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Caffeic acid phenethyl ester: Inhibition of metastatic cell behaviours *via* voltage-gated sodium channel in human breast cancer *in vitro*

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ABSTRACT

Caffeic acid phenethyl ester, derived from natural propolis, has been reported to have anti-cancer properties. Voltage-gated sodium channels are upregulated in many cancers where they promote metastatic cell behaviours, including invasiveness. We found that micromolar concentrations of caffeic acid phenethyl ester blocked voltage-gated sodium channel activity in several invasive cell lines from different cancers, including breast (MDA-MB-231 and MDA-MB-468), colon (SW620) and non-small cell lung cancer (H460). In the MDA-MB-231 cell line, which was adopted as a 'model', long-term (48 h) treatment with 18 μM caffeic acid phenethyl ester reduced the peak current density by 91% and shifted steady-state inactivation to more hyperpolarized potentials and slowed recovery from inactivation. The effects of long-term treatment were also dose-dependent, 1 μM caffeic acid phenethyl ester reducing current density by only 65%. The effects of caffeic acid phenethyl ester on metastatic cell behaviours were tested on the MDA-MB-231 cell line at a working concentration (1 μM) that did not affect proliferative activity. Lateral motility and Matrigel invasion were reduced by up to 14% and 51%, respectively. Co-treatment of caffeic acid phenethyl ester with tetrodotoxin suggested that the voltage-gated sodium channel inhibition played a significant intermediary role in these effects. We conclude, first, that caffeic acid phenethyl ester does possess anti-metastatic properties. Second, the voltage-gated sodium channels, commonly expressed in strongly metastatic cancers, are a novel target for caffeic acid phenethyl ester. Third, more generally, ion channel inhibition can be a significant mode of action of nutraceutical compounds.

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1. Introduction

Caffeic acid phenethyl ester (CAPE), a natural plant polyphenol compound isolated from honeybee propolis, has been shown to have anti-epileptic, anti-inflammatory, and hepato-, neuro- and cardio-protective effects (Chan et al., 2013; Tolba et al., 2013). In addition, there is increasing evidence that CAPE has anti-cancer properties, including against human breast cancer (BCa) (Chan et al., 2013; Silva-Carvalho et al., 2014; Watanabe et al., 2011; Weng and Yen, 2012; Wu et al., 2011). As such, CAPE could act through many different pathways (Chan et al., 2013; Weng and Yen, 2012). It is a known inhibitor of β-catenin, c-myc, NF-κB, PI3K/AKT (Chan et al., 2013; Wu et al., 2011), matrix metalloproteinases MMP-2/9 (Lee et al., 2008) and VEGF (Wu et al., 2011). The related compounds caffeic acid and caffeic acid 3,4-dihydroxy-phenethyl ester also have anti-cancer properties and are known to act through ERK1/2 (*e.g.* Han et al., 2010; Yang et al., 2014).

We, and others, have demonstrated that up-regulation of functional voltage-gated Na^+ channel (VGSC) expression occurs during progression of many cancers including breast, cervical, colon, prostate, and non-small cell lung, both *in vitro* and/or *in vivo* (*e.g.* Baptista-Hon et al., 2014; Brisson et al., 2011; Campbell et al., 2013; Diaz et al., 2007; Driffort et al., 2014; Fraser et al., 2005; Grimes et al., 1995; Hernandez-Plata et al., 2012; House et al., 2010; Laniado et al., 1997; Nelson et al., 2015a,b; Roger et al., 2003; Yildirim et al., 2012). Different VGSC isoforms have been shown to be functionally up-regulated in several cancer types. For example, for human prostate and non-small cell lung cancer it is Nav1.7 (Campbell et al., 2013; Laniado et al., 1997), whilst for cervical cancer it is Nav1.6 (Hernandez-Plata et al., 2012). Finally, in the cases of human colon and BCa, the predominant VGSC is the neonatal splice variant of Nav1.5 (nNav1.5) (Baptista-Hon et al., 2014; Brackenbury et al., 2007; Fraser et al., 2005). The latter is a clear example of oncofoetal gene expression (*e.g.* Ben-Porath et al., 2008; Monk and Holding, 2001).

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The VGSC activity promotes a range of cell behaviours involved in metastasis – metastatic cell behaviours (MCBs) – including motility, secretion, adhesion and invasion (*e.g.* Fraser et al., 2003a; Gillet et al., 2009; Hernandez-Plata et al., 2012; House et al., 2010; Mycielska et al., 2003; Palmer et al., 2008). Thus, targeting the VGSCs may have therapeutic potential (Djamgoz and Onkal, 2013; Nelson et al., 2015a,b; Yang et al., 2012). Interestingly, VGSC expression/activity may also be a target for nutraceutical compounds with anti-cancer properties (Ingólfsson et al., 2014). These include polyunsaturated fatty acids (Isbilen et al., 2006) and resveratrol (Fraser et al., 2014b).

It is not known if CAPE would also affect VGSC activity, although effects on some other ion channels have been described (*e.g.* Chiang et al., 2015; Choi et al., 2013; Shin et al., 2012). In the present study, using cell lines from several cancers, we aimed to determine possible effect of CAPE on VGSC activity known to occur in these cells. Then, using the strongly metastatic BCa cell line MDA-MB-231 as a model, we elucidated whether any such effect might lead to change in MCBs.

2. Materials and methods

2.1. Tissue culture

Experiments were carried out on the following cell lines: BCa (MDA-MB-231 and MDA-MB-468), colon cancer (SW620) and non-small cell lung cancer (H460). BCa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% foetal bovine serum (FBS) and 4 mM L-glutamine. Colon and non-small cell lung cancer cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and 4 mM L-glutamine. All cells were maintained at 37 °C, 5% CO_2 and 100% humidity (Fraser et al., 2005).

2.2. Chemicals and treatment regimes

CAPE was dissolved in dimethyl sulphoxide (DMSO) and aliquoted. Stock solutions (10 mg/ml) were diluted to the required concentration immediately prior to use. The final working concentration of DMSO for 1 μM CAPE was 0.03%. Cells were treated either 'short-term' (acute application) or 'long-term' (up to 72 h). For the latter, solutions were changed every 24 h. In each case, control/untreated cells were maintained in parallel with the corresponding DMSO concentration. All chemicals were obtained from Sigma–Aldrich Chemicals (Poole, UK) except tetrodotoxin (TTX) and CAPE which were purchased, respectively, from Alomone Labs (Jerusalem, Israel) and Tocris (Bristol, UK). The final working concentration of DMSO was also added to the TTX-treated dishes to allow direct comparison with the CAPE control dishes which contained DMSO.

2.3. Assays of MCB

Cell viability and proliferation. Cellular viability (toxicity) and proliferation were quantified as described previously (Fraser et al., 1999, 2003a; Grimes et al., 1995). Briefly, cells were seeded into 35 mm plates at 3.5×10^4/plate (for toxicity) or 24-well plates at 2×10^4/well (for proliferation) and allowed to settle overnight. The cells were incubated under control conditions or treated with CAPE, with a change of medium every 24 h. Cell viability was determined by Trypan blue exclusion assay whilst proliferation was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each biological repeat (treatment and control) was performed in triplicate.

Lateral motility. Cells were initially cultured at a density of 5×10^5 cells/ml and wound-heal assays were carried out as described previously (Fraser et al., 2003a) with the exception that cells were pre-treated for 24 h with CAPE and/or TTX. Wounds were measured at four time points: 0, 12, 24 and 36 h. Motility index (MoI), representing lateral motility of the cells, at a given time, was defined as follows:

$$\text{MoI} = 1 - (\text{final width}/\text{initial width})$$

For analysis, the mean values of the 'wound' widths were determined for given dishes (45 data points maximum per dish). The overall data from the biological repeats were then averaged and normalized for comparison ($n = 14–25$).

Matrigel invasion. Cells were plated at a density of 2×10^4 on Transwell filters, with 8 μm pores, that had been pre-coated with 50 μl, 1.25 mg/ml Matrigel® (Becton Dickinson) in a 24-well plate. The inserts were coated one day prior to the experiment and left in the incubator overnight. Cells were also pre-treated with CAPE and/or TTX for 24 h. On the day of the experiment, coated inserts were initially rehydrated with serum-free medium for 2–3 h. The serum-free medium was then removed and 1% FBS containing medium (+treatments) was added to the upper chamber of the insert and 5% FBS containing growth medium (+treatments) added to the lower chamber. Cells were then seeded and left for 16 h. At the end of the assay, non-invaded cells were removed from the upper side of the filters by swabbing with cotton buds. Inserts were then placed into clean wells with ice-cold 100% methanol for 15 min. Inserts were then incubated with crystal violet (0.5 g/ml in 0.25% methanol) for 15 min. Finally, excess crystal violet was washed away gently with distilled water and inserts were dried before counting the invaded cells on the lower side. For analysis, using ×400 magnification, the mean number of invaded cells was determined from 12 fields of view per insert. These data was then averaged and normalized for comparison ($n = 8–12$).

2.4. Electrophysiology and pharmacology

Experiments were performed on cells (in at least three separate dishes) that had been in culture for 1–3 days. Details of the patch pipettes, solutions and the whole-cell recording protocols were as described previously (Fraser et al., 2003b; Grimes et al., 1995; Laniado et al., 1997). Patch pipettes (tip resistances, ~5 MΩ) were filled with a solution designed to block the outward K^+ currents; the composition was as follows (in mM): NaCl 5, CsCl 145, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10 and EGTA 11, adjusted to pH 7.4 with 1 M CsOH. The estimated intracellular free calcium concentration was ~15 nM (Laniado et al., 2001). Whole-cell membrane currents were recorded from cells that appeared 'isolated' in culture, using an Axopatch 200B amplifier (Axon Instruments, CA, USA). Analogue signals were filtered at 10 kHz using a lowpass Bessel filter and series resistance errors were compensated by >90%. Electrophysiological signals were sampled at 50 kHz and digitized using an interface (Digidata 1200). Data acquisition and analysis of whole-cell currents were performed using pClamp software (Axon Instruments). A holding potential of −100 mV was applied, unless indicated otherwise. Standard voltage-clamp protocols were used to study the electrophysiological and pharmacological properties of the VGSC currents. CAPE was bath-applied. For experiments studying the long term effects of CAPE incubation, CAPE was removed by perfusion of physiological saline solution prior to recording from the cells. For each cell, steady-state availability (I/I_{max}) was plotted as a function of membrane potential, and fit with a Boltzmann function (I, current recorded during the prepulse; I_{max}, current during the corresponding control pulse). The time course of recovery from inactivation (I_t/I_c) was plotted as a function of recovery time and fitted to a single exponential function (I_t, current recorded during the test pulse; I_c, current during the corresponding control pulse).

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