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Stimulation of Dichlorofluorescin Oxidation by Capsaicin and Analogues in RAW 264 Monocyte/Macrophages: Lack of Involvement of the Vanilloid Receptor

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ABSTRACT. In studies into the oxidative burst in RAW 264 monocyte/macrophages, it was observed that capsaicin, a vanilloid receptor agonist, stimulated dichlorofluorescin (DCFH) oxidation in a concentration-dependent manner, which could be blocked by capsazepine, a vanilloid receptor antagonist. However, by use of a number of vanilloid agonists (including *N*-octyl-3-chloro-4-hydroxyphenylacetamide, 4m), we demonstrated that there was no relationship between vanilloid agonist potency and the capacity to stimulate DCFH oxidation. The oxidative burst stimulators Tween 20 and phorbol myristyl acetate (PMA) also stimulated reactive oxygen species generation, which again was inhibited by capsazepine. Use of the selective inhibitor diphenyliodonium iodide ruled out a role for plasma membrane NAD(P)H oxidase as the site of capsaicin- and 4m-stimulated DCFH oxidation. However, this DCFH oxidation was modulated by a number of inhibitors of mitochondrial respiration. Rotenone enhanced DCFH oxidation induced by capsaicin and 4m, whilst malonic acid and potassium cyanide inhibited this response. 2,4-Dinitrophenol, an inhibitor of oxidative phosphorylation, was without effect. The antioxidant trolox c inhibited DCFH oxidation stimulated by capsaicin, 4m, and PMA, whereas *N*-acetylcysteine, a precursor of glutathione, was without effect. Capsazepine inhibited DCFH oxidation in unstimulated cells and in cells treated with menadione, a redox-cycling quinone. Capsazepine was also a potent antioxidant when measured in a Fe3+ reduction assay. We concluded that DCFH oxidation stimulated by vanilloid analogues was not mediated via a vanilloid receptor, but rather by impairment of mitochondrial electron transport. BIOCHEM PHARMACOL **59**;5:563–572, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. capsaicin; capsazepine; vanilloid; antioxidant; dichlorofluorescein; ferric reducing antioxidant power

Capsaicin is found in chilli peppers and other members of the genus *Capsicum*; it is responsible for the 'hot' sensation produced by these fruits [1, 2]. Capsaicin stimulates sensory nerve fibres, causing neuropeptide release, pain sensations, and vasodilation, with increased vascular permeability leading to erythema and oedema [3–5]. Capsaicin produces many of these effects through a membrane-bound vanilloid receptor, stimulation of which leads to sodium and calcium influx, which results in neuronal depolarisation [6, 7]. The structural features responsible for the potency of capsaicin at this receptor have been defined by studies on a large series of capsaicin analogues [8–11], a free phenolic group being essential for agonist activity [8]. Capsaicin-mediated sensory depolarisation may be inhibited by capsazepine, a vanilloid receptor antagonist [12]. However, capsazepine is relatively non-specific in its action in that it inhibits nicotinic receptors and blocks calcium channels [13, 14].

Capsaicin stimulates vanilloid receptors at submicromolar concentrations, and the IC_{50} for capsaicin-mediated guinea pig ileum contractions is approximately 300 nM. In HEK 293 cells transfected with a vanilloid receptor, up to 30 μM capsaicin was required to obtain a maximum response [6]. However, at higher concentrations (10–20 μM), capsaicin exhibits a number of non-selective actions, for instance, interacting with lipid membranes to form a non-selective ion channel [15]. Capsaicin is also reported to inhibit the NADH oxidase of rat liver plasma membrane [16] and transformed HL60 cells [17]. This feature is thought to account for the cytostatic action of capsaicin [17]. Additionally, capsaicin is reported to inhibit NADH-ubiquinone oxidoreductases in bovine mitochondria [18].

While carrying out investigations on chemical modulators of the oxidative burst in cells of the RAW 264 monocyte/macrophage cell line, we found that capsaicin

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stimulates oxidation of the probe DCFH* to the fluorescent product DCF. The observation that capsaicin stimulates DCFH oxidation conflicts with a report describing antioxidant attributes of capsaicin [19]. Identification of this novel activity of capsaicin led us to investigate the cellular site of DCFH oxidation stimulated by capsaicin and its analogues and to determine whether or not these effects of capsaicin are mediated via a vanilloid receptor.

MATERIALS AND METHODS

Chemicals

Capsazepine and olvanil were obtained from Tocris Cookson Ltd. DCFH-DA was obtained from Eastman Ltd. and PAV from Fluka. DPII was from Aldrich Chemical Co., Inc. Capsaicin, curcumin, piperine, PMA, menadione, rotenone, malonic acid, DNP, DCF, and vanillic acid were supplied by Sigma Chemical Co. Potassium cyanide and neutral red were obtained from BDH. The compounds 2c, 2h, 2j, 2m, and 4m were gifts from the Novartis Institute for Medical Research. All other chemicals were obtained from local suppliers and were of analytical grade or better.

Tissue Culture Plastic and Media

All serum, supplements, and tissue culture media were obtained from Life Technologies, Inc. Cell culture flasks (175 cm^2) were obtained from Nunc and Costar 24-well tissue culture plates from Corning.

Cells and Culture Media

Cells of the murine leukaemic monocyte/macrophage cell line RAW 264 (ECACC number 85062803) were obtained from the European Collection of Animal Cell Cultures. Cells were grown in 175-cm^2 flasks with Dulbecco's modified Eagle's medium supplemented with glutamine (2 mM), pyruvic acid (1 mM), foetal bovine serum (10% v/v), penicillin/streptomycin (10,000 IU/L), and fungizone (2 mg/L). Cells were incubated with air/CO_2 (5%) in a humidified atmosphere and maintained at 37°. Cells were used between passages 4 and 16 and were regularly split such that confluency in flasks was avoided. To subculture cells, they were washed with PBS, then incubated with PBS containing EDTA (5 mM) for 20 min. The detached cells were spun down and resuspended in fresh Dulbecco's modified Eagle's medium at 2×10^6 cells/mL. For the experiments reported in this paper, cells were plated onto 24-well plates at 2×10^6 cells/well, and left for 2 hr to adhere to the plastic.

Cell Treatments

After cells had adhered to the tissue culture plastic, culture medium was removed and cells were washed once with HBSS. Cells were exposed to HBSS containing DCFH-DA (20 μM) with capsaicin (analogues), PMA, and various inhibitors for 4 hr. Potassium cyanide and malonic acid were dissolved directly into HBSS. PMA and DPII were prepared in dimethylsulphoxide at 1000 times the required concentration and added at 0.1% v/v into HBSS to give the required concentration. All other chemical additions were prepared at 200 times the required concentration in methanol and added at 0.5% v/v to give the final desired concentration.

DCFH Oxidation as a Measure of Oxidative Stress

DCFH-DA enters cells passively and is de-acetylated by esterase action to the non-fluorescent DCFH. DCFH reacts with ROS to form the fluorescent product DCF [20]. We observed that DCF and DCFH are not retained by RAW 264 cells; leakage of DCF/DCFH out of cells has been reported for other cell lines [20, 21]. To counteract this problem, we adopted the approach of Buxser *et al.* [20] and measured DCF formation with the continuous presence of DCFH-DA in the extracellular medium. Consequently, the DCF measured represents the sum total of intra- and extracellular DCF production. Royall and Ischiropoulos [21] reported that DCFH-DA spontaneously and rapidly autoxidises to DCF in complete tissue culture media. We found the spontaneous oxidation rate of DCFH-DA to DCF to be slower in HBSS (at about 1% of the total DCFH-DA added over 4 hr). For this reason, all studies on DCFH-DA oxidation were carried out in HBSS rather than complete culture media.

DCFH-DA was dissolved in methanol at 4 mM; this was diluted 200-fold in HBSS to give DCFH-DA at 20 μM. Cells were exposed to DCFH-DA (with various additions) for 4 hr. After this time, the fluorescence was read at wavelengths of 485 nm excitation and 530 nm emission on a Cytoflour 2350 fluorescence plate reader (Perspective Biosystems). The conversion of DCFH-DA to DCF was determined by reference to a DCF standard (20 μM). The fluorescence of DCF was linear over the concentration range 0–20 μM. After determination of DCF production, the chemicals were removed and cells were washed with PBS. Neutral red uptake was employed to determine cell viability using the method of Borenfreund and Puerner [22]. None of the manipulations described here led to an altered neutral red uptake, and for this reason these data are not shown. Fluorescence produced by the conversion of DCFH to DCF was calculated as nmol/2×10^6 cells/4 hr. Preliminary studies indicated that a confluent monolayer, as achieved by plating the cells at 2×10^6 cells per well, was

* *Abbreviations:* 2c, *N*-(3,4-dimethoxybenzyl) nonamide; 2h, *N*-(3,4-methylenedioxybenzyl) nonamide; 2j, *N*-(3-hydroxy-5-methoxybenzyl) nonamide; 2m, *N*-(2-methoxy-4-hydroxybenzyl) nonamide; 4m, *N*-octyl-3-chloro-4-hydroxyphenylacetamide; DCFH-DA, dichlorofluorescin diacetate; DCFH, dichlorofluorescin; DCF, dichlorofluorescein; DNP, 2,4-dinitrophenol; DPII, diphenyliodonium iodide; FRAP, ferric reducing antioxidant power; HBSS, Hanks' balanced salt solution; PAV, pelargonic acid vanillylamide; PMA, phorbol myristyl acetate; and ROS, reactive oxygen species.

essential to achieve reproducibly consistent levels of DCF generation. A 4-hr time point was chosen because the difference between treatments and untreated control were most marked at this time. The background (non-stimulated) rate of DCF production was higher with increased cell passage number, although the relative responses to capsaicin and other agents was consistent between cells of differing passages.

Preparation of Cell Homogenates from RAW 264 Cells

RAW 264 cells were removed from tissue culture flasks using PBS/EDTA (5 mM) as described above. Cells were re-suspended in 0.2 M Tris–sucrose–EDTA (pH 7.4) and sonicated twice for 5 sec using an MSE "Soniprep 150" sonic probe. Homogenates were frozen at −80° until required. The cell protein content was 0.19 mg/10^6 cells.

Effects of Capsaicin and Capsazepine on NADH Oxidase Activity in Homogenates of RAW 264 Cells

The activity of NADH oxidase in homogenates of RAW 264 cells was determined using a method modified from Morre *et al.* [17]. Incubations (1 mL) consisted of: 0.1 M Tris/HCl buffer pH (7.4), magnesium sulphate (5 mM), RAW 264 homogenate (0.92 mg protein/mL) and capsaicin (10 μM), rotenone (10 μM), capsazepine (3 μM) in methanol, or DMSO as a solvent control at 10 μL/mL. The reaction was started by the addition of NADH (150 μM), and the rate of loss of NADH was recorded at 340 nm and 25° using a Unicam SP4 UV-visible spectrometer. The NADH oxidase activity of homogenates was calculated as nmol NADH oxidised/min/mg protein using an extinction coefficient for NADH of 6200 M^{-1} cm^{-1}. The protein content of cell homogenates was determined by the method of Lowry *et al.* [23].

FRAP of Capsaicin and Analogues

One parameter of antioxidant potential is measured by the capacity of antioxidants to reduce Fe^{3+} to Fe^{2+} [24], which can be measured by the formation of the highly coloured ferrous–tripyridyltriazine complex. Antioxidants (50 μM) were incubated at room temperature for 6 min with 2,4,6-tripyridyl-*s*-triazine (1 mM), ferric chloride 2 mM, and acetate buffer (300 mM, pH 3.6) in a volume of 200 μL. Production of the ferrous–tripyridyltriazine complex, following reduction of the ferric–tripyridyltriazine complex by antioxidants, was determined at 570 nm (with 655 nm reference) on a Biorad 450 microplate reader. Ferrous chloride (0–150 μM) was added separately to generate a standard curve for ferrous–tripyridyltriazine. The extent of conversion of Fe^{3+} to Fe^{2+} by antioxidants was calculated by reference to this standard curve.

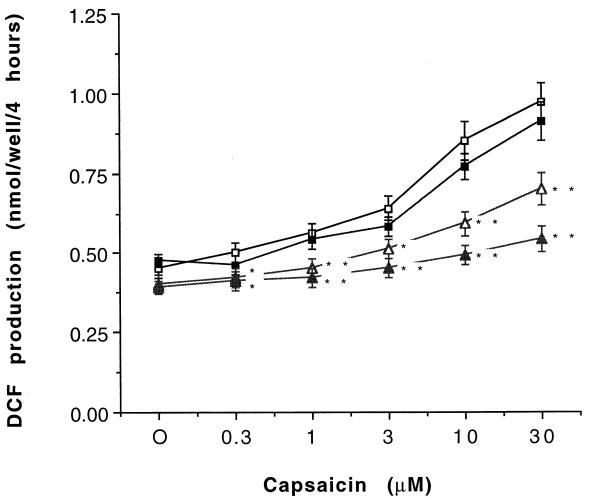

FIG. 1. DCFH oxidation in response to capsaicin. Cells were exposed to HBSS containing DCFH-DA (20 uM) in the presence of capsaicin (0–30 μM) without capsazepine (□) or with capsazepine at 0.3 (■), 1.0 (△), or 3.0 (▲) μM. Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. Dose–response curves for capsaicin and capsaicin + capsazepine were compared for statistically significant differences using 2-way ANOVA followed by Dunnett's post hoc test. Capsazepine significantly inhibited responses to capsaicin (*$p < 0.05$, **$p < 0.01$).

Presentation of Results and Statistical Analysis

Results are presented as means ± SEM of 4–10 independent determinations. Dose–response curves were compared using two-way ANOVA. When 3 or more treatments were compared to a single control, one-way ANOVA followed by Dunnett's multiple range test was used. When multiple comparisons were made with no fixed control, analysis was carried out using one-way ANOVA followed by the Tukey–Kramer test. Statistically significant differences from controls are indicated as *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

RESULTS

Effect of Capsazepine on Capsaicin-Mediated DCF Oxidation

Capsaicin (0–30 μM) stimulated the oxidation of DCFH-DA to DCF in RAW 264 monocyte/macrophages in a concentration-dependent fashion, which was inhibited by increasing concentrations (0–3 μM) of capsazepine, a vanilloid antagonist (Fig. 1).

Effect of Structural Analogues of Capsaicin on DCF Oxidation

A number of structural analogues of capsaicin were examined for their ability to stimulate DCF oxidation in RAW 264 cells. A 10-μM concentration was chosen, as preliminary experiments demonstrated that this concentration was

not cytotoxic (assessed by neutral red uptake) over the 4-hr exposure time and, with capsaicin, produced around 70% maximum response. PAV, which differs in structure from capsaicin by lacking an unsaturated bond and a branched end on the long alkyl group (Fig. 2), had a similar potency to capsaicin in stimulating DCF oxidation (Table 1). Given this similarity, we examined DCF oxidation by some analogues of PAV, supplied by Novartis. Compounds were selected on the basis of their ability to activate the vanilloid receptor, as measured by Ca^{45} uptake into cultured dorsal root ganglion neurones [8]. Structures of these analogues are illustrated in Fig. 2. Compound 4m was selected for similar potency to capsaicin, compound 2c for being less potent than capsaicin, and 2j, 2h, and 2m were selected as for their inactivity with respect to vanilloid receptor activation. In addition, the ability of olvanil (a novel analgesic), piperine (a constituent of black pepper), curcumin (a constituent of turmeric), and resiniferatoxin (an ultrapotent vanilloid agonist [7]) to stimulate DCF oxidation was investigated. Capsaicin analogues that possessed a free phenolic group (capsaicin, PAV, olvanil, resiniferatoxin, 4m, 2m, and 2j; see Fig. 2) all stimulated DCFH oxidation, a very marked response being obtained with compound 4m (Table 1). By contrast, compounds in which the phenolic group was occupied by a methyl or a bridging group (piperine, 2c, 2h) were inactive. Vanillic acid, which possesses the vanillyl moiety without an alkyl chain, failed to stimulate DCFH oxidation (Table 1). Oleamide, which possesses a long alkyl chain like olvanil but lacks the aromatic moiety, also failed to stimulate DCFH oxidation (Table 1).

DCFH Oxidation Mediated by PMA and Tween 20: Inhibition by Capsazepine

PMA is known to stimulate the cell membrane NADPH oxidase in monocytes and neutrophils, leading to the generation of hydrogen peroxide. Surfactants such as Tween 20 are also known to stimulate NADPH oxidase activity [25]. PMA and Tween 20 stimulated DCF oxidation in RAW 264 cells, and in both cases this oxidation was inhibited by capsazepine (3 μM) (Figs. 3 and 4).

Effect of DPII on DCFH Oxidation Mediated by Capsaicin, 4m, and PMA

DPII is a potent inhibitor of cell membrane NADPH oxidase [25]. DPII (1 and 10 μM) inhibited DCF oxidation mediated by PMA. However, DCF oxidation mediated by capsaicin or 4m was unaffected (Fig. 5).

Effect of the Antioxidants Trolox c and N*-Acetylcysteine on DCFH Oxidation*

Trolox c is a vitamin E analogue and a potent antioxidant [26], whereas *N*-acetylcysteine is an electrophile scavenger and acts to elevate cellular glutathione [27]. Trolox C was a potent inhibitor of DCFH oxidation mediated by capsaicin, 4m, and PMA. By contrast, *N*-acetylcysteine had no effect on DCFH oxidation (Fig. 6).

Effect of Capsazepine on DCF Oxidation Mediated by Menadione

Menadione stimulates peroxide synthesis in many cell types following metabolism by cellular reductases [28]. We wondered whether capsazepine could be acting non-selectively as an antioxidant and so we examined the effects of capsazepine on menadione-mediated DCF oxidation. Capsazepine inhibited spontaneous DCF oxidation and that stimulated by menadione (Fig. 7).

Effect of Mitochondrial Inhibitors on DCFH Oxidation Mediated by Capsaicin, 4m, and PMA

Rotenone, an inhibitor of mitochondrial NADH oxidase associated with complex 1 [29], stimulated DCFH oxidation mediated by capsaicin and 4m, but did not affect DCFH oxidation mediated by PMA (Fig. 8a). Malonate (an inhibitor of complex II succinate dehydrogenase; [29]) and potassium cyanide (an inhibitor of complex III cytochrome oxidase; [29]) inhibited DCFH oxidation mediated by capsaicin and 4m, but led to a small but significant stimulation of DCFH oxidation mediated by PMA (Fig. 8, b and c). DNP (which uncouples oxidative phosphorylation from ATP production; [29]) had little effect on the DCFH oxidation mediated by capsaicin and 4m, but led to a small but significant increase in DCFH oxidation mediated by PMA (Fig. 8d).

Effect of Capsaicin Analogues and Capsazepine on FRAP

Capsazepine was a potent reducer of Fe^{3+} to Fe^{2+}, its activity exceeding that of trolox c. Capsaicin was similar in potency to trolox c, whereas curcumin and olvanil were less potent. Piperine, and the Novartis compounds 2c, 2j, 4m, and 2h, were virtually inactive in this antioxidant model, whereas compound 2m exhibited a low antioxidant activity (Table 2).

Inhibition of NADH Oxidase by Capsaicin in Homogenates of RAW 264 Cells

The control activity (in the absence of capsaicin) was 4.05 ± 0.17 nmol NADH oxidised/mg protein/min, whilst that in the presence of 10 μM capsaicin was 2.00 ± 0.11 nmol NADH oxidised/mg protein/min (values are means ± SEM of 4–10 separate experiments).

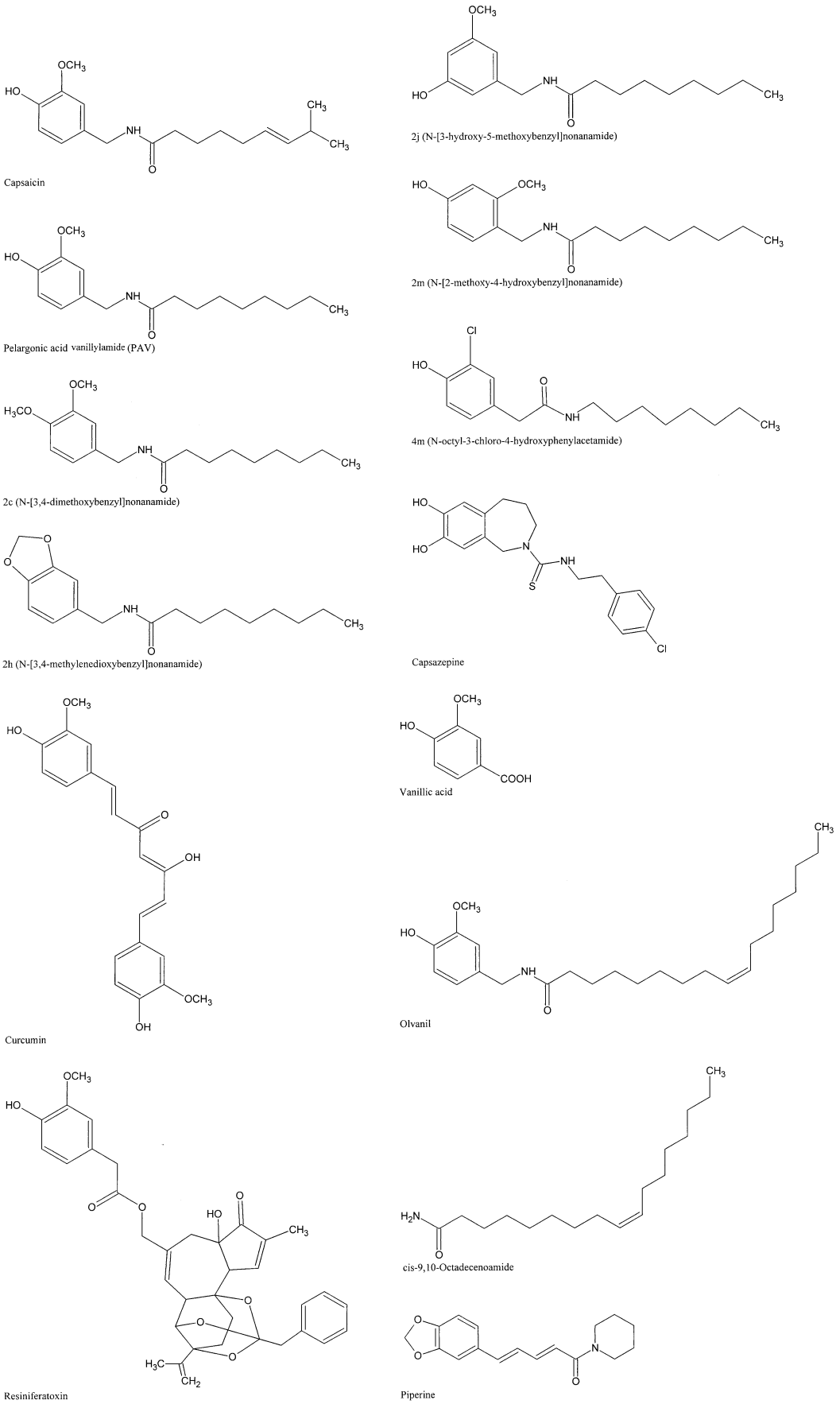

FIG. 2. Structures of capsaicin, structural analogues, capsazepine, and Novartis compounds used in these studies.

TABLE 1. Effect of capsaicin analogues on DCF production in RAW 264 cells

Capsaicin analogue (10 μM)	DCF production (nmol/well/4 hrs)	+ capsazepine 3 μM
Untreated control	0.431 ± 0.04	0.130 ± 0.01
Capsaicin	0.831 ± 0.06*	0.135 ± 0.01
PAV	0.909 ± 0.06*	0.141 ± 0.01
Olvanil	1.021 ± 0.11*	0.120 ± 0.02
2h	0.390 ± 0.04	0.126 ± 0.01
2m	1.167 ± 0.06*	0.139 ± 0.02
4m	2.661 ± 0.12*	0.184 ± 0.02
2j	0.790 ± 0.03*	0.143 ± 0.01
2c	0.455 ± 0.03	0.133 ± 0.01
Vanillic acid	0.446 ± 0.03	ND
Piperine	0.480 ± 0.04	ND
9,10-Octadecenoamide	0.439 ± 0.04	ND
Curcumin	0.446 ± 0.02	ND
Resiniferatoxin	0.841 ± 0.14*	0.203 ± 0.02

Cells were exposed to HBSS containing DCFH-DA (20 μM) in the presence of capsaicin analogue with or without capsazepine. Results represent means ± SEM of 4–6 independent determinations. Compounds were compared to the untreated control for statistically significant differences using one-way ANOVA followed by Dunnett's multiple range test. ND = not determined.

*$P < 0.01$.

DISCUSSION

Stimulation of DCFH Oxidation by Capsaicin: Involvement of a Vanilloid Receptor?

Initial studies indicated that capsaicin, a vanilloid receptor agonist, stimulated the oxidation of DCFH to DCF in RAW 264 cells (Fig. 1). This response was taken as evidence for generation of ROS, an interpretation confirmed by the blockade of this response by trolox c, a well-characterised antioxidant (Fig. 6). This response was inhibited by capsazepine (Fig. 1), a vanilloid receptor antagonist. These observations led us to consider whether

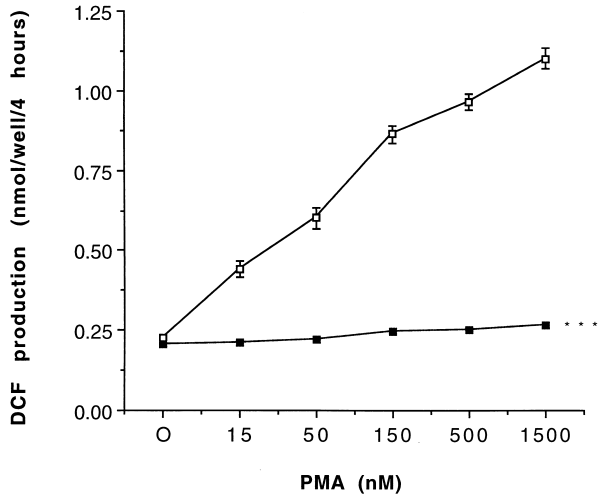

FIG. 3. DCFH oxidation in response to PMA. Cells were exposed to HBSS containing DCFH-DA (20 μM) in the presence of PMA (0–1500 nM) with (■) or without (□) capsazepine (3 μM). Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. Dose–response curves for PMA alone and PMA + capsazepine were compared for statistically significant differences using 2-way ANOVA. Capsazepine significantly inhibited DCF production stimulated by PMA (*$P < 0.001$).**

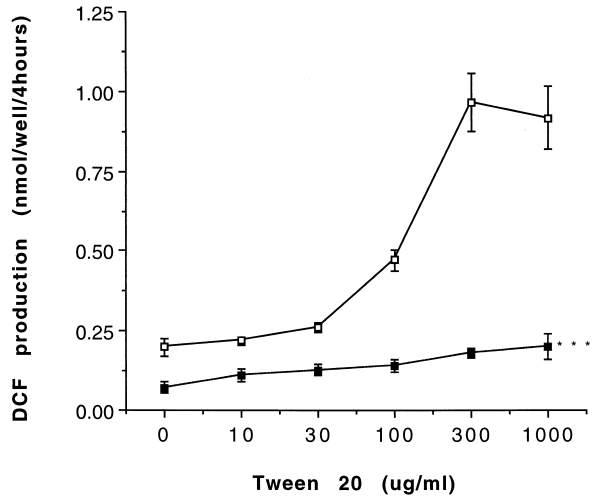

FIG. 4. DCFH oxidation in response to Tween 20. Cells were exposed to HBSS containing DCFH-DA (20 μM) in the presence of Tween 20 (0–1000 μg/mL) with (■) or without (□) capsazepine (3 μM). Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. Dose–response curves for Tween 20 alone and Tween 20 + capsazepine were compared for statistically significant differences using 2-way ANOVA. Capsazepine significantly inhibited DCF production stimulated by Tween 20 (*$P < 0.001$).**

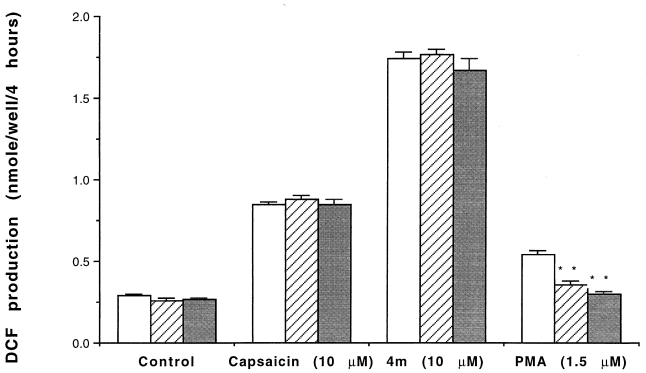

FIG. 5. Effect of DPII on the responses elicited by capsaicin, 4m, and PMA. Cells were exposed to HBSS containing DCFH-DA (20 uM) in the presence of capsaicin (10 μM), 4m (10 μM), PMA (1.5 μM), or with no additions (control), in the absence (□) or presence of 1.0 (▨) or 10 (■) μM DPII. Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. For each chemical stimulator of DCF production, the effect of DPII on DCF production was compared statistically to each chemical control using one-way ANOVA followed by the Tukey–Kramer post hoc test. DPII significantly inhibited DCF production stimulated by PMA ($P < 0.01$), but did not affect DCF production stimulated by capsaicin or 4m.**

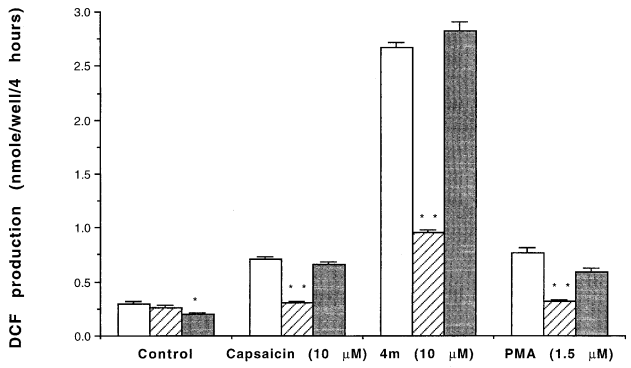

FIG. 6. Effect of trolox c and *N*-acetylcysteine (NAC) on responses elicited by capsaicin, 4m, and PMA. Cells were exposed to HBSS containing DCFH-DA (20 μM) in the presence of capsaicin (10 μM), 4m (10 μM), PMA (1.5 μM), or with no additions (control), in the absence (□) or presence of 10 μM trolox c (▨) or 1 mM NAC (■). Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. For each chemical stimulator of DCF production, the effects of trolox C and NAC were compared statistically to the chemical control using one-way ANOVA followed by the Tukey–Kramer post hoc test. Trolox C inhibited capsaicin-, 4m-, and PMA-stimulated DCF production ($P < 0.01$), but did not affect unstimulated rates of DCF oxidation. NAC failed to modify chemically stimulated DCF production, but did significantly reduce control DCF production (*$P < 0.05$).**

this stimulation of ROS generation was mediated through a vanilloid receptor. However, a number of observations argue against this. Firstly, RAW 264 cells responded to capsaicin only at concentrations above 3 μM, a concentration that is 10-fold higher than that reported as the EC_{50} for

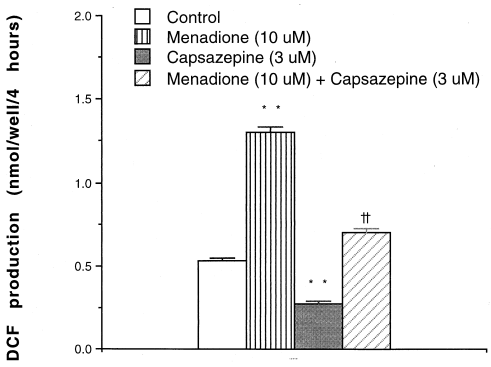

FIG. 7. DCFH oxidation in response to menadione, and the effect of capsazepine. Cells were exposed to HBSS containing DCFH-DA (20 μM) in the presence of menadione (10 μM), or capsazepine (3 μM) added alone or simultaneously. Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. Effects of menadione, capsazepine alone and together on DCF production were assessed using one-way ANOVA followed by the Tukey–Kramer post hoc test. Menadione significantly stimulated ($P < 0.01$), and capsazepine significantly inhibited (**$P < 0.01$), DCF production. Capsazepine significantly inhibited DCF production that was stimulated by menadione (††$P < 0.01$).**

stimulation of vanilloid receptors on guinea pig ileum and cultured dorsal root neurones [8]. Furthermore, capsazepine was 10-fold less potent at inhibiting capsaicin-mediated DCFH oxidation when compared to its effects on the vanilloid receptor of dorsal root neurones [12]. Secondly, the profile of stimulation of DCFH oxidation elicited by the Novartis compounds did not match the profile of these compounds as agonists at the vanilloid receptor [8]. Thus, the order of potency in regard to DCFH oxidation was 4m > 2m > 2j = capsaicin > 2h = 2c (non-effective), whereas that in regard to receptor agonism was 4m = capsaicin > 2c > 2j = 2h = 2m (inactive) [8]. Finally, the inhibitory effect of capsazepine on DCFH oxidation was also demonstrated in the absence of capsaicin, whilst piperine, a potent vanilloid receptor agonist [30], was without effect on DCFH oxidation. Together, these points suggest that the stimulation of DCFH oxidation by capsaicin is not mediated by a vanilloid receptor and further, that the inhibitory effects of capsazepine may be mediated through an antioxidant activity.

Structural Requirements for Stimulation of DCFH Oxidation

Capsaicin is composed of a hydroxy-methoxybenzyl nucleus coupled to a long alkylamide side chain (Fig. 2). Both components appear to be important in the oxidant activity, although the nature of the side chain is less important. Thus, vanillic acid (which lacks the side chain) is inactive, whilst PAV, olvanil, and resiniferatoxin (all of which contain the hydroxy-methoxybenzyl nucleus linked to different side chains) are active. Furthermore, oleamide (which comprises the side chain of olvanil but lacks the hydroxy-methoxybenzyl nucleus) is inactive. The phenyl moiety is essential for activity, as evidenced by the lack of activity of 2h (hydroxy-methoxy-benzene nucleus replaced by a methylenedioxy bridge) and 2c (dimethoxy groups in place of hydroxy-methoxy groups). However, the hydroxy and methoxy groups need not be in adjacent positions, as evidenced by the retention of activity with 2j and 2m. Finally, the methoxy group does not seem to be essential, as evidenced by retention of activity with 4m, in which the methoxy group is replaced with a chloro group (and the amide linkage in the side chain is reversed). Taken together, these observations point to a phenyl nucleus linked to a keto-alkyl side chain as being the key structural features responsible for the oxidant activity of capsaicin.

Cellular Location of Capsaicin-Mediated ROS Generation

Activity of the cell membrane-bound NAD(P)H oxidase is a mechanism by which neutrophils and macrophages produce superoxide and hydrogen peroxide in response to various stimuli, including PMA and detergents such as Tween 20 [25]. These stimuli increased the level of DCFH oxidation in a concentration-dependent manner in RAW

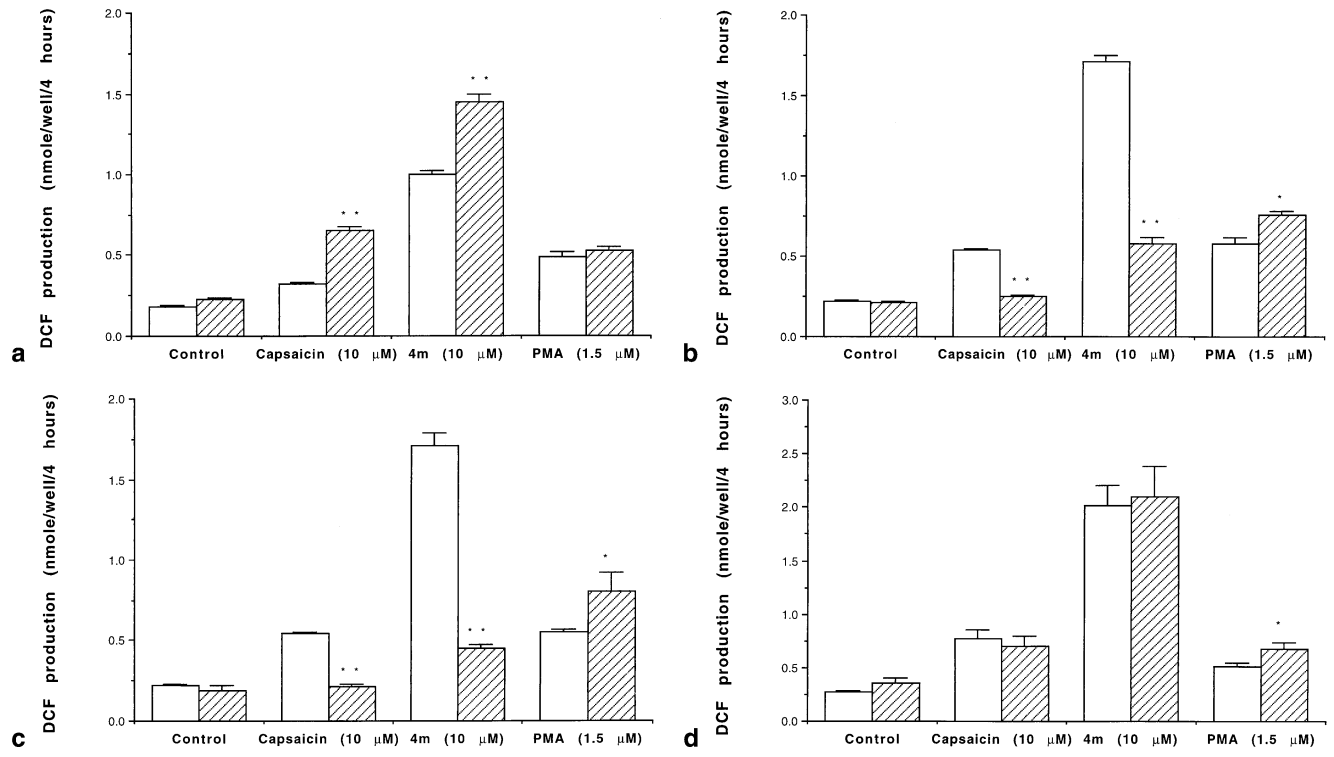

FIG. 8. Effect of mitochondrial inhibitors on responses elicited by capsaicin, 4m, and PMA. Cells were exposed to HBSS containing DCFH-DA (20 μM) in the presence of capsaicin (10 μM), 4m (10 μM), PMA (1.5 μM), or with no additions (control), in the absence (□) or presence (▨) of inhibitor. The inhibitors tested were: (a) rotenone (1 μM); (b) malonic acid (5 mM); (c) potassium cyanide (5 mM); and (d) 2,4-dinitrophenol (DNP, 30 μM). Cells were incubated for a further 4 hr, after which time the production of DCF was determined. Results represent means ± SEM of 4–6 independent determinations. The effect of mitochondrial inhibitors on DCF production was compared statistically to each chemical control using one-way ANOVA followed by the Tukey–Kramer post hoc test. Rotenone significantly enhanced DCF production that was stimulated by capsaicin and 4m ($P < 0.01$), but controls and PMA-stimulated DCF production were unaffected. Malonic acid and potassium cyanide (Figs. 7b and 7c, respectively) significantly inhibited DCF production stimulated by capsaicin and 4m ($P < 0.01$), but significantly enhanced DCF production stimulated by PMA (*$P < 0.05$). By contrast, DNP significantly enhanced DCF production stimulated by PMA ($P < 0.05$), but failed to modify DCF production in controls or that stimulated by capsaicin or 4m.**

264 cells (Figs. 3 and 4). This enzyme activity is inhibited by DPII [25]. DPII inhibited the DCFH oxidation stimulated by PMA, but failed to inhibit that stimulated by capsaicin or 4m (Fig. 4). These findings suggest that stimulation of cell membrane NAD(P)H oxidase activity is not the mechanism by which capsaicin (and 4m) elicit an elevation of ROS generation.

It has been reported [18] that capsaicin inhibits mitochondrial NADH-ubiquinone oxidoreductases. This property could explain the capsaicin-mediated oxidative activity, in that inhibition of this enzyme is known to lead to NADH accumulation [29] which, in turn, leads to ROS generation through NADH autoxidation [31]. To investigate this possibility, the effects of rotenone, a well-known inhibitor of NADH quinone oxidoreductase [32], were studied. Rotenone augmented DCFH oxidation mediated by capsaicin and 4m, while not affecting that mediated by PMA or that occurring in the absence of any applied stimulators (Fig. 7a). Two other mitochondrial inhibitors, malonate and potassium cyanide, inhibited ROS generation stimulated by capsaicin and 4m, but were without effect on the basal level of ROS generation, and actually enhanced that mediated by PMA (Fig. 7, b and c). DNP, a mitochondrial uncoupler, did not affect the DCFH oxidation in controls or that stimulated by capsaicin, but did enhance that stimulated by PMA (Fig. 7d). The small increase in PMA-stimulated DCFH oxidation in the presence of malonate, cyanide, and DNP may be due to an elevated level of cytosolic NADH, as mitochondrial inhibition is known to lead to a compensatory increase in glycolysis, leading to accumulation of cytosolic NADH [33].

Collectively, these data suggest that capsaicin (and 4m) stimulate ROS generation by interacting at many sites in the respiratory chain, but that intact oxidative phosporylation is not a prerequisite for this, as DNP did not modify the effects of capsaicin or 4m. Furthermore, we were able to confirm the inhibitory effect of capsaicin on NADH oxidase activity in cell homogenates, this suggesting one site of interaction in the respiratory chain. These observations tend to suggest that the prime site for capsaicin-mediated DCFH oxidation is mitochondrial (intracellular), whereas the site of PMA-mediated oxidation by NAD(P)H oxidase is extracellular. However, the rapid efflux of DCF and

TABLE 2. Ferric reducing activity of capsazepine, capsaicin analogues, and trolox c

Treatment	Fe^{2+} production (nmol/mL)	
Trolox c	113 ± 2.7	
Capsaicin	93.0 ± 8.0	
Capsazepine	150 ± 7.1*	⇳
Piperine	0.51 ± 0.22*	⇳
Olvanil	41.0 ± 8.7*	⇳
Curcumin	57.0 ± 4.2*	⇳
2c	0.52 ± 0.18*	⇳
2j	0.56 ± 0.22*	⇳
4m	0.83 ± 0.32*	⇳
2m	4.38 ± 0.98*	⇳
2h	0.60 ± 0.19*	⇳
Ascorbate	85.0 ± 1.9†	⇳

Antioxidants (50 μM) were incubated at room temperature for 6 min with 2,4,6-tripyridyl-*s*-triazine (1 mM), ferric chloride (2 mM), and acetate buffer (300 mM, pH 3.6) in a volume of 200 μL. Production of ferrous–tripyridyltriazine complex, following reduction of ferric–tripyridyltriazine complex by antioxidants, was determined at 570 nm (with 655-nm reference) on a Biorad 450 microplate reader. Ferrous chloride (0–150 μM) was added separately to generate a standard curve for ferrous–tripyridyltriazine. The extent of conversion of ferric to ferrous iron by antioxidants was calculated by reference to this standard curve. Results for each antioxidant represent means ± SEM of 4 separate determinations. Data are compared statistically to trolox c, which is used as an antioxidant reference standard.

*$P < 0.01$, †$P < 0.05$, repeated measures ANOVA, followed by Dunnett's multiple range test.

DCFH from cells [20, 21] means that it is not possible to explore these proposals using this protocol.

Antioxidant Activity of Capsazepine and Related Compounds

Given the apparent lack of involvement of the vanilloid receptor in the ROS generation induced by capsaicin, it is reasonable to conclude that capsazepine inhibition of this response is mediated by an antioxidant activity. Further evidence for this conclusion was obtained from a number of experiments. Firstly, capsazepine potently inhibited the DCFH oxidation stimulated by PMA and Tween 20 (Figs. 3 and 4). Secondly, capsazepine inhibited the ROS generation mediated by menadione (Fig. 7), a compound which undergoes redox cycling via various intracellular reductases to generate superoxide and hydrogen peroxide [28]. These results indicate that capsazepine can inhibit ROS generation mediated by a number of different mechanisms—activation of membrane NAD(P)H oxidase (PMA, Tween 20), mitochondrial dysfunction (capsaicin), and redox cycling (menadione)—which argues for a general antioxidant effect rather than a specific receptor-mediated event. Finally, capsazepine displayed powerful direct antioxidant activity in a cell-free test system based on the ability of antioxidants to act as reductants (Table 2). It is likely that the antioxidant activity of capsazepine resides in the catechol moiety, as other catechols such as caffeic and ferulic acids are also reported to display antioxidant activity [34].

Interestingly, some of the compounds previously demonstrated to enhance DCFH oxidation in RAW 264 cells also demonstrated antioxidant activity in the Fe^{3+} reduction assay (Table 2). The structural requirements for these two effects were clearly different, with an *o*-methoxy-phenyl pairing being the principal feature required for effective antioxidant activity. Other examples of this anti-/pro-oxidant duality have been reported, e.g. for flavonoids [35, 36]. Thus, it may be that capsaicin has an inherent antioxidant capability which is overcome in intact cells by a pro-oxidant activity mediated through modulation of mitochondrial respiration.

In conclusion, this study shows that capsaicin and various analogues can stimulate DCFH oxidation, probably through modulation of mitochondrial respiration. A free phenolic group and a large lipophilic region (usually an alkyl chain) are essential for this activity. Capsazepine inhibits DCFH oxidation, probably by acting as an antioxidant. It appears that the classical vanilloid receptor is not involved in any of the DCFH oxidations described in this study.

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