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TRPC5 Channel Sensitivities to Antioxidants and Hydroxylated Stilbenes*[S]

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Transient receptor potential canonical 5 (TRPC5) forms cationic channels that are polymodal sensors of factors including oxidized phospholipids, hydrogen peroxide, and reduced thioredoxin. The aim of this study was to expand knowledge of the chemical-sensing capabilities of TRPC5 by investigating dietary antioxidants. Human TRPC5 channels were expressed in HEK 293 cells and studied by patch clamp and intracellular Ca^{2+} recording. GFP- and HA-tagged channels were used to quantify plasma membrane localization. Gallic acid and vitamin C suppressed TRPC5 activity if it was evoked by exogenous hydrogen peroxide or lanthanide ions but not by lysophosphatidylcholine or carbachol. Catalase mimicked the effects, suggesting that lanthanide-evoked activity depended on endogenous hydrogen peroxide. *Trans*-resveratrol, by contrast, inhibited all modes of TRPC5, and its effect was additive with that of vitamin C, suggesting antioxidant-independent action. The IC_{50} was ~10 μM. Diethylstilbestrol, a related hydroxylated stilbene, inhibited TRPC5 with a similar IC_{50}, but its action contrasted sharply with that of resveratrol in outside-out membrane patches where diethylstilbestrol caused strong and reversible inhibition and resveratrol had no effect, suggesting indirect modulation by resveratrol. Resveratrol did not affect channel surface density, but its effect was calcium-sensitive, indicating an action via a calcium-dependent intermediate. The data suggest previously unrecognized chemical-sensing properties of TRPC5 through multiple mechanisms: (i) inhibition by scavengers of reactive oxygen species because a mode of TRPC5 activity depends on endogenous hydrogen peroxide; (ii) direct channel blockade by diethylstilbestrol; and (iii) indirect, antioxidant-independent inhibition by resveratrol.

Since the discovery of the *Drosophila melanogaster* transient receptor potential (*TRP*) gene, it has emerged that mammalian genomes contain 28 homologous genes encoding proteins that are capable of forming a large variety of homo- or hetero-multimeric cationic channels, usually with permeability to sodium and calcium (1, 2). Unlike many other types of ion channel, activity is not gated by changes in voltage and there is no fast neurotransmitter-dependent gating. Instead, the channels respond relatively slowly to various chemical and physical factors. In the case of some types of TRP[2] channel, there is extensive knowledge of the chemical-sensing capabilities, and roles as sensors of chemical or temperature changes in the environment have been proposed (2).

A subtype of TRP channels that is relatively poorly understood are the TRPC (canonical) channels, which have the closest amino acid sequence similarity to *Drosophila* TRP (3). Humans contain six TRPC-expressing genes, and mice contain seven. A TRPC protein common to both species is TRPC5 (4). Early studies noted high expression of TRPC5 in the brain, but it was subsequently detected in many, but not all, cell and tissue types. TRPC5 has been a focus of numerous studies at least partly because it is readily overexpressed and unambiguously distinguished from background channels in experimental settings (5–7). There is also increasing evidence for key roles in native cells, including in growth cone formation, potentiation of innate fear responses, synoviocyte secretion, cardiac development in diabetes, and endothelial cell and vascular smooth muscle cell remodeling (8–14). In many of these contexts, TRPC5 forms channels with other TRPC proteins (*e.g.* TRPC1). A specific physiological stimulator has not emerged; instead there are multiple nonspecific stimulators, including receptor agonists (*e.g.* carbachol and ATP), endogenous lipids (*e.g.* lysophosphatidylcholine (LPC)), redox factors, mild acidification, and toxic metal ions (4, 11, 15–18). It has been suggested that one function of TRPC5 may be as a sensor of adverse signals (19), but the chemical-sensing profile of the channels is still unfolding and requires further investigation.

TRPC5 sensitivity to redox factors is shown by the effects of exogenous hydrogen peroxide (H_2O_2) and the redox protein thioredoxin (11, 15). There is also sensitivity to oxidized

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[2] The abbreviations used are: TRP, transient receptor potential; TRPC, transient receptor potential channel; Tet, tetracycline; SBS, standard bath solution; LPC, lysophosphatidylcholine; 2-APB, 2-aminoethoxydiphenylborate; DMSO, dimethyl sulfoxide.

phospholipids (14). To expand knowledge of these sensing capabilities, we hypothesized that there may be effects of antioxidant chemicals, including those present in the diet. Although we previously found no effect of vitamin E (α-tocopherol) (20), antioxidants are not necessarily equivalent. Additional dietary antioxidants include vitamin C, gallic acid, and resveratrol, which are components of some fruits, vegetables, and beverages, including green tea and red wine. Resveratrol has attracted exceptional attention because of its apparent capacity to protect against non-contagious diseases and explain benefits of the "Mediterranean diet" (21). Reports show, for example, intriguing effects on cardiovascular disease indicators, including flow-mediated dilatation and endothelial nitric oxide synthase activity, and measures of type-2 diabetes, cancer, and neurological disorder (21–23). One of the suggested mechanisms of action of resveratrol is as an activator of the sirtuin enzymes, which impact on insulin secretion and lipid mobilization. However, the mechanism has been challenged, and alternative effects through diverse membrane proteins have been suggested (24).

The data of this study suggest an H_2O_2-dependent mode of TRPC5 activity that can be suppressed by dietary scavengers of reactive oxygen species, such as gallic acid and vitamin C. Resveratrol, however, acted differently, and investigation of its mechanism of action led to identification of a novel TRPC5 inhibitor based on the stilbene chemical backbone.

EXPERIMENTAL PROCEDURES

Cell Culture and TRP Channel Expression—HEK-293 cells stably incorporating tetracycline-regulated expression of human TRPC5 have been described (7). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 +GlutaMAX-1 (Invitrogen) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin at 37 °C in a 5% CO_2 incubator; 400 μg ml^{-1} zeocin and 5 μg ml^{-1} blasticidin S were included in the culture medium to maintain selection of tetracycline-regulated expression. To induce channel expression, cells were incubated with 1 μg ml^{-1} tetracycline (Sigma) for 24–72 h prior to experiments (Tet+). Non-induced cells without addition of tetracycline (Tet−) were used as control. Freshly discarded human saphenous vein segments were obtained anonymously and with informed consent from patients undergoing open heart surgery in the Leeds General Infirmary. Approval was granted by the Leeds Teaching Hospitals Local Research Ethics Committee. Proliferating vascular smooth muscle cells were prepared using an explant technique and grown in Dulbecco's modified Eagle's medium +GlutaMAX (catalog number 31966, Invitrogen). The medium was supplemented with 10% fetal calf serum, 100 units/ml penicillin/streptomycin (Sigma) at 37 °C in a 5% CO_2 incubator. Experiments were performed on cells passaged 3–5 times.

Intracellular Ca^{2+} Measurement—Induced (Tet+) and non-induced (Tet−) cells were plated in poly-D-lysine-coated black 96-well plates (Corning). Cells were incubated for 1 h in 4 μM fluo-4 AM in standard bath solution (SBS) at 37 °C in the presence of 0.01% pluronic acid (Invitrogen) and freshly prepared 2.5 mM probenecid. SBS contained (in mM): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$, and 1.5 $CaCl_2$; the pH was titrated to 7.4 with NaOH. Cells were washed three times with SBS before measurements were made at room temperature (21 ± 2 °C) on a 96-well fluorescence plate reader (FlexStation II^{384}, Molecular Devices). Fluo-4 was excited at 485 nm, and emission was collected at 525 nm. Readings were made every 10 s to indicate the change in intracellular calcium concentration ($[Ca^{2+}]_i$) as the change (Δ) in fluo-4 fluorescence (F, in arbitrary units). For simplicity of presentation, fluo-4 fluorescence values were divided by 10^4 (F^*). Pretreatments with agents were for 15 min at room temperature prior to recordings and maintained throughout. Control cells were treated with ethanol, methanol, or dimethyl sulfoxide (DMSO) (vehicle) as appropriate.

Whole-cell and Outside-out Patch Recording—Current recordings were made at room temperature under voltage clamp using the whole-cell or outside-out patch configurations of the patch clamp technique. Signals were amplified and sampled using an Axopatch 200B amplifier and pCLAMP 8 software (Molecular Devices). Cells were investigated on glass coverslips at 20–30% density. The extracellular solution was SBS, and the patch pipette solution contained (in mM): 135 CsCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Na_2ATP, and 0.1 Na_2GTP, titrated to pH 7.2 with NaOH ("1 EGTA pipette"); or 17 $CaCl_2$, 2 $MgCl_2$, 8 NaCl, 40 EGTA, 10 HEPES, 66 L-glutamic acid, 66 CsOH, 1 Na_2ATP, and 0.1 Na_2GTP ("40 EGTA pipette," 100 nM calculated free Ca^{2+}). For outside-out patch recording, the extracellular solution was SBS, and the patch pipette solution contained (in mM): 135 CsCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Na_2ATP, and 0.1 Na_2GTP, titrated to pH 7.2 with NaOH. For experiments requiring no extracellular Ca^{2+}, $BaCl_2$ replaced the $CaCl_2$ in SBS. All solutions were filtered using a 0.2-μm filter (Sartorius). Voltage ramps were applied from −100 to +100 mV every 10 s from a holding potential of 0 mV. Data were filtered at 2 kHz and digitally sampled at 4 kHz. Agents were applied using a continuous gravity flow perfusion system through a chamber containing ~200 μl of ionic solution; solution exchange was complete in ~1 min.

Labeling of Plasma Membrane TRPC5—Wild-type HEK 293 cells were transfected with TRPC5-GFP (11) using FuGENE HD 48 h prior to experiments. Live cells plated onto coverslips were treated with 0.1% ethanol in SBS for 5 min followed by 0.1% ethanol or 30 μM resveratrol (in ethanol vehicle) for 7.5 min. Images were collected every 30 s at room temperature using a Zeiss LSM 510-META laser scanning inverted microscope with an oil-immersed ×63 objective lens (NA = 1.40). Images were analyzed using ImageJ software (National Institutes of Health). Alternatively, wild-type HEK 293 cells were transfected with TRPC5 that was hemagglutinin (HA) epitope-tagged in the second extracellular loop **VKY**YPYDVPDYA**NGS** (single-letter amino acid code; bold indicates TRPC5, and underline indicates HA). After treatment with resveratrol or vehicle, cells were labeled with anti-HA antibody (Covance) detected with Cy3-conjugated donkey anti-mouse secondary (1:300) (Jackson ImmunoResearch) and visualized using a deconvolution microscope (Applied Precision Instruments, Seattle, WA) containing an Olympus IX-70

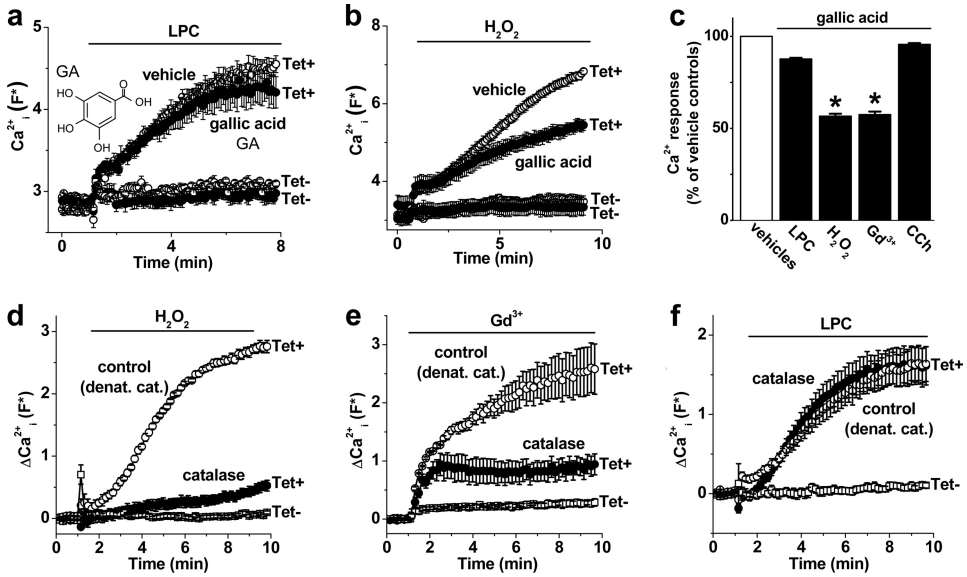

FIGURE 1. **Selective inhibition by gallic acid and catalase.** *a–f*, intracellular Ca^{2+} measurements from non-induced (Tet−) and TRPC5-expressing (Tet+) cells incubated with 1 mM gallic acid (*GA*) (*a–c*), 500 units·ml^{-1} catalase (*d–f*), or vehicle control. *White symbols* are for vehicle controls, and *black symbols* are for test agents. *a* and *b*, representative experimental traces for responses to 5 μM LPC (*a*) or 1 mM H_2O_2 (*b*) in the presence or absence of gallic acid (its chemical structure is in the *inset* in *a*). *c*, for the types of experiments illustrated in *a* and *b*, mean summary data for Tet+ cells where responses to each stimulator are normalized to their own controls in the absence of gallic acid ($n/N = 3/9$ for each). Carbachol (*CCh*) was used at 10 μM, and Gd^{3+} was used at 50 μM. *, $p < 0.05$. *d–f*, representative experimental traces for responses to 1 mM H_2O_2 (*d*), 50 μM Gd^{3+} (*e*), and 5 μM LPC (*f*) in the presence of catalase or its denatured catalase control (*denat. cat.*). Each data set is for $N = 8$ and representative of three independent experiments.

inverted microscope fitted with a ×100 UPLAN objective (NA 1.35). For quantification of HA labeling at the cell surface (see Fig. 5, *c–e*), 13 images were analyzed from three independent experiments. In each case, the image was derived from 12 planes within a z-stack, and a rectangle across the cell and over the nucleus, rather than a single line scan, was used to generate a fluorescence profile and therefore peak intensities at the outer edges of the cell. Peak intensities in the resveratrol group were normalized to the matched vehicle controls.

Chemicals—Unless specified, chemicals were from Sigma. Stocks of chemicals were reconstituted in an appropriate vehicle. Fluo-4 AM (Invitrogen) was dissolved at 1 mM in DMSO; pluronic acid F-127 (Invitrogen) was stored at 10% w/v in DMSO at room temperature; probenecid was freshly prepared in 1 M NaOH at 0.5 M and diluted 1:200 to give a working concentration of 2.5 mM; resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) was dissolved at 30 or 100 mM in ethanol; 2-aminoethoxydiphenylborate (2-APB) was dissolved at 75 mM in DMSO; LPC was dissolved at 50 mM and diethylstilbestrol at 50 or 10 mM in methanol; 17β-estradiol was dissolved at 10 mM in ethanol; gallic acid and vitamin C were dissolved at 1 M; and gadolinium chloride (Gd^{3+}) was dissolved at 100 mM in H_2O. Stock solutions were diluted 1:1000 into the recording solution, giving a final working solvent concentration of 0.01% v/v. The stock of 200,000 units·ml^{-1} catalase was prepared in SBS and denatured (boiled for 10 min) catalase was the control.

Data Analysis and Presentation—Mean data are presented as means ± S.E., where the *n* represents the number of independent experiments and the *N* represents the number of wells when 96-well plates were used. Statistical comparisons were made on data collected in pairs (test and control groups) using *t* tests with $p < 0.05$ indicating significant difference (*) in Figs. 1, 4, and 6 and $p > 0.05$ indicating no significant difference (*NS*) in Figs. 4 and 5. Each test group had its own control even if only one control bar is shown in the figures. Comparisons between multiple groups were made using analysis of variance. The Hill equation was fitted, and data were presented and analyzed using Origin software (MicroCal Inc.).

RESULTS

Inhibition of H_2O_2-dependent Activity by Gallic Acid and Vitamin C—The data of Fig. 1 show that cells induced to express human TRPC5 exhibited Ca^{2+} influx in response to LPC, hydrogen peroxide (H_2O_2), gadolinium (Gd^{3+}), or carbachol (*CCh*). The dietary antioxidant gallic acid (Fig. 1*a*, *inset*) lacked effect on LPC (Fig. 1, *a* and *c*) and carbachol (Fig. 1*c*) responses but inhibited H_2O_2 (Fig. 1, *b* and *c*) and Gd^{3+} responses (Fig. 1*c*). The effect of another dietary antioxidant, vitamin C, was similar (supplemental Fig. I, *a* and *b*). Because of the selective effects on H_2O_2 and Gd^{3+} responses, we investigated the hypothesis that Gd^{3+} responses depend on endogenous H_2O_2 production, using catalase to catalyze the conversion of H_2O_2 to water. Consistent with this hypothesis, catalase inhibited H_2O_2 and Gd^{3+} responses but not LPC responses (Fig. 1, *d–f*). The data suggest that gallic acid and vitamin C suppress only TRPC5 activity that depends on H_2O_2, lacking effect on other modes of channel activity.

General and Antioxidant-independent Inhibition by Resveratrol—Resveratrol (*Resv.*) is a biphenolic compound based on the stilbene backbone (Fig. 2*a*) and carries antioxidant capability (25). Like gallic acid and vitamin C, resveratrol inhibited H_2O_2 (Fig. 2*b*) and Gd^{3+} responses (supplemental Fig. Ic) of TRPC5. In contrast, however, it also inhibited responses to LPC (Fig. 2*c*) and carbachol (supplemental Fig. Ic). Fur-

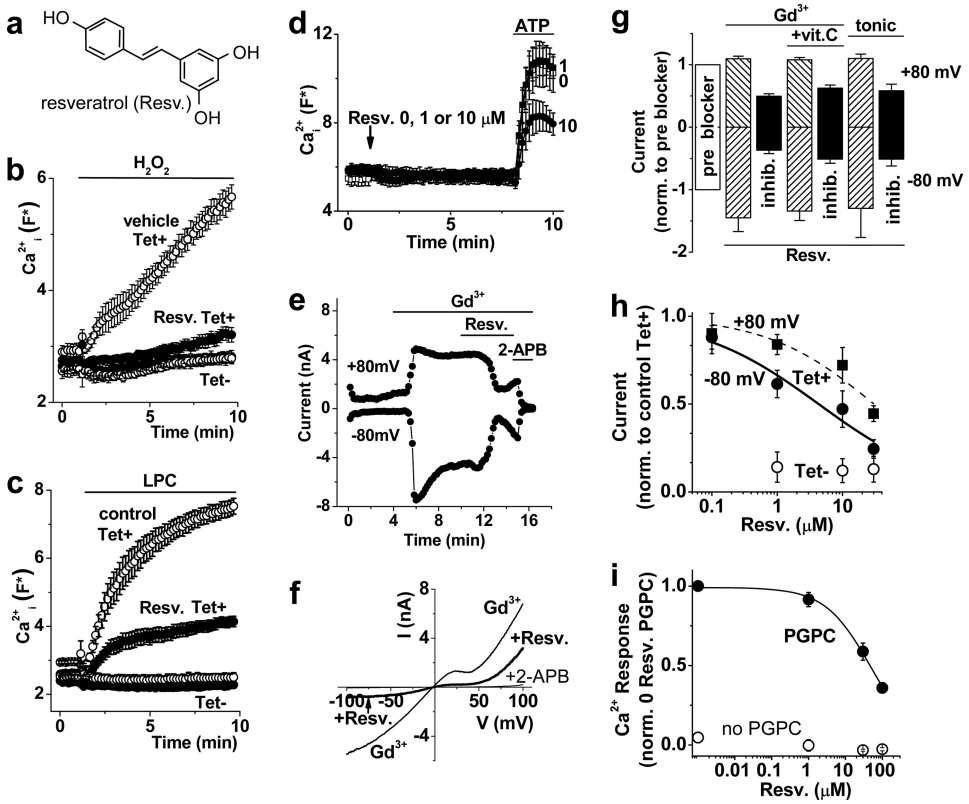

FIGURE 2. **General inhibition by resveratrol.** *a*, chemical structure of resveratrol (*Resv.*). *b–h*, recordings were made from TRPC5-expressing (Tet+) cells (*d–g*) and Tet+ or non-induced (Tet−) cells (*b*, *c*, and *h*). *b–d*, intracellular Ca^{2+} measurements showing responses to 1 mM H_2O_2 (*b*), 5 μM LPC (*c*), or 100 μM ATP (*d*) in the presence of resveratrol or its vehicle control ($N = 8$ each). Resveratrol was applied at 50 μM (*b* and *c*) or 1 or 10 μM (*d*). *e–h*, whole-cell current measurements from induced cells (*e–g*) and induced (Tet+) or non-induced (Tet−) cells (*h*). *e*, example time-series plot at +80 and −80 mV, showing the effect of 30 μM Gd^{3+}, then 30 μM Gd^{3+} plus 30 μM resveratrol, and then 75 μM 2-APB. *f*, current-voltage relationships (I-Vs) for the experiment shown in *e*. *g*, mean normalized (*Norm.*) data comparing stimulation (*hatched bars*) and inhibition (*inhib.*; *black bars*) of current by 30 μM resveratrol in the presence of 30 μM Gd^{3+} ($n = 10$), 30 μM Gd^{3+} plus 1 mM vitamin C ($n = 21$), or no additional agent and thus showing the effect on tonic (constitutive) TRPC5 activity ($n = 10$). All inhibitory effects were statistically significant when compared with their controls, but only the stimulatory effect in Gd^{3+} alone was significant. *h*, mean concentration-response data for resveratrol inhibition of TRPC5-mediated current evoked by 30 μM Gd^{3+} ($n = 5–16$/point). Curves are Hill equations with mid-points at 4.4 μM at −80 mV and 30.4 μM at +80 mV. Tet− (no TRPC5 expression) data are shown for comparison. *i*, normalized mean intracellular Ca^{2+} measurement data for vascular smooth muscle cells stimulated by 3 μM 1-palmitoyl-2-glutaryl phosphatidylcholine (*PGPC*) (*black circles*) or vehicle control (*open circles*). Inhibition of 1-palmitoyl-2-glutaryl phosphatidylcholine responses occurred with resveratrol (the IC_{50} of the fitted Hill equation is 46.0 μM) ($n/N = 3/9$).

thermore, it inhibited basal (Fig. 2, *b–d*) and ATP receptor-activated TRPC5 (Fig. 2*d*; supplemental Fig. II). The data suggest that resveratrol is a general inhibitor of all modes of TRPC5 activity.

The inhibitory effects of resveratrol on TRPC5 also occurred in whole-cell patch clamp recordings (Fig. 2*e*), showing inhibition of the signature TRPC5 current-voltage relationship (I-V) (Fig. 2*f*). These experiments also revealed a delay to the onset of action of resveratrol (Fig. 2*e*; supplemental Fig. III, *a* and *b*), and an initial, transient, stimulatory effect was observed in 64% of recordings (Fig. 2*g*; supplemental Fig. IV*a*). Tonic as well as Gd^{3+}-evoked TRPC5-dependent currents were inhibited by resveratrol (Fig. 2*g*).

Consistent with an antioxidant-independent mechanism of action, 30 μM resveratrol had an additive inhibitory effect in the continuous presence of 1 mM vitamin C (Fig. 2*g*; supplemental Fig. III*a*). Resveratrol did not completely inhibit TRPC5 at achievable concentrations (Fig. 2, *e–h*); additional blockade occurred with the nonspecific TRPC5 inhibitor 2-APB (Fig. 2, *e* and *f*). No recovery of channel activity occurred on wash-out of resveratrol (supplemental Fig. III*c*). Concentration-response experiments suggested an IC_{50} of 4–30 μM, depending on voltage (Fig. 2*h*). Similar, but slightly higher, concentrations of resveratrol inhibited endogenous TRPC5-containing channels (Fig. 2*i*).

The data suggest that resveratrol has complex modulator effects at TRPC5 that result in sustained but incomplete inhibition. The effect is independent of the antioxidant capability of resveratrol.

Inhibition by Diethylstilbestrol—Diethylstilbestrol (*DES*) is another biphenolic stilbene with a chemical structure that is similar to that of resveratrol (Fig. 3*a*; *cf.* Fig. 2*a*). It also inhibited TRPC5 in Ca^{2+} measurement (Fig. 3, *b* and *c*) and patch clamp recordings (Fig. 3, *d–f*). It was notable, however, that its effect was stronger and more consistent than that of resveratrol, with complete channel inhibition at 50 μM diethylstilbestrol and an IC_{50} of 4–9 μM (Fig. 3, *c* and *f*).

The data suggest that diethylstilbestrol is also a TRPC5 inhibitor but with a potentially different mechanism of action when compared with resveratrol. The variable initial stimulatory effect of resveratrol was, however, also observed with diethylstilbestrol (supplemental Fig. IV*b*).

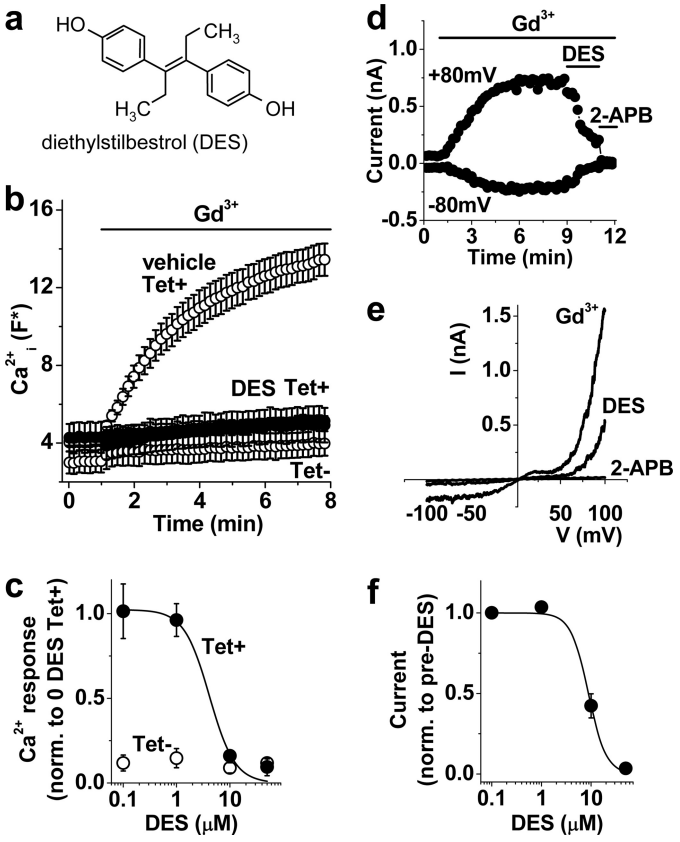

FIGURE 3. **Strong inhibition by diethylstilbestrol.** *a*, chemical structure of diethylstilbestrol (*DES*). *b–f*, recordings were made from TRPC5-expressing (Tet+) cells (*d–f*) and Tet+ or non-induced (Tet−) cells (*b* and *c*). *b*, intracellular Ca^{2+} measurements showing responses to 50 μM Gd^{3+} in the presence of 10 μM diethylstilbestrol or its vehicle control ($N = 8$ each). *c*, as for *b* but concentration-response data (the IC_{50} of the fitted Hill equation is 3.6 μM) ($n/N = 3/9$). *norm.*, normalized. *d–f*, whole-cell current measurements from cells induced to expression TRPC5 and using the 40 EGTA patch pipette solution. *d*, example time-series plot at +80 and −80 mV, showing the effect of 30 μM Gd^{3+}, then 30 μM Gd^{3+} plus 10 μM diethylstilbestrol, and then 30 μM Gd^{3+} plus 75 μM 2-APB. *e*, I-Vs for the experiment of *d*. *f*, mean concentration-response data for diethylstilbestrol inhibition of TRPC5-mediated current evoked by 30 μM Gd^{3+} at −80 mV ($n = 6–9$/point; the IC_{50} of the fitted curve is 9.0 μM).

Direct Blockade by Diethylstilbestrol but Not Resveratrol—To investigate whether the actions of resveratrol and diethylstilbestrol were on TRPC5 channel activity at the plasma membrane, we performed outside-out patch recordings, applying compounds to the external face of the channels. In patches excised from cells overexpressing TRPC5, basal macroscopic currents were observed that were enhanced by application of Gd^{3+} (Fig. 4*a*). Consistent with the currents being explained by multiple TRPC5 channels, the I-Vs showed characteristics that were similar to those seen in whole-cell recordings of TRPC5-dependent currents, albeit with milder inflection (Fig. 4, *b* and *c*). The currents gradually ran down during recordings, leading to reduced basal currents and relatively stronger effects of Gd^{3+} (*e.g.* Fig. 4*a*, *section ii*) that gave greater confidence in the purity of the TRPC5 signal.

Although we occasionally observed what appeared to be slow effects of resveratrol on these excised patch currents, the effects were not reproducible within recordings (Fig. 4*a*, *section i*; *cf. section* ii), and analysis of all data failed to show statistically significant effects (Fig. 4*d*). Furthermore, we observed clear examples of the complete lack of effect of resveratrol (Fig. 4*c*). By contrast, diethylstilbestrol had a reliable, rapid, and strong effect that was clearly reversible on wash-out (Fig. 4, *a–d*); a high level of statistical significance was obtained (Fig. 4*d*). Therefore, outside-out patch recordings clarified the distinction between actions of resveratrol and diethylstilbestrol, with resveratrol being devoid of direct channel blocking effect in contrast to diethylstilbestrol, which behaves as a direct blocker of TRPC5 channels in the plasma membrane.

Lack of Effect of Resveratrol on Channel Surface Density—Because resveratrol was not a direct blocker of TRPC5 and there was a delay to its onset of action (supplemental Fig. III), we hypothesized that it may act by altering the surface density of the channels, either by suppressing forward trafficking (26) or by enhancing internalization or degradation. Therefore, we generated TRPC5 channel, TRPC5-[HA], with an HA epitope tag in the second extracellular loop to distinguish surface-exposed TRPC5 from other TRPC5 in the cells. We observed that TRPC5-[HA] had function that was similar to that of wild-type TRPC5; there was stimulation by lanthanides (*e.g.* Fig. 5*a*) and inhibition by 2-APB (Fig. 5*a*), and the signature TRPC5 I-V was present (Fig. 5*b*). As predicted, antibody to the HA tag detected surface localized TRPC5-[HA] in non-permeabilized cells, without labeling of intracellular TRPC5 (Fig. 5, *c* and *d*). Visual inspection suggested that resveratrol had no effect on the quantity of surface TRPC5-[HA] (Fig. 5, *c* and *d*), and quantitative analysis suggested a trend toward increased surface TRPC5-[HA], but there was no statistically significant difference.

To investigate the hypothesis further, we transfected cells with a TRPC5 construct containing green fluorescent protein (GFP) at the intracellular C terminus (11), which was observed in the vicinity of the plasma membrane and intracellular structures (Fig. 5*f*). TRPC5 was similarly at the cell surface in the presence of the vehicle control or resveratrol (Fig. 5*f*). Using this approach, we were also able to follow the fluorescence in real time, comparing exposure to resveratrol with the vehicle control (Fig. 5*g*). In both cases, we observed the fade of the GFP signal, but the fade in response to resveratrol was small and not significantly different from the vehicle control (Fig. 5*g*). The data suggest that resveratrol has no significant effect on the quantity of TRPC5 at the plasma membrane, at least within the time required for inhibition of TRPC5 function by resveratrol.

Ca^{2+} Dependence of the Action of Resveratrol—The above data suggest that resveratrol acts indirectly to suppress channel activity at the plasma membrane. In an effort to shed light on the mechanism, we compared responses in the presence and absence of extracellular Ca^{2+} because TRPC5 shows marked Ca^{2+} sensitivity and association with various Ca^{2+}-sensor proteins (4, 27, 28). The effect of resveratrol was smaller when Ba^{2+} replaced Ca^{2+} in the bath solution (Fig. 6, *a–c*), and there was significantly less delay to its onset of action (supplemental Fig. III, *b* and *c*). Similarly, when there was strong intracellular Ca^{2+} buffering at 100 nM, the effect of resveratrol was suppressed (Fig. 6*c*). In contrast, the action of diethylstilbestrol was unaffected (Fig. 6*c*). The data suggest

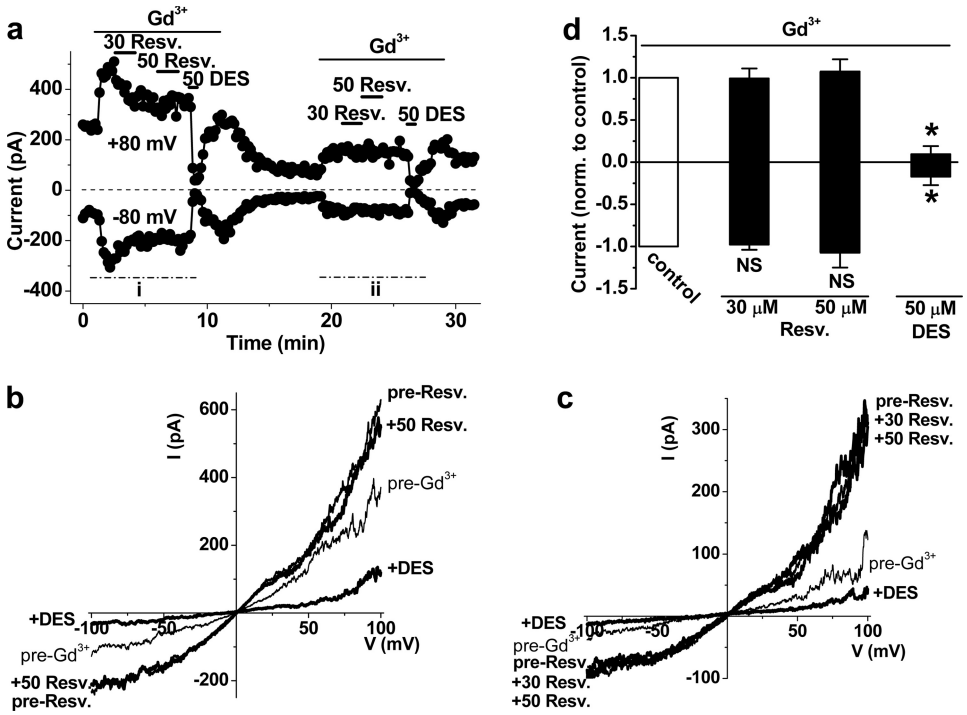

FIGURE 4. **Differential effects in outside-out patches.** Recordings were made from outside-out patches excised from TRPC5-expressing (Tet+) cells. *a*, example time-series plot of Gd^{3+}-induced current and the effects of bath-applied resveratrol (*Resv.*) and diethylstilbestrol (*DES*) at the concentrations indicated (μM). *b* and *c*, for the experiment shown in *a*, I-V values for the time periods *i* (*b*) and *ii* (*c*). *d*, normalized (*norm.*) mean data for the experiment type illustrated in *a* ($n = 6$, 4 and 5). Statistical comparisons were made with the paired controls (only one control bar is shown). Similar results were obtained in 13 additional patches for diethylstilbestrol. *, $p < 0.05$; *NS*, no significant difference.

that the action of resveratrol on TRPC5 involves a Ca^{2+}-sensing mechanism such that the mechanism is more active when there is more available Ca^{2+}, *i.e.* in the presence of extracellular Ca^{2+} and when Ca^{2+} buffering from the patch pipette is modest (1 mM EGTA).

DISCUSSION

This study expands knowledge of the chemical-sensing capabilities of TRP channels, showing novel chemical modulations of TRPC5 activity that arose via indirect and direct mechanisms. Firstly, there was differential inhibition by dietary antioxidants that arose indirectly because of scavenging of endogenously produced H_2O_2, which had the unexpected capacity to stimulate only lanthanide-evoked channel activity. Secondly, there was general inhibition of all modes of TRPC5 by resveratrol through an indirect Ca^{2+}-sensitive effect without impact on channel surface density. Thirdly, there was direct inhibition by diethylstilbestrol, revealing a relatively potent novel blocker of the channels. The first two effects are consistent with known redox- and Ca^{2+}-sensing functions of TRPC5. The third effect suggests additional chemical interaction with the stilbene diethylstilbestrol.

TRPC5 has been previously shown to be stimulated by exogenous H_2O_2, and a similar effect has been described for TRPC6 (15, 29). Therefore, it was anticipated that exogenous H_2O_2 would stimulate TRPC5 and that antioxidants would suppress this effect, as observed for gallic acid and vitamin C. However, it was unanticipated that the antioxidants would suppress stimulation of TRPC5 by the lanthanide Gd^{3+}, especially because there was no general suppression of TRPC5 activity (as shown by the resistance of LPC- or carbachol-evoked activity, consistent with previous vitamin E results (20)). Importantly, we showed that catalase had the same differential effect, suggesting that the effect arose because of reduced bioavailability of endogenous H_2O_2, generated possibly by mitochondrial Complex I of the cells. We hypothesize that differential sensitivity of Gd^{3+}-stimulated TRPC5 occurs because Gd^{3+} is not a direct channel activator but a stabilizer of the channel open state, making its stimulatory effects uniquely proportionate to constitutive channel activity. The implication is, therefore, that constitutive TRPC5 activity arises because endogenous H_2O_2 stimulates the channels, much as exogenous H_2O_2 acts, but at a lower level. In this way, Gd^{3+} responses depend on endogenous H_2O_2. On a separate but related matter, it is evident that the inhibitory effects of antioxidants contrast with the stimulatory effects of dithiothreitol, tris(2-carboxyethyl)phosphine, and reduced thioredoxin, which are reducing agents that stimulate TRPC5 channels by breaking a cysteine bridge in the turret (11). We suggest that these cysteine residues are not involved in the action of H_2O_2 (11, 30).

As a precautionary technical note, the described effects of dietary antioxidants such as gallic acid may lead to complications in interpreting TRPC5 data when mannitol is used as an osmotic substitute because mannitol is also an antioxidant. High concentrations of mannitol are commonly used in electrophysiological studies in variable amounts, as if inert. Mannitol is also used in experiments seeking to identify stretch-sensitive channels because it is removed, in large amounts,

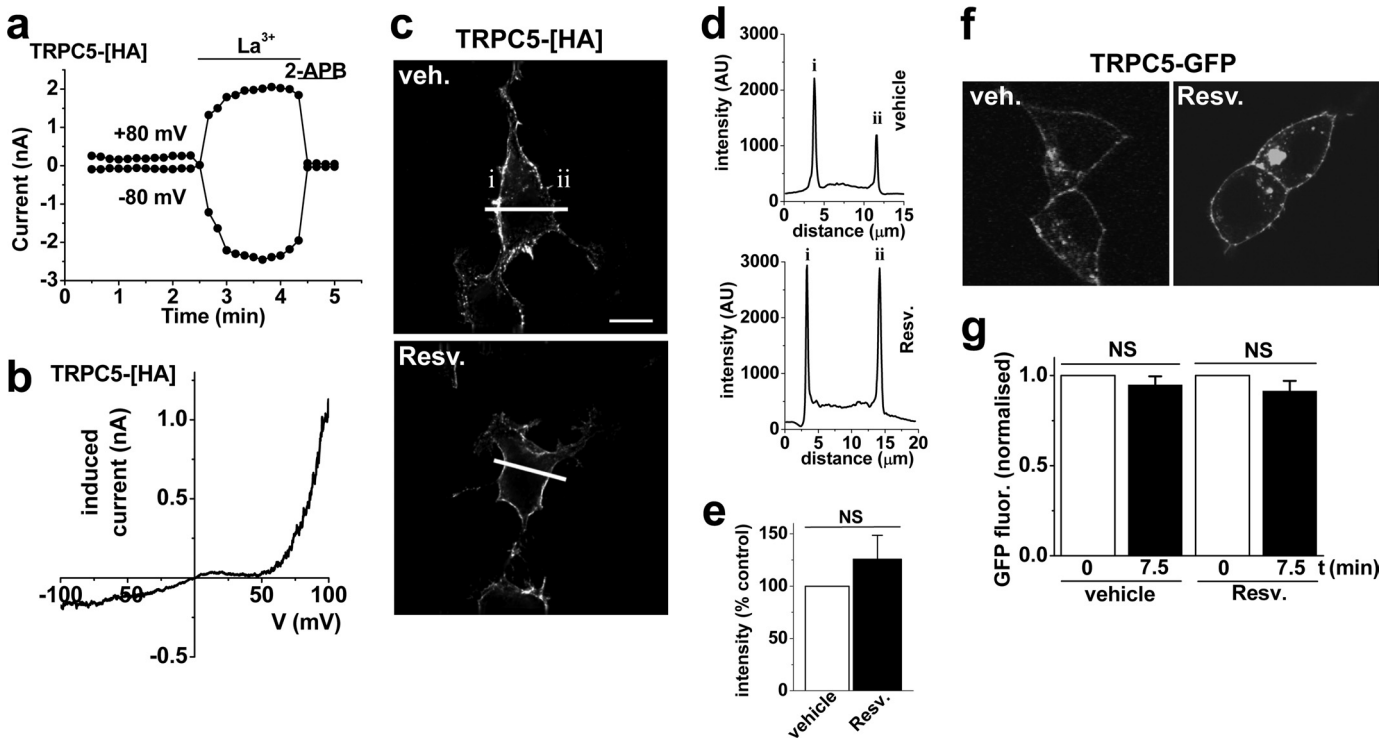

FIGURE 5. **Lack of effect of resveratrol on TRPC5 surface density.** TRPC5-[HA] (*a–e*) or TRPC5-GFP (*f* and *g*) constructs were transiently transfected in HEK 293 cells. *a* and *b*, example whole-cell current data showing: a time-series plot for responses to 100 μM lanthanum (La^{3+}) and 75 μM 2-APB (*a*) and an I-V for La^{3+}-induced current (*b*). *c*, example cell images for anti-HA labeling after 7.5 min in 30 μM resveratrol (*Resv.*) or vehicle control (*veh.*). *White lines* are scan rectangles used for the analysis in *d*. The *scale bar* is 10 μm. *d* and *e*, as for *c* but line-scan intensity analysis showing peaks of HA-labeling at the edges of the cells (*i* and *ii*, as in *c*). *AU*, arbitrary units. *f*, example images after 7.5 min in 30 μM resveratrol or vehicle. *g*, mean data showing GFP fluorescence intensity at the cell surface before and after application of the vehicle (0.1% v/v ethanol) or 30 μM resveratrol in ethanol (n/N = 3/9 for each). *NS*, no significant difference in *e* and *g*.

during live cell recordings to evoke osmotic shock. TRPC5 has been suggested to be a stretch-activated channel, largely based on mannitol experiments but also using direct mechanical deformation (31). We have observed inhibition of TRPC5 activity by mannitol.[3]

It has been suggested that the beneficial effects of resveratrol might arise through diverse and relatively nonspecific effects on enzymes, receptors, transporters, and ion channels (24). However, resveratrol is often investigated at quite high concentrations, above those achieved in the plasma *in vivo* (up to 2 μM). Therefore, distinctions should be made between effects that occur at low (<5 μM) and high (>5 μM) concentrations. In a general screen that included a set of ion channels, Pacholec *et al.* (24) indicated the effects of 10 μM resveratrol only on the L-type Ca^{2+} channel (~50% inhibition). Another study provided more detailed information and found that 10 μM resveratrol inhibited the T-type but not L-type Ca^{2+} channel (32). A small inhibitory effect of 3 μM resveratrol was observed on ATP-sensitive K^+ current of a pancreatic β-cell line, where as ≤10 μM resveratrol lacked effect on voltage-dependent K^+ current (33). Non-ion channel targets of resveratrol include the estrogen receptor, where resveratrol acts as an agonist at 3–10 μM (34). On TRPC5, we observed a small inhibitory effect of 1 μM resveratrol and greater effect at higher concentrations (IC_{50} 4 μM at −80 mV). Therefore, the effect of resveratrol on TRPC5 can be considered to be relatively potent and potentially relevant to plasma concentrations of resveratrol.

One context in which TRPC channels have been suggested to be active is cells with depleted Ca^{2+} stores, contributing to the so-called store-operated Ca^{2+} entry signals (35). This subject is hotly debated because calcium release-activated calcium (CRAC) channels also contribute to such signals, and the pore-forming subunits of these channels are generated by Orai1 proteins rather than TRPCs. Some investigators propose that TRPC channels make no contribution to such signals (36), whereas others, for example, suggest that the Ca^{2+} store sensor, STIM1, activates Orai1 and TRPC channels (37). Intriguingly, resveratrol and diethylstilbestrol have both been described previously as potent inhibitors of store-operated Ca^{2+} entry and CRAC channel currents (38–40). Therefore, it would seem either that there must be similar resveratrol and diethylstilbestrol sensitivities of Orai1 and TRPC channels or that the functions of the channels are linked. Dobrydneva *et al.* (40) did not identify distinctions between the actions of resveratrol and diethylstilbestrol, but they did not perform excised membrane patch recordings that would enable separation of direct from indirect effects.

Diethylstilbestrol is a synthetic estrogen-like compound that acts at all three estrogen receptors. The effect of diethylstilbestrol on TRPC5 did not, however, relate to nuclear estrogen receptors because the effects of diethylstilbestrol occurred promptly in excised membrane patches and 17β-estradiol was not active at TRPC5 (supplemental Fig. IV). In

[3] J. Naylor and D. J. Beech, unpublished observations.

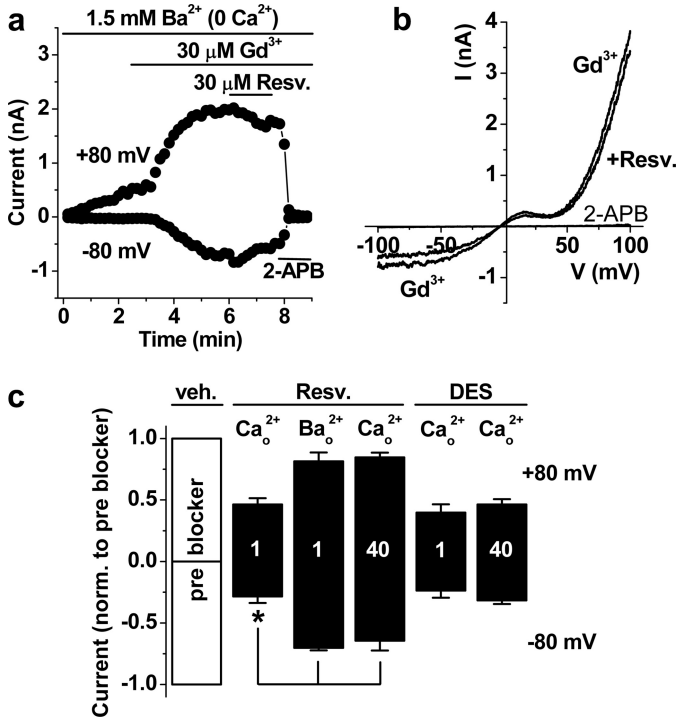

FIGURE 6. **Differential dependence on Ca^{2+}.** Recordings were made using whole-cell voltage clamp applied to TRPC5-expressing (Tet+) cells. *a*, example time-series plot showing Gd^{3+} induction of TRPC5-mediated current in extracellular solution containing Ba^{2+} in place of Ca^{2+}. Resveratrol (*Resv.*) and 2-APB were bath-applied at 30 and 75 μM, respectively. The 1 EGTA patch pipette solution was used. *b*, as for *a* but showing I-Vs. *c*, mean normalized (*Norm.*) data for the inhibitory effects of: 30 μM resveratrol when using the 1 EGTA pipette solution with Ca^{2+} bath solution ($n = 14$), 1 EGTA pipette with Ba^{2+} bath ($n = 5$), and 40 EGTA pipette with Ca^{2+} bath ($n = 6$); and 10 μM diethylstilbestrol (*DES*) when using the 1 EGTA pipette with Ca^{2+} bath ($n = 6$) and 40 EGTA pipette with Ca^{2+} bath ($n = 10$). *, $p < 0.05$. *veh.*, vehicle.

the past, diethylstilbestrol was used clinically in an effort to avert miscarriages, but its use ceased because of concerns over efficacy and long term associations with cancer (41). Therefore, although diethylstilbestrol may be a framework for developing novel TRPC5 inhibitors, caution would naturally be needed to avoid estrogen-related or other adverse effects.

In summary, this study reveals previously unrecognized chemical-sensing features of TRPC5 channels, which include stimulation by endogenous H_2O_2 and relatively potent, but indirect, inhibition by the important dietary factor resveratrol. The findings suggest that healthy dietary factors, such as gallic acid, vitamin C, and resveratrol, may act in part by suppressing TRPC channel activity, thus potentially suppressing unwanted cellular remodeling. Diethylstilbestrol, although chemically similar to resveratrol, acted differently as a direct channel blocker and may provide a basis for pharmacological TRP channel inhibitors.

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