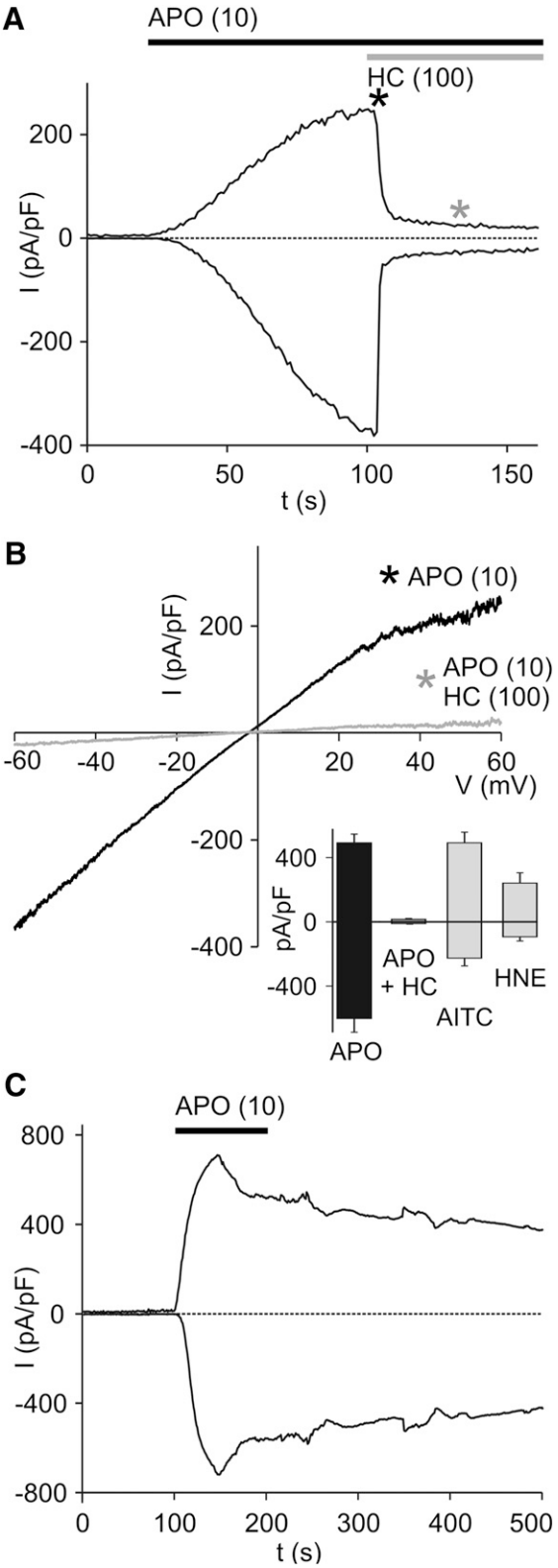

Fig. 3. Apomorphine (APO) activates TRPA1 currents in whole-cell patch clamp recordings. (A) Example whole-cell recording of a $HEK293_{TRPA1}$ cell at V_h = +60 mV and V_h = −60 mV. Additions of apomorphine (10 μM) and HC-030031 (100 μM) are indicated by the horizontal bars. Data were extracted from voltage ramps such as in (B). (B) 500-ms voltage ramps (V_h = −60 mV to V_h = +60 mV) were applied every second. Voltage ramps displayed were taken at the time points indicated in (A) (black asterisk, 100 s; gray asterisk, 135 s). Note the weak flickery block at positive membrane potentials. Inset, statistical analysis as mean ± S.D. of several experiments such as in (A) after stimulation with 10 μM apomorphine and after block with 100 μM HC-030031 (APO + HC). For comparison, values for 10 μM AITC-stimulated currents (AITC) and 10 μM 5-hydroxynonenal–evoked currents (HNE) are displayed. (C) Example whole-cell recording using a similar protocol as in (A). We added 10 μM apomorphine as indicated by the bar, followed by 300-s washout. After 300-s washout of apomorphine 85% ± 3% (V_h = −60 mV; n = 3) and 77% ± 9% (V_h = +60 mV; n = 3) of the apomorphine-evoked TRPA1 current persisted.

experiments (Fig. 2). All single $HEK293_{TRPA1}$ cells measured showed a robust increase in intracellular calcium after addition of 20 μM apomorphine, which was approximately 20% lower than the AITC-induced response (Fig. 2C). After preincubation with the TRPA1-specific blocker HC-030031, apomorphine-induced rises in intracellular calcium were abolished (Fig. 2, B and C).

Apomorphine Is a Concentration-Dependent Bimodal Modulator of TRPA1 Currents. To further characterize the activation of TRPA1 by apomorphine, we next performed whole-cell patch clamp experiments on $HEK293_{TRPA1}$ cells. Experiments were carried out in calcium-free extracellular solution to prevent calcium-induced channel rundown. Addition of 10 μM apomorphine to the bath solution elicited large currents, which could be blocked by HC-030031 (Fig. 3, A and B). I/V curves showed that the inward current was slightly more pronounced than the outward current in contrast to the typical weak inward rectification which is seen with classic TRPA1 activators such as AITC and 4-hydroxynonenal (Fig. 3B, inset). The activation of TRPA1 by apomorphine was mostly irreversible over a washout period of 300 s (Fig. 3C). Moreover, as evident from the voltage ramp in Fig. 3B, a weak block was evident at higher positive membrane potentials. To further investigate this effect, we applied apomorphine at higher concentrations (Fig. 4). Our results showed that 100 μM apomorphine blocked TRPA1 currents in a voltage- and time-dependent manner (Fig. 4, A and B), exhibiting a fast kinetic for the block of the outward current, accompanied by a slow rundown of the inward current. In contrast to the apomorphine-evoked activation of TRPA1, which was only weakly reversible, the block at high concentrations could be relieved upon washout (Fig. 4A, inset). AITC-activated currents could also be modulated by 100 μM apomorphine (Fig. 4, C and D). Whereas 10 μM AITC evoked typical outwardly rectifying I/V curves (Fig. 4D, gray line), addition of 100 μM apomorphine caused a voltage-dependent block of the outward current and an initial potentiation of the AITC-induced inward current (Fig. 4D, black line), which was followed by a slow decline of the current within several minutes.

Mechanism of TRPA1 Activation by Apomorphine. To examine whether apomorphine acts on TRPA1 in a membrane-delimited fashion, we conducted electrophysiological experiments on excised patches in the inside-out configuration. Application of apomorphine to the cytosolic side of the channel caused a robust activation of TRPA1 channels (Fig. 5, A and B), which could be blocked by the TRPA1 antagonist HC-030031, indicating that no intracellular components (e.g., an elevation of intracellular calcium) contribute to the activation of TRPA1 by apomorphine. We next analyzed the modulation of TRPA1 by high concentrations of apomorphine at the single-channel level. Current traces were recorded in the inside-out configuration. TRPA1 was first stimulated with 10 μM apomorphine followed by the addition of 100 μM

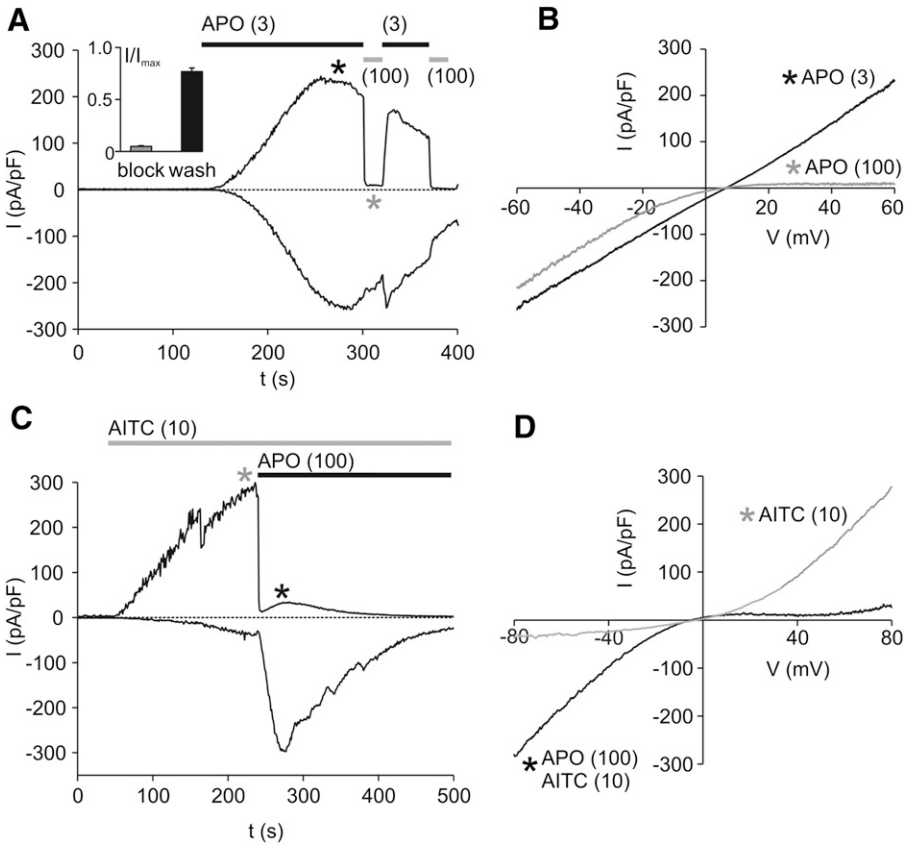

Fig. 4. TRPA1 currents can be concentration-dependently modulated by apomorphine (APO). (A) Example whole-cell recording similar to that in Figure 3A. TRPA1 was repetitively exposed to apomorphine at concentrations of 3 and 100 μM as indicated by the bars. Inset shows statistical evaluation of current amplitudes normalized to activation by 3 μM apomorphine (I/I_{max}) after addition of 100 μM apomorphine (gray bar; $n = 5$) and after washout with 3 μM apomorphine (black bar; $n = 5$). (B) Voltage ramps were taken at the timepoints indicated in (A) (black asterisk, 280 s; gray asterisk, 310 s). (C) Whole-cell recording similar to that in (A). TRPA1 currents were stimulated with 10 μM AITC, followed by the addition of 100 μM apomorphine as indicated by the bars. (D) Voltage ramps similar to that in (B). Data displayed was taken at the time points indicated in (C) (gray asterisk, 235 s; black asterisk, 280 s).

apomorphine. High apomorphine concentrations strongly reduced the single-channel open probability by provoking a flickering channel block (Fig. 6, A and B). An average single-channel conductance of 86 $\pm$ 8 pS could be deducted from several recordings, which is in line with previously reported values for TRPA1 (Fig. 6C).

Apomorphine can undergo autooxidation forming semiquinone and quinone derivatives and redox cycling of these forms can produce reactive oxygen species (El-Bachá et al., 1999). Because TRPA1 is a target for oxidative stress (Andersson et al., 2008) we tested whether hydrogen peroxide generation contributes to the activation of TRPA1 by apomorphine. Preincubation of TRPA1 cells with catalase prevented hydrogen peroxide–induced activation, as expected, but did not affect apomorphine activation (Fig. 7A). Moreover, as shown above, TRPM2, which is a target for hydrogen peroxide, proved to be insensitive to apomorphine treatment (Fig. 1B). It is well known that intracellular cysteine residues within TRPA1 serve as nucleophiles, providing a target for electrophilic attacks by reactive compounds (Hinman et al., 2006; Macpherson et al., 2007). Such reactions can be alleviated by nucleophiles (e.g., by reduced glutathione). When we included 10 mM reduced glutathione in the intracellular solution in whole-cell patch

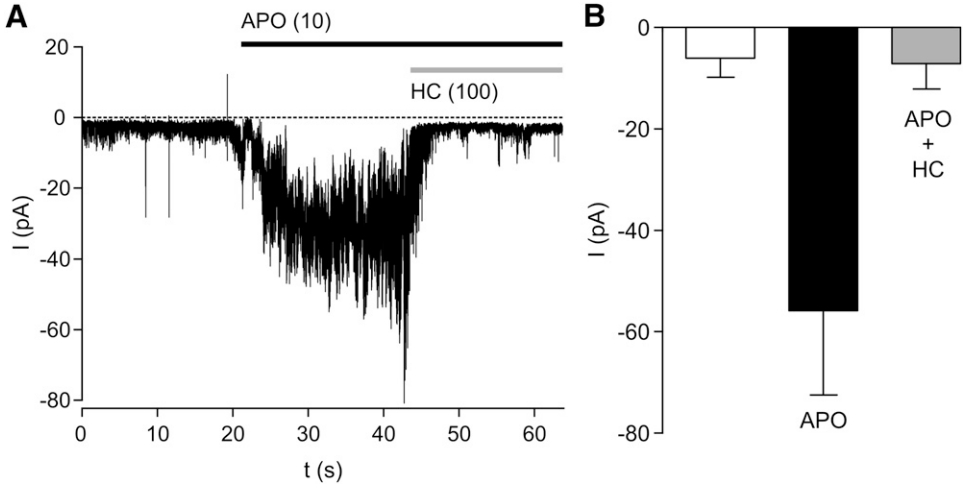

Fig. 5. TRPA1 channels are activated by apomorphine (APO) in a membrane-delimited manner. (A) Example recording obtained in an inside-out membrane patch from a $HEK293_{TRPA1}$ cell at $V_h = -70$ mV. Addition of apomorphine (10 μM) and HC-030031 (100 μM) is indicated by the horizontal bars. (B) Statistical analysis of several experiments such as in (A). Data represent means $\pm$ S.E. of five independent experiments.

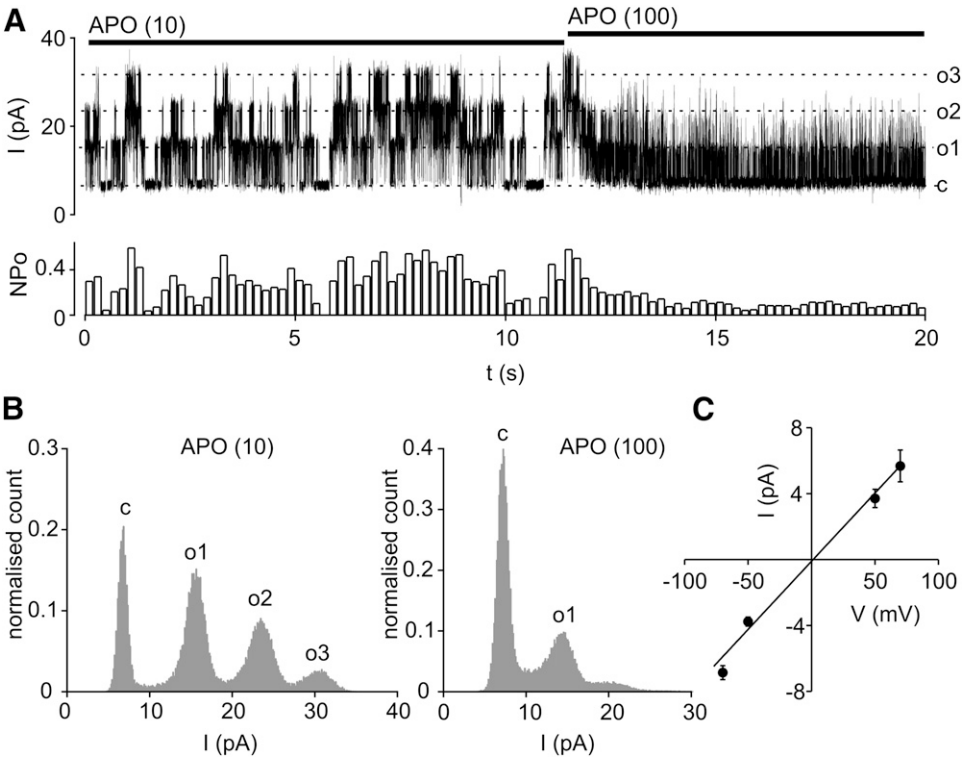

Fig. 6. High concentrations of apomorphine (APO) reduce the open probability of TRPA1 channels in inside-out patches. (A) Example trace of an inside-out patch of a $HEK293_{TRPA1}$ cell containing at least three TRPA1 channels (o1–o3 are indicated by the dotted lines) at V_h = +80 mV. Apomorphine was added at a concentration of 10 μM and 100 μM as indicated by the bars. Calculated NP_o of the same recording over the time course is depicted below the current trace (NP_o was averaged for 200-ms intervals for each bar). (B) Corresponding amplitude histograms for a 10-s recording with 10 μM (left) and 100 μM (right) apomorphine of the same patch. (C) Plot of the single-channel amplitudes pooled from four independent inside-out recordings at different holding potentials. A unitary conductance of 86 pS could be determined from the I/V plot.

clamp experiments, the apomorphine-induced TRPA1 activation was strongly impeded (Fig. 7B).

Apomorphine Induces TRPA1-Dependent Calcium Influx in DRG Neurons. We next assessed whether apomorphine can stimulate TRPA1 in cultured DRG neurons. Single-cell calcium imaging revealed that application of 20 μM apomorphine evoked an increase in intracellular calcium in a subset of DRG neurons that corresponded to 28% of all viable neurons as assessed by the activation of voltage-dependent calcium channels after depolarization of the cells with 50 mM KCl. To confirm the contribution of TRPA1-expressing neurons to the apomorphine-induced calcium signal, we challenged the neurons with an additional application of AITC. None of the apomorphine-negative neurons displayed a further rise in intracellular calcium when cells were additionally stimulated with AITC (Fig. 8, A and B). We repeated the same set of experiments but with AITC addition preceding the apomorphine treatment (Fig. 8, C and D). Again, none of the AITC-insensitive neurons responded to an additional apomorphine stimulation, indicating that apomorphine and AITC activate the same subset of DRG neurons. To further corroborate the assumption that TRPA1 is responsible for the apomorphine-induced calcium rise, we preincubated the neurons with 100 μM HC-030031. Afterward, only 3% of the neurons showed a small signal after apomorphine application, compared with 28% responsive neurons without the TRPA1-specific blocker. From our experiments, we conclude that TRPA1 is responsible for the apomorphine-induced rise in intracellular calcium in DRG neurons.

QGP-1 Cells Release 5-HT via an Activation of TRPA1 by Apomorphine. In addition to its expression in sensory neurons, TRPA1 is widely expressed in the digestive system not only in peripheral nerve fibers but also in 5-HT–releasing ECs and cholecystokinin-releasing endocrine cells of the gastrointestinal mucosa (Purhonen et al., 2008; Nozawa et al., 2009). We therefore tested whether apomorphine can activate TRPA1 in the QGP-1 cell line, a human pancreatic endocrine cell line that was found to highly express TRPA1 and EC cell marker genes (Doihara et al., 2009c). As expected, apomorphine caused strong rises in intracellular calcium that could be completely prevented when QGP-1 cells were preincubated with HC-030031 (Fig. 9, A and B). Responses to apomorphine were significantly lower compared with AITC, and the EC_{50} values for apomorphine- and AITC-induced activation were approximately 10-fold higher than in the heterologous expression system (Fig. 9C). We next examined whether QGP-1 cells can release 5-HT in response to apomorphine, which has already been described for AITC and cinnamaldehyde (Doihara et al., 2009b). For maximal response, we chose a high agonist concentration of 300 μM, which was previously shown to be effective for AITC-induced release of 5-HT in QGP-1 cells (Doihara et al., 2009c). Stimulation of QGP-1 cells with apomorphine provoked a rise in the 5-HT concentration in the supernatant of QGP-1 cells comparable with AITC. This release was at least partly be mediated by TRPA1 activation as preincubation of the cells with HC-030031 potently reduced the amount of released 5-HT (Fig. 9C).

Discussion

We found that TRPA1 is activated by apomorphine with an EC_{50} of 7.1 μM. Other sensory TRP channels that are expressed in DRG neurons remained unaffected by apomorphine. However, it has to be taken into account that except for TRPM8 and TRPA1, only the rodent orthologs were

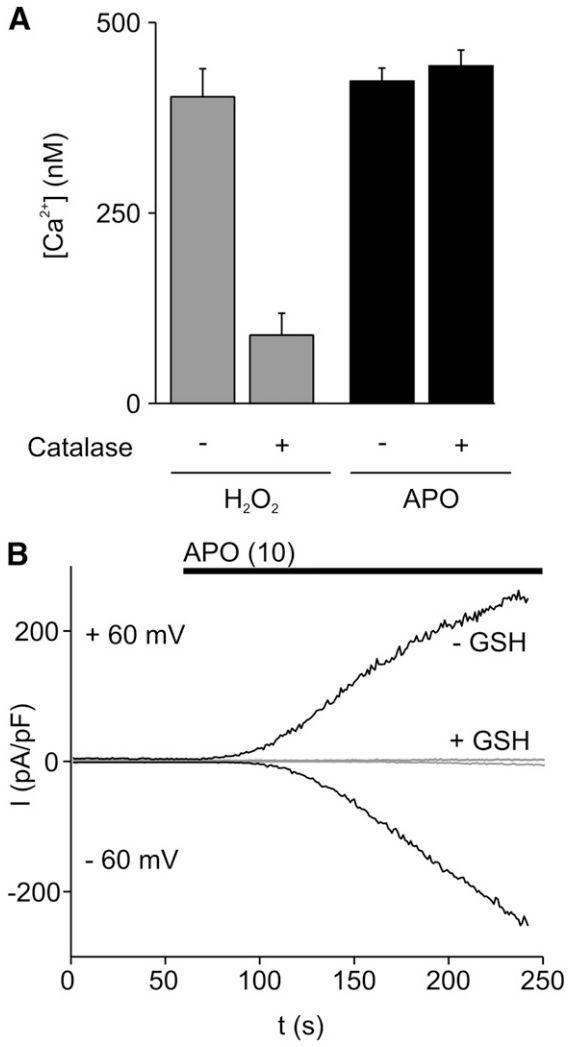

Fig. 7. TRPA1 activation by apomorphine (APO) is not caused by hydrogen peroxide generation and can be inhibited by intracellular reduced glutathione. (A) Statistical analysis of single-cell analysis of $[Ca^{2+}]_i$ of Fura-2–loaded $HEK293_{TRPA1}$ cells, stimulated with 500 μM hydrogen peroxide or 20 μM apomorphine in the absence (−) and presence (+) of catalase (data represent mean of three independent experiments ± S.D.). (B) Example whole-cell recordings of a $HEK293_{TRPA1}$ cell with 10 mM reduced glutathione in the patch pipette (+GSH; gray line) and of a control recording without glutathione (−GSH; black line) at V_h = +60 mV and V_h = −60 mV. Data were extracted from voltage ramps as previously described. Statistical analysis of four similar experiments including 10 mM glutathione in the pipette revealed current densities of 3.6 ± 1.1 pA/pF (V_h = +60 mV) and −12.2 ± 6.5 pA/pF (V_h = −60 mV) after 3 min of stimulation with apomorphine.

investigated in this study. The activation of TRPA1 by apomorphine concentrations in the low micromolar range produced a robust current in electrophysiological recordings, which did not decline within the timeframe observed (usually up to 10 min). Apomorphine could activate TRPA1 by several possible mechanisms. Apomorphine undergoes autooxidation to its semiquinone and quinone derivatives. The generation of reactive oxygen species during redox cycling of these derivatives with molecular oxygen may therefore underlie the channel activation. Because neither catalase could impede apomorphine gating of TRPA1, nor the redox-gated TRPM2 channel showed sensitivity toward apomorphine, we conclude that the generation of oxidative stress during redox cycling is not responsible for the apomorphine-induced activation of TRPA1. Nonetheless, formation of electrophilic quinone and semiquinone derivatives appears to be required for TRPA1 activation, because reduced glutathione, which acts as a nucleophile, could prevent apomorphine-induced activation of TRPA1. The quinone and semiquinone derivatives of apomorphine presumably form irreversible conjugates with reactive N-terminal cysteines of the TRPA1 protein, due to their character as electrophilic Michael acceptors (Moreira et al., 2003). A similar mode of action has been described for a variety of other TRPA1 activators (Hinman et al., 2006; Macpherson et al., 2007).

Apomorphine-stimulated whole-cell I/V curves demonstrated a slight inward rectification, in contrast to the weak outward rectification that is evident immediately after activation with other classic TRPA1 activators such as AITC and 5-hydroxynonenal. Recent evidence suggests that TRPA1 undergoes dynamic pore dilation during sustained agonist application, resulting in a transformation of the initially strong outward rectification toward an almost linear I/V shape (Chen et al., 2009; Banke et al., 2010). It may thus be possible that binding of apomorphine to TRPA1 either causes a rapid transition to a fully dilated pore or directly forces TRPA1 to adapt a conformation that is associated with a dilated permeation pathway. As a consequence, apomorphine may induce a more pronounced depolarization of TRPA1-expressing cells compared with other TRPA1 agonists. Moreover, apomorphine showed a concentration- and voltage-dependent bimodal action on TRPA1 currents in the heterologous expression system, activating the channel at low and blocking the channel at high drug concentrations. The block of the outward current was fast and accompanied by a slow rundown of the inward current, which sometimes showed an initial potentiation (data not shown). The AITC-induced response could be modulated in a similar way with the initial potentiation of the inward current being more pronounced. Under physiologic conditions, apomorphine behaves as a cationic drug (Subramony, 2006), making it unlikely that the block of the outward current is caused by apomorphine being electrostatically forced into the pore. It seems more likely that apomorphine acts as a gating modifier of TRPA1, which, at high concentrations, reduces TRPA1 single-channel open times, as can be seen from the current traces in excised inside-out patches. A similar bimodal action has been described for the modulation of TRPA1 by menthol and lidocaine (i.e., an activation at low and a block at high concentrations) (Karashima et al., 2007; Leffler et al., 2011).

The role of TRPA1 in nociception has been intensely studied applying TRPA1 knockout animals (Bautista et al., 2006; Kwan et al., 2006). For example, it has been shown that TRPA1 is a key player in the mechanism of formalin-induced pain models by exciting sensory neurons through a direct activation of TRPA1 (McNamara et al., 2007). In humans, injections of apomorphine for the treatment of motor fluctuations in patients with Parkinson's disease are very commonly accompanied by local reactions at the injection site, such as subcutaneous nodules, erythema, itching, and painful sensations. For subcutaneous administration, apomorphine is applied at a typical dose of 2 mg in 0.2 ml volume but presumably quickly dilutes within the surrounding tissue, making it difficult to estimate local apomorphine concentrations at the injection site. However, in cultured DRG

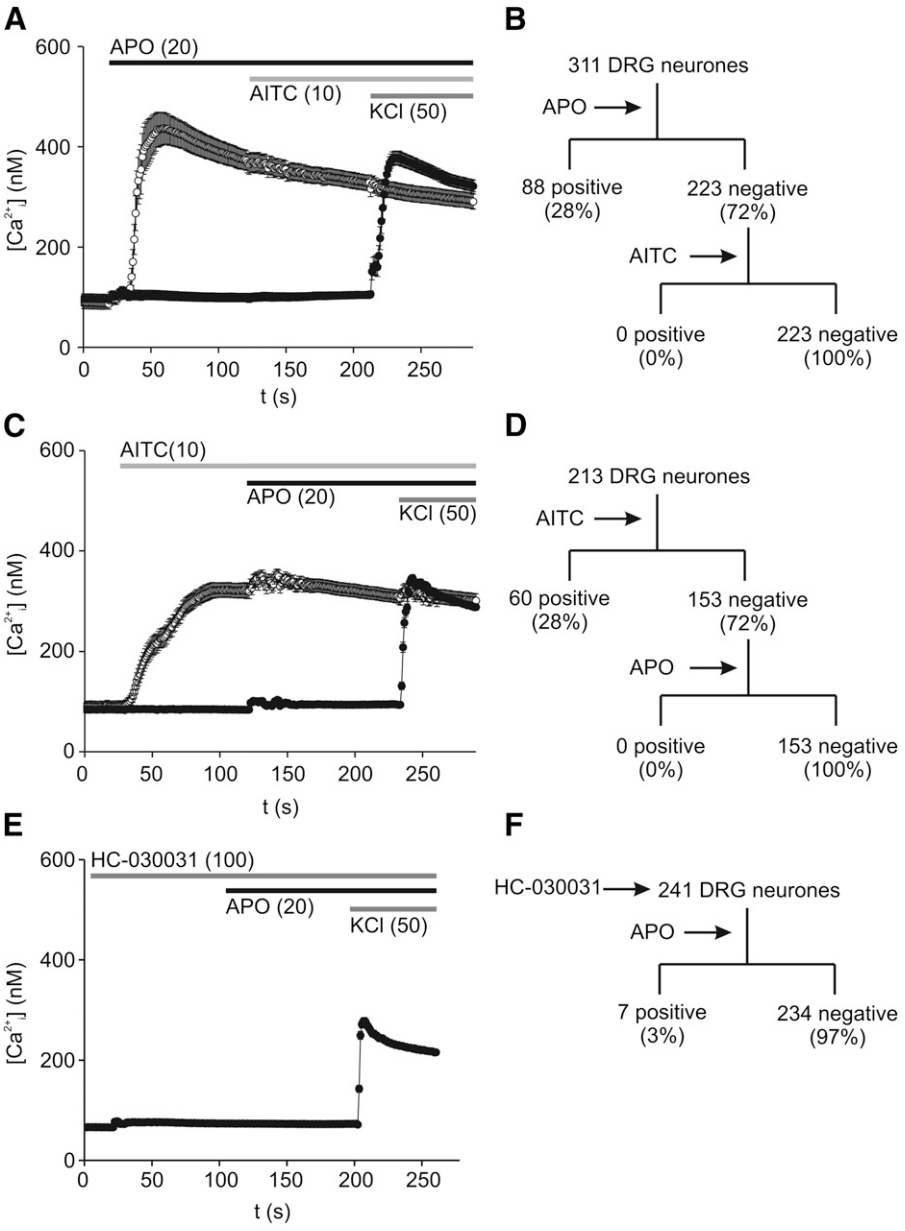

Fig. 8. Apomorphine (APO) activates TRPA1 channels in DRG neurons. (A) Representative examples of time-lapse analyses of two different populations of Fura-2–loaded DRG neurons after addition of apomorphine (20 μM) followed by the addition of AITC (10 μM), and KCl (50 mM) as indicated by the bars. Only a subpopulation of DRG neurons (open symbols) responded to the addition of apomorphine with a rapid increase in intracellular calcium, whereas a second population (black symbols) was insensitive toward apomorphine addition. (B) Statistical analysis for several experiments such as in (A); 28% of the DRG neurons responded to apomorphine and none of the apomorphine-negative neurons subsequently responded to AITC. (C) Similar experiment as in (A) but with AITC addition preceding apomorphine addition. (D) Statistical analysis of several experiments such as in (C); 28% of the DRG neurons were AITC-sensitive and none of the AITC-negative neurons additionally responded to apomorphine. (E) Similar experiment as in (A) and (C) but with DRG neurons being preincubated with HC-030031 (100 μM). (F) Statistical analysis of several experiments such as in (E). Only 3% of HC-030031–treated neurons showed an increase in $[Ca^{2+}]_i$ after apomorphine addition. For each experimental condition, coverslips of four individual animals were used on different days.

neurons, TRPA1 could be activated at a 1000-fold dilution from the concentration injected in vivo. Therefore, it seems likely that apomorphine injection can induce a TRPA1-mediated excitation of nociceptive neurons *in vivo,* possibly contributing to local reactions at the injection site. This conclusion is further supported by a study that demonstrated a hyperalgesic effect of subcutaneously administered apomorphine on formalin-induced pain in rodents (Pelissier et al., 2006).

TRPA1 is also present in the gastrointestinal tract, not only in sensory afferent fibers and in the enteric nervous system (Penuelas et al., 2007) but also in 5-HT–releasing ECs (Nozawa et al., 2009). TRPA1 agonists, such as AITC and cinnamaldehyde, release 5-HT from purified primary ECs and from cultured EC cell lines (Nozawa et al., 2009). In dogs, for example, intragastric administration of AITC stimulates frequent contractions of the gastric antrum and jejunum, triggering vomiting within 5 min after AITC administration (Doihara et al., 2009a). Taken together, these data indicate a possible role for TRPA1 as a sensor molecule for the regulation of various gastrointestinal reactions [see Holzer (2011a,b) for a review].

In humans and in dogs, administration of apomorphine is accompanied by severe nausea and emesis (Lefebvre et al., 1981; Axelsson et al., 2006). In general, mechanisms by which drug-induced emesis can be triggered are diverse. In addition to a direct binding of drugs to the CTZ, nausea can also be caused by a direct stimulation of vagal afferents (Andrews et al., 1990) and/or by the release of 5-HT from ECs, which subsequently stimulates vagal afferents via 5-HT receptors [see Hesketh (2008) for a review]. It is believed that apomorphine directly binds to dopamine receptors within the CTZ, causing nausea and emesis. Considering the expression of TRPA1 on vagal afferents and in ECs, a second

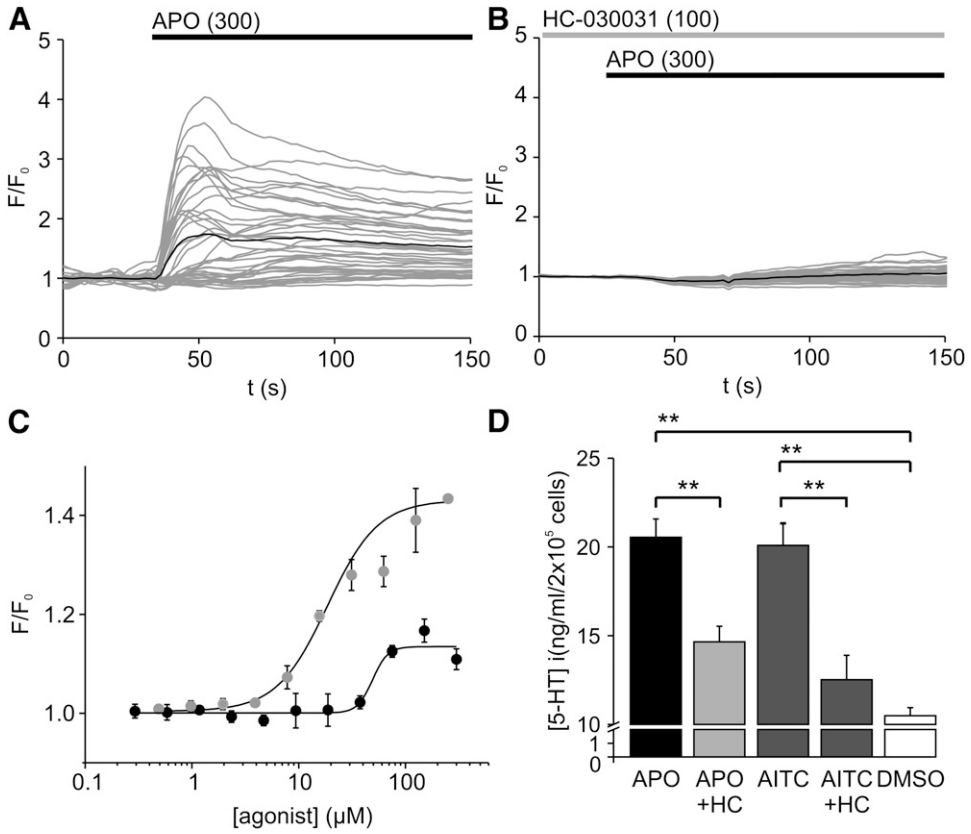

Fig. 9. Apomorphine (APO) elevates $[Ca^{2+}]_i$ in the QGP-1 cell line and stimulates 5-HT release. (A) Time-lapse analysis of calcium signals in Fluo-4–loaded QGP-1 cells of individual cells (gray traces) and the mean response (black trace) upon stimulation with apomorphine (300 μM). (B) Similar experiments as in (A) but in the presence of HC-030031 (100 μM) prior to stimulation with apomorphine (300 μM). (C) Concentration response curves using Fluo-4–loaded QGP-1 cells revealed EC_{50} values of 68 μM for apomorphine (black symbols) and 19 μM for AITC (gray symbols). (D) The release of 5-HT into culture supernatants of QGP-1 cells was determined by enzyme-linked immunosorbent assay. Open bar, dimethylsulfoxide control; black bar, apomorphine (300 μM); light gray bar, apomorphine (300 μM) together with HC-030031 (100 μM); gray bars, AITC (300 μM) and AITC (300 μM) together with HC-030031 (100 μM). Data represent means ± S.D. (n = 4). $^{**}P < 0.01$.

mechanism involving a local activation of TRPA1 in the gastrointestinal tract may also contribute to the apomorphine-induced emesis. However, it must be taken into account that plasma levels of apomorphine in dogs that are emetogenic may only lead to a minor activation of TRPA1 (maximal plasma concentration of 370 nM) (Youssef et al., 1999). Taken together, our results demonstrate that TRPA1 is a molecular target for apomorphine, indicating that an activation of TRPA1 might underlie local reactions at the injection site and might also contribute to the emetic side effects during apomorphine therapy by activating and/or sensitizing TRPA1 in EC cells and on vagal afferents.

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Authorship Contributions

Participated in research design: Schulze, Schaefer, Hill.

Conducted experiments: Schulze, Urban, Oehler, Hill.

Performed data analysis: Schulze, Urban, Oehler, Hill.

Wrote or contributed to the writing of the manuscript: Schulze, Schaefer, Hill.

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