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ORIGINAL CONTRIBUTION

Dietary polyphenols identified as intracellular protein kinase A inhibitors

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Abstract *Background* Dietary plants contain several thousands different polyphenols that can potentially influence normal and pathological cellular processes through modulation of intracellular signaling pathways. A few polyphenols have been shown to be potent inhibitors of protein kinases. *Aims of study* To identify possible dietary protein kinase A (PKA) inhibitors we designed a method for screening of substances in crude mixtures of food items for modulation of intracellular PKA activity that enables high-throughput testing of a large number of compounds and extracts. *Methods* Luciferase was mutated to render it sensitive to phosphorylation by PKA (luciferase^{PKA}) and transfected into a human hepatoma cell line (HepG2). Cells were then treated with extracts from dietary plants, including berries, fruits and spices, and intracellular PKA-activity was assessed by change in bioluminescence in live cells by imaging. *Results* Several extracts were found to inhibit PKA activity in a 96-well platform high-throughput screen. Green tea, crowberry, clove and cinnamon extracts were found to reduce intracellular cAMP levels consistent with their ability to increase luminescence from luciferase^{PKA}. Also pomegranate extract inhibited intracellular PKA and was used to estimate cellular association of polyphenols by HPLC and LC–MS. Pomegranate extract contains several anthocyanins, including delphinidin-3 glucoside. Delphinidin aglycone was found to inhibit cellular PKA activity in a concentration dependent manner. The inhibitory activity was found to be structure specific as a closely related compound to delphinidin had no activity. *Conclusion* The current work identify phytochemicals in crude extracts which modulate cell signaling through PKA in a way that facilitate high through-put screening to help elucidate how plant based diet reduce risks of chronic diseases.

Key words polyphenols – protein kinase A – inhibitors – high-throughput screening – delphinidin

EJN 749

Introduction

Dietary phytochemicals are claimed to be at least partly responsible for the beneficial effect of a diet rich in plant material such as fruits, vegetables and spices. Phytochemicals, such as the roughly 8,000 different polyphenols found in the human diet, may have a plethora of different effects on cells, of which only a few have been characterized [27]. Their antioxidant properties have been extensively studied, but it is still unclear whether dietary antioxidants can contribute significantly to the cellular antioxidant defense. Additionally, there are considerable uncertainties regarding bioavailability of polyphenols and their access to intracellular processes and signaling pathways [18].

Several phytochemicals, including some of those found in the human diet, have been employed as protein kinase modulators in experimental cell biology to study the various intracellular signaling pathways [2, 11]. This suggests that at least some of the cellular effects of complex mixtures of dietary phytochemicals may be explained by their modulation of protein kinases. Indeed, it has been shown that extracts used as dietary supplements contain substances which inhibit several protein kinases, including protein kinase A (PKA) [22]. PKA is very important in regulation of various physiological process through phosphorylation of substrates in response to cAMP synthesized by adenylate cyclase. A few examples of such processes are lipolysis, glycogen and glucose metabolism, synaptic transmission between neurons and spermatogenesis. To test the hypothesis that extracts from dietary plants can modulate kinase mediated cell signaling, we have now developed a tool to screen for intracellular protein kinase modulation. Luciferase, which can be detected in intact cells and animals by imaging of bioluminescence [5, 21] was engineered to contain a target sequence for PKA to yield a PKA sensitive luciferase (luciferase^{PKA}) [20, 25, 26]. A construct encoding PKA-sensitive luciferase was transfected into human hepatocytes and the cells were subsequently treated with extracts from fruits, berries and spices, or potential protein kinase inhibitors found in such extracts. We show that of several extracts tested crowberry, clove, green tea, pomegranate and cinnamon extracts inhibit basal PKA activity in a human hepatocarcinoma cell line, HepG2. Crowberry, cinnamon, clove and green tea extracts were tested for effects on intracellular cAMP levels, and were found to reduce cAMP levels at low concentrations. These are all from polyphenol rich plants and extract from another polyphenol-rich plant, pomegranate, was shown to contain delphinidin-3-glucoside among other anthocyanins, and delphinidin glucosides were identified by HPLC in lysate from extract treated cells. Evidence for the PKA inhibitory effect of pomegranate extract and delphinidin was further strengthened by a conventional in vitro assay based on phosphorylation of kemptide [14]. The method described here demonstrates for the first time the possibility to screen for effects of physiologically relevant metabolism of dietary compound and their possible metabolic derivatives on protein kinase activity.

Materials and methods

Delphinidin 3-glucoside, delphinidin and pelargonidin were obtained from Polyphenols AS (Sandnes, Norway). Acetonitrile for LC–MS analysis was LiChrosolv grade from Merck (Darmstadt, Germany) and water was of Milli-Q quality (Millipore Corp., Ireland). Formic acid was pro-analysis from Prolabo (Paris, France). Forskolin was from Sigma-Aldrich (St. Louis, MO). Luciferin was from BioSynth (Staad, Switzerland).

Preparation of plant extracts

All items used for extract preparation were purchased in regular grocery stores in Oslo. Spice extracts were prepared by addition of 10 ml water and 10 ml methanol to 10 g of dry material and mixed vigorously for 15 s. The mixture was the placed in an ultrasound water bath for 30 min at 0°C. In the case of fruit and berries only 10 ml of methanol was used. Undissolved material was removed by centrifugation at 3,000×*g* for 15 min and the pellet was re-extracted using the same procedure. Excess liquid in pooled supernatants was evaporated under nitrogen and volumes adjusted with DMSO (spices) or PBS (fruit and berry extracts) to final concentration 2 g wet-weight plant material per milliliter. All extracts were sterile filtered and stored at −70°C under argon until used in experiments. Estimates of dry weight phytochemicals in the various extracts was obtained by removal of liquid by lyophilization.

cAMP measurements

Intracellular cAMP was measured by an enzyme immunoassay (cAMP EIA, Amersham Biosciences, UK) essentially as recommended by the manufacturer. Briefly, HepG2 cells were cultured in 96 well plates (10^4 cells per well) and treated for 24 h with different extracts. Culture medium was then replaced with lysis buffer and a fraction of the lysate was

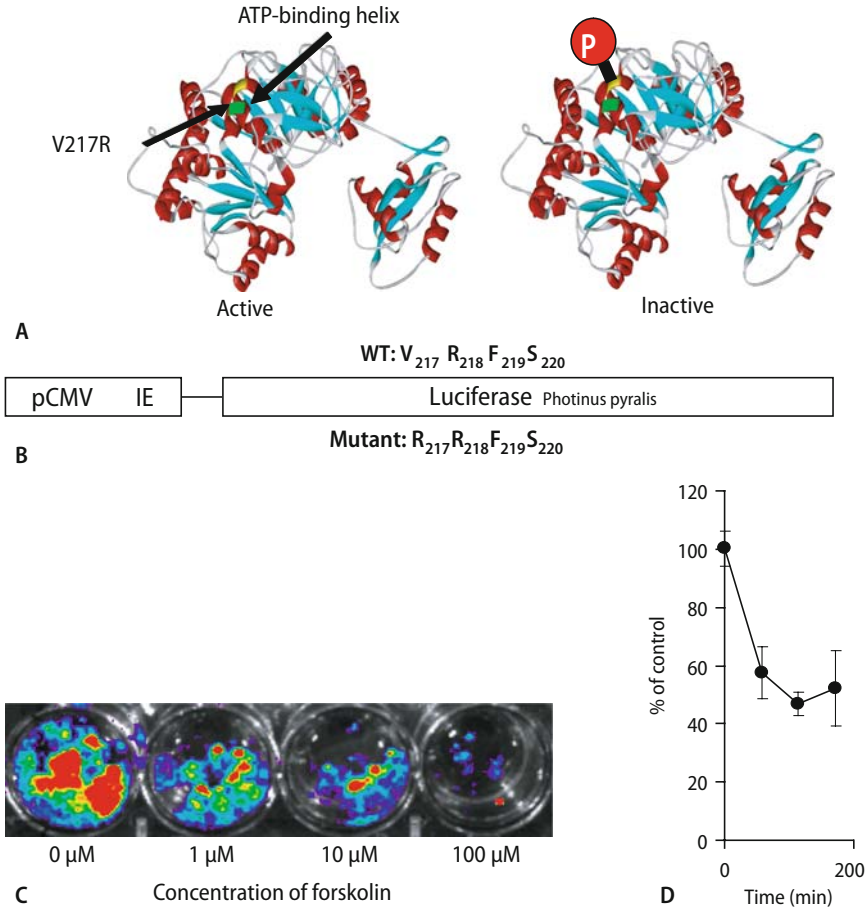


Fig. 1 Schematic illustration of lucifease^{PKA} and its response to PKA stimulation. **a** Illustration of crystal structure of luciferase with β-sheets and α-helices are shown [15]. **b** The mutant luciferase construct with phosphorylation site on luciferase is indicated. **c** Wells with cells transfected with the pLuciferase^{PKA} were treated with increasing concentration of forskolin and bioluminescence detected by imaging. **d** Transfected β-adrenergic responsive 293T cells were treated with 10 µM isoproterenol and analyzed for bioluminescence by imaging and photon quantization after increasing time periods. Data is presented as % of luminescence at time 0 min of means ± SD of triplicates

transferred to wells precoated with donkey anti-rabbit Ig and rabbit anti-cAMP Ig. Bound cAMP in samples and standard solutions were then measured spectrophotometrically in a competition assay with peroxidase labeled cAMP and peroxidase-conjugated substrate (tetramethylbenzidine).

Mutagenesis of luciferase and construction of reporter plasmid: (pLuciferase^{PKA})

Luciferase from Photinus pyralis was genetically modified to code for the amino acid sequence $R_{217}R_{218}F_{219}S_{220}$, instead of the wild type sequence $R_{217}V_{218}F_{219}S_{220}$ in the ATP binding helix (Figs. 1a, [6]). The modified luciferase (luciferase^{PKA}) was obtained by the following procedure: Luciferase was amplified with PCR primers 5′-CGAAACAAAACAAA CTA-3′ and 5′-GGGGCATGCGAGAATCTCCTGCAG GCAGTTCTATG-3′ with pTAL-luc (BD Biosciences, San Jose, CA) as template. The PCR product was digested with BglII and SphI and ligated into the same sites in pTAL-luc (BD Biosciences, San Jose, CA, USA). This construct was digested with NcoI and XbaI and the fragment was ligated into pGL3-Control (Promega, Madison, WI, USA) digested with the same enzymes to obtain pGL3-PKA-Luc expressed from the SV40 promoter. Introduction of the mutation was verified by sequencing. Luciferase^{PKA} expressed from CMV promoter was engineered by digestion of pGL3-PKA-luc and pCDNA3 (Invitrogen, Carlsbad, CA) with HindIII and XbaI. The 1,655 bp fragment from pGL3-PKA-luc was ligated into pCDNA3 using T4 DNA ligase (Promega, Madison, WI, USA) to obtain pLuciferase^{PKA} (Fig. 1b).

Cell culture and transfections

HepG2 cells (passage 80–110) and 293T cells were routinely cultured in Dulbeccos Modified Eagles Medium and RPMI 1640, respectively (Gibco, Carlsbad, CA, USA) with 10% fetal calf serum and penicillin/streptomycin. Cells were cultured in 12 or 24 well plates and transfections with pLuciferase^{PKA} were performed with Lipofectamine 2000 (Invitrogen,

Carlsbad, CA, USA) essentially as described by the manufacturer. Briefly, 0.8–1.6 μg DNA and Lipofectamin 2000 were diluted separately in OptiMem (Gibco, Carlsbad, CA, USA) and mixed after 10–15 min incubation. After additional 15 min the mixture was added to cells cultured in DMEM with 10% FCS without antibiotics. The transfection mix was left in the cell medium for approx. 20 hours before treatment of cells with PKA modulators or extracts.

PKA-assay

Activity of PKA in cell homogenates was measured essentially as described by Kemp et al. [14]. Briefly, cells from each well were harvested in PBS and lysed in the presence of 0.5% Triton-X 100, 5 mM EDTA, 50 mM NaF, 10 mM Na-pyrophosphate, 1 mM PMSF, 1 mM Na-vanadate, 100 mM NaCl, 50 mM Tris–HCl, pH 7.4. The homogenate was centrifuged for 15 min at 10,000 rpm, and 10 μl of the supernatant was added to a mixture of ATP, Kemptide (Sigma-Aldrich, St. Louis, MO) and ^{32}P-γATP. The mixture was incubated for 9 min at 30°C, before application of the reaction mix to pieces of P81 chromatographic paper (Whatman, Brentford, UK) for binding. The paper pieces were washed 5 times in 75 mM phosphoric acid and dried before scintillation counting.

Luminescence measurements

After treatment of transfected cells with extracts or pure substances in 12 or 24 well plates, luciferin was added to each well (100 μg/ml final concentration). Cells were incubated for 4 min before imaging for 1 min with IVIS 100 Imaging system (Xenogen, Alameda, CA, USA). Photons emitted per second (flux) was measured during imaging and quantified with the software Living Image version 2.2 (Xenogen, Alameda, CA, USA).

Measurement of total phenol content

Total phenol content in dietary plant extracts was estimated using the Folin-Ciocalteau assay as described [28]. Briefly, extracts were diluted sufficiently to give linearity in the spectrophotometric absorption, before 100 μl Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, MO) was added and incubated for 5 min. Then, Na_2CO_3 was added and the sample incubated for another 2 h, before absorption at 765 nm was measured. A standard curve using 0–500 μg/ml gallic acid (Sigma-Aldrich, St. Louis, MO) was generated in the same manner and used for calculation of gallic acid equivalents in the extracts.

Statistical analyses

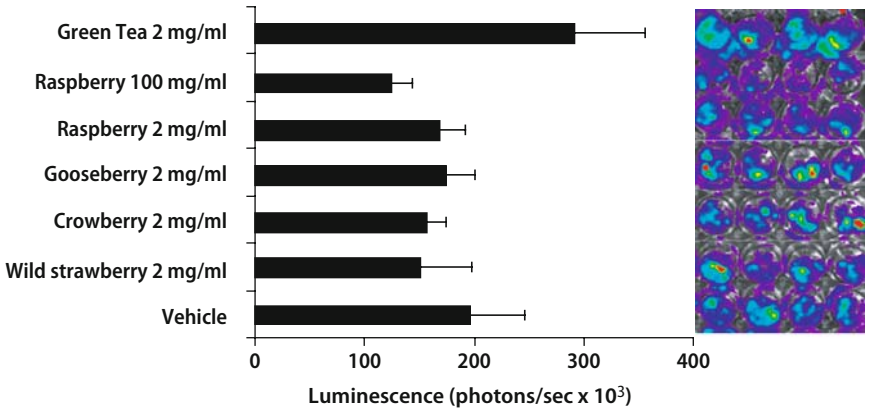
All statistical analyses were performed using the Student *t* test.

Results

Screening of crude mixtures of dietary phytochemicals for effects on intracellular PKA activity

Genetically engineered luciferase, illustrated schematically in Fig. 1a, b, was tested for response to established PKA modulators, such as forskolin (HepG2 cells) and isoproterenol (293T cells). Luminescence from transfected HepG2 cells in response to forskolin was found to be as expected and in accordance with results from Waud et al. [32], i.e. the adenylate cyclase activator forskolin decreased luminescence in a dose dependent manner (Fig. 1c). To prove that luciferase^{PKA} also responded to a physiological stimulus, transfected 293T cells were treated with the β-adrenergic agonist isoproterenol. Isoproterenol dramatically reduced luminescence from the cells, Fig. 1d.

In subsequent experiments HepG2 cells were used, as these express cytochrome P450 enzymes that can possibly metabolize polyphenols [13] similar to liver cells in vivo. Various food plants suspected to contain high amounts of polyphenols were used for production of extracts. Transfected HepG2 cells were treated with an arbitrary high or low concentration of water/methanol extracts of dietary plants. Several extracts altered luminescence from luciferase^{PKA}, some extracts increased luminescence relative to control (vehicle) (clove and allspice extract), whereas others reduced luminescence to almost undetectable levels (high concentration of green tea and wild strawberry extract), Fig. 2. To estimate a possible correlation between PKA inhibition and polyphenol content, all extracts were submitted to the Folin-Ciocalteau assay. As shown in Fig. 3, the highest total phenol content was found in extracts from clove and green tea. Clove and a low concentration of green tea extract gave the highest induction of luminescence (2–3 fold higher than control, Fig. 2), suggesting that these extracts contain one or several substances that inhibit phosphorylation of luciferase^{PKA}, possibly polyphenols. This initial screen did neither take into account the possibility of cytotoxic effects of extract phytochemicals nor their potential direct effect on the enzymatic activity of luciferase itself. Indeed, several extracts were found to be cytotoxic (as judged by



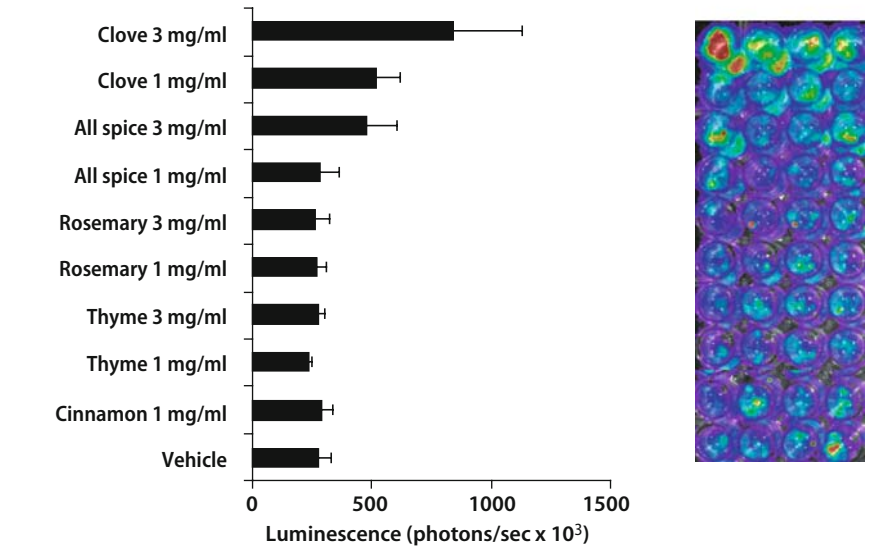


Fig. 2 Screening of extracts from dietary plants for effect on luciferase^{PKA} activity. **a** HepG2 cells in 96 well plates were transfected with pLuciferase^{PKA} 24 h prior to treatment with the indicated wet weight concentrations of extracts for 20 h (wet weight starting material per ml cell medium). Luciferin was then added to the medium and cells were imaged with IVIS100. The *graphs* represent mean luminescence from quadruplicates ±SD in the associated images. *Top panel* shows data obtained with berry and green tea extracts and lower panel that obtained with spices

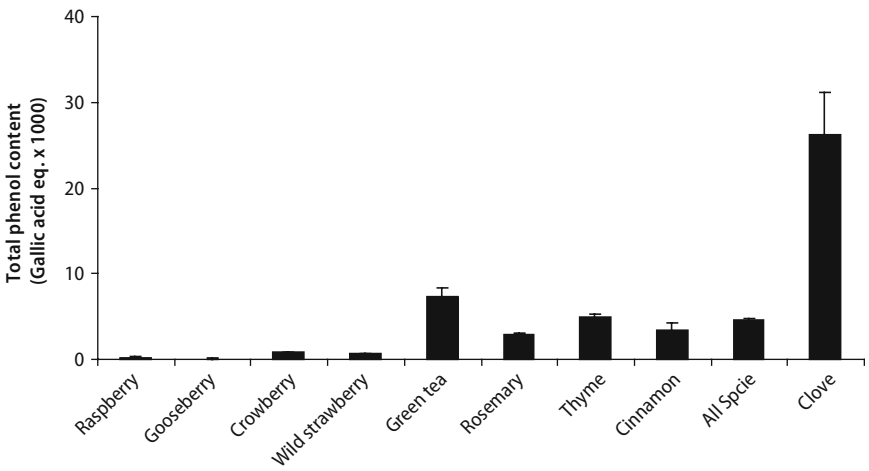


Fig. 3 Total phenol content in dietary plant extracts. Equal volumes of plant extracts were analyzed for reactivity in the Folin-Ciocalteau assay and expressed as equivalents of gallic acid reactivity in the same assay, $n = 3$

trypan blue exclusion) and some also directly interfered with luciferase activity, particularly green tea extract at high concentration (data not shown). Based on these analyses clove, cinnamon, green tea and crowberry extracts were tested further at concentrations where such effects were not pronounced (less than 10%).

Reduced PKA activity in HepG2 cells as suggested by changes in luminescence in Fig. 2 could be explained by phytochemical interference with PKA di-

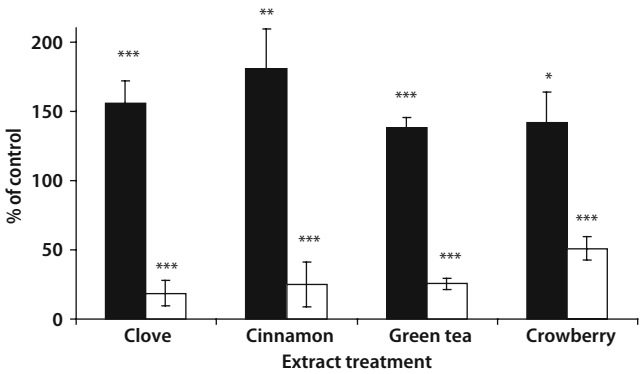


Fig. 4 Effect of dietary plant extracts on luciferase^{PKA} activity and intracellular cAMP. Transfected HepG2 cells in 24 (luminescence measurement, filled bars) or 96 well plates (cAMP measurements, open bars) were treated with 127 μg/ml clove extract, 60 μg/ml cinnamon extract, 190 μg/ml green tea extract or 110 μg/ml crowberry extract for 24 h (concentrations are given as μg dry weight phytochemicals per ml cell culture medium). The medium was removed and replaced with fresh medium with luciferin (luminescence measurements) or lysis buffer (cAMP measurements). Cells and lysates were then processed as described in Materials and methods. Data are presented as mean % luminescence or cAMP of non-treated cells (vehicle only) ±SD, $n = 3$ each in triplicates (luminescence) or $n = 1$ in triplicate (cAMP). ***$P < 0.001$, **$P < 0.01$, $P < 0.05$

rectly or indirectly by reduction in intracellular cAMP. To clarify this, clove, cinnamon, green tea and crowberry extracts were tested simultaneously for their effect on luciferase^{PKA} and intracellular cAMP. Low, non-cytotoxic concentrations of these extracts resulted in approximately 50–75% increase in luminescence and decrease in intracellular cAMP to 25–50% of untreated cells, Fig. 4.

In a similar screen of dietary plants as that illustrated in Fig. 2, also pomegranate extract was found to increase luminescence in HepG2 cells. The increase in luminescence was comparable to that seen with clove, cinnamon, green tea and crowberry extracts. Pomegranate is rich in polyphenols such as anthocyanins [7] and we suspected that one or several of these might contribute to PKA inhibition. Therefore pomegranate extract was subjected to HPLC-DAD/ESI-MS/MS ion trap analysis [1, 10, 29] to estimate the composition with respect to anthocyanins. Five peaks in the resulting chromatogram were confirmed by spectra analysis and LC–MS/MS to be delphinidin 3,5-diglucoside, cyanidin 3,5-diglucoside, delphinidin 3-glucoside, cyanidin 3-glucoside and pelargonidin 3-glucoside (Table 1). The pomegranate extract was also analyzed by LC–MS after incubation for 24 h in cell culture medium alone. At this time point no delphinidin was detected, suggesting physical degradation under these conditions (data not shown).

Effect of anthocyanidins on intracellular PKA activity

Pomegranate seed coat contains glucosides of delphinidin, cyanidin and pelargonidin. Du et al. [7] found that delphinidin-3,5-diglucoside was the most abundant anthocyanin in pomegranate juice but this may vary with variety and culture condition. Anthocyanidins (anthocyanin aglycones) are structurally related to flavonoids which have been used extensively as protein kinase inhibitors in experimental systems. We therefore tested delphinidin and pelargonidin with respect to PKA inhibition in HepG2 cells. We found that delphinidin resulted in increase in luminescence from luciferase^{PKA} suggesting inhibition of basal PKA activity in HepG2 cells, Fig. 5a. Delphinidin-3-glucoside at the same concentrations was less effective (data not shown) and cyanidin could not be tested under similar conditions due to low solubility. Pelargonidin, which compared to delphinidin has two additional hydroxyl-groups in one of the phenol rings had no effect. Petunidin and peonidin, two polyphenols closely related to delphinidin, were also without effect (data not shown).

Luminescence was found to respond to delphinidin in a dose dependent manner (Fig. 5b) and the manifestation of the effect was found to increase over time with a maximum at approximately 24 h after addition to the cells (data not shown).

Table 1 Characterization of of pomegranate extract by HPLC-DAD and LC/ESI-MS^n

Peak no.	t_R (min)	UV spectra data (nm)	MS/MS data[a]		Identification
			Molecular ion (*m/z*)	Fragments from MS^2 and MS^3(*m/z*)	
1	3.3	529, 384, 260	627 (M^+)	465 (M-hexose)303 (delphinidin)	Delphinidin 3,5-diglucoside
2	4.3	516, 379, 259, 234	611 (M^+)	449 (M-hexose)287 (cyanidin)	Cyanidin 3,5-diglucoside
3	10.6	518, 266	465 (M^+)	303 (delphinidin)	Delphinidin 3-glucoside
4	13.1	516, 279	449 (M^+)	287 (cyanidin)	Cyanidin 3-glucoside
5	15.8	502, 429, 330, 283	433 (M^+)	271 (pelargonidin)	Pelargonidin 3-glucoside

[a]Conditions for LC are described in [1]

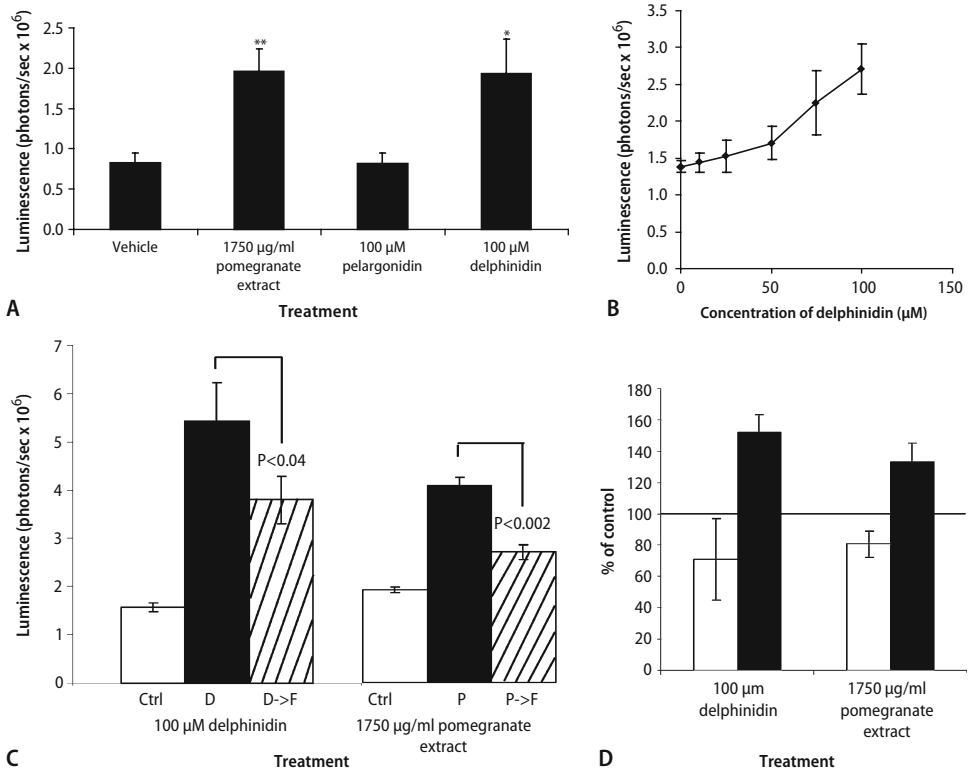


Fig. 5 Effect of anthocyanidins on luciferase^{PKA}. In all experiments HepG2 cells in 24 well plates were transfected with pLuciferase^{PKA} and treated as indicated after 20 h. All luminescence measurements were performed by imaging after 4 min incubation with luciferin. **a** Cell were treated as indicated and results are presented as flux (photons/s) in an average of 3 replicates ±SD in a typical experiment. **b** HepG2 cells were incubated for 20 h with the indicated concentrations of delphinidin dissolved in DMSO (all samples adjusted to 0.1% of the vehicle DMSO). Luminescence is presented as flux (photons/s). Each point is an average of three independent samples ±SD. **c** HepG2 cells were incubated with delphinidin (*left panel*, *black bar*, **d**) or pomegranate extract (*right panel*, *black bar*, P) or without (*white bars*, Ctrl)) After 20 h incubation, forskolin (20 µM final concentration, striped bars, D- > F and P- > F) or vehicle (*white and black bars*) was added and cells incubated for 30 min at 37°C. Luminescence is presented as mean flux (photons/s) of triplicates ±SD in a typical experiment. The *P*-values refer to comparison of luminescence with or without forskolin treatment. $^{**}P < 0.01$, $^{*}P < 0.05$. **d** HepG2 cells were treated with or without the indicated concentration of pomegranate extract or delphinidin before luciferin was added and luminescence was measured by imaging with (black bars). Cells were then harvested and lysed in PKA-assay lysis buffer and aliquots of each lysate were then submitted to measurements of PKA-mediated phosphorylation of kemptide as described in materials and methods (*white bars*). Luminescence (*black bars*) is presented as average % of control (no treatment) of 3 replicates ±SD. Kemptide phosphorylation is presented as average of three replicates measured in triplicates ±SD

The increase in luminescence seen with extracts from the cells without previous stimulation of PKA suggests that at least in HepG2 cells, luminescence from luciferase^{PKA} is to some extent inhibited by a basal PKA mediated phosphorylation. To test whether extract mediated PKA inhibition could be overcome by stimulation with forskolin, cells were treated with delphinidin or pomegranate extract with or without subsequent treatment with forskolin. Fig. 5c shows that both extract and delphinidin inhibition of PKA can be partly overcome by treatment of cells with forskolin.

Correlation between luciferase^{PKA} activity and PKA phosphorylation of kemptide

To provide evidence that the increase in luminescence is linked to intracellular PKA activity, the effect of pomegranate extract and delphinidin was measured by a conventional assay in which PKA phosphorylates a synthetic peptide containing a PKA target sequence (kemptide). Cells were transfected with luciferase^{PKA} and treated with extract or delphinidin as indicated and luminescence was measured by imaging. After imaging the same cells were lysed and cell homogenates were assessed for PKA activity. Fig. 5d demonstrates that delphinidin and pomegranate extracts inhibit PKA activity as assessed by both the increase in luminescence as well as the decrease in phosphorylation of kemptide.

Identification of cell-associated delphinidin

To our knowledge few analytical studies have shown that anthocyanins/cyanidins or metabolites are present in or otherwise associated with cells. To test if delphinidin added to the cell medium was taken up or associated with cells by other means, HepG2 cells

were incubated with the aglycone delphinidin, lysed and the supernatant analyzed by HPLC-UV/ESI-MS/MS [1, 10]. Our studies show that delphinidin added to the cell culture medium associated with HepG2 cells in a time dependent manner with maximum of intact delphinidin after 30 min (not shown). The presence of delphinidin in the cell extracts was confirmed by comparison with both the retention time and product ion spectrum of standard delphinidin (not shown). The fairly long incubation time used to obtain PKA inhibition compared to cell association may allow metabolism of delphinidin and it can be speculated that the effect on protein kinase A is mediated by some of the metabolites rather than delphinidin itself. This possibility was substantiated by the observation that cell associated delphinidin increased at first for subsequently to decrease to non-detectable levels after 180 min. Simultaneously, other peaks not found in the delphinidin standard also appeared that could possibly represent metabolites of delphinidin. The mechanisms behind this is unclear, both unspecific degradation and intracellular metabolism are possible and requires further studies.

Discussion

We have in the present study clearly demonstrated that the PKA-sensitive luciferase can be utilized for screening of PKA-modulatory activity in mixtures of substances and pure compounds. We found that delphinidin and extracts of pomegranate, clove, cinnamon, crowberry and green tea quite potently inhibit PKA in HepG2 cells. The strong implication of this is that substances in berry extracts are able to get transported across the cell membranes and modulate important intracellular signaling pathways. Indeed, HPLC analyses demonstrated that delphinidin associates with cells in a time dependent manner. The hydrophobic nature of anthocyanidins could possibly promote accumulation in lipid membranes, thus approaching concentrations of anthocyanidins used in studies such as the present in vitro work. Luciferase^{PKA} is thus useful for identification of PKA modulators particularly in mixtures of dietary components such as fruit and vegetable extracts that may or may not require to be metabolized to gain biological activity. Our results suggest that the effect on PKA is mediated by reduced cAMP. Reduced cAMP can again be explained by either increased phosphodiesterase or reduced adenylate cyclase activity and further studies are required to clarify this issue.

Pomegranate extract contains delphinidin 3-glucoside that can possibly be responsible for PKA inhibition. However, the concentration of pure delphinidin required to obtain comparable inhibition is far higher than that found in the extract. The explanation can be that pomegranate extract contains additional inhibitors not tested in this study. Alternatively, delphinidin 3-glucoside in the extract is more stable than delphinidin and thus allows extended accumulation by the cells. The PKA inhibitory effect of the other extracts warrants further studies to identify their active constituents. It will also be interesting to see if extracts exert additive or synergistic effect on PKA.

The observed increased luminescence can be explained by either increased dephosphorylation of existing luciferase^{PKA} or translation of luciferase^{PKA} that does not become phosphorylated due to PKA inhibition. We can not distinguish between these possibilities but the time lapse between delphinidin association with cells and increased luminescence is consistent with the latter possibility.

Only a fraction of the delphinidin added to cell culture medium could be accounted for by HPLC analysis. This may be related to the previously observed instability of delphinidin glucosides. Such high instability has also been observed for anthocyanins in urine samples after freezing [9] and it has been suggested that instability may have contributed to underestimation of anthocyanins in biological specimens [18].

We observed a clear relationship between structure of anthocyanidins and PKA inhibition. Only delphinidin of the anthocyanidins tested had inhibitory activity and this substance has 3 hydroxyl groups on the B-ring of the anthocyanidin structure. Pelargonidin has one hydroxyl group, petunidin has two hydroxyl groups and one methyl ether group, and peonidin has one hydroxyl and one methyl ether group on the B-ring. One possible explanation for the PKA inhibitory activity of delphinidin is that it competes with ATP for the binding site on the kinase. Such binding has been shown for a structurally related compound, quercetin, which in crystallization experiments was found in the ATP binding pocket of PI-3 kinase [31]. It is possible that the three-hydroxyl group configuration is favorable for binding to the ATP-binding pocket and that the more bulky methyl ether groups found in peonidin and petunidin prevents binding and inhibitory activity. Alternatively, as suggested by Kosuge et al., polyphenols may bind to proline-rich regions in PKA β-subunits and thereby inhibiting its activity as shown with galloyl pedunculagin [15].

A number of other studies have also shown that polyphenols have modulatory activity on other kinases. Tea polyphenols such as epigallocatechin gallate has been shown to inhibit the epidermal growth factor receptor (EGFR) signaling pathway by direct

inhibition of ERK1/2 and Akt [24]. Also Meiers and coworkers [19] have shown by using a MAPK dependent reporter construct that delphinidin inhibits EGFR signaling. Anthocyanins in fruits have been shown to possess both antioxidant activity [23] and modulatory activity towards the MAPK pathway (11). Whereas the role of the antioxidant properties of anthocyanins/anthocyanidins on disease development is unclear, the latter effect has been suggested to contribute to the anticarcinogenic action of fruits through inhibition of phosphorylation of ERK, JNK and MEK1/2 [12]. Delphinidin has also been shown to inhibit angiogenesis through modulating the effect of VEGF on p27 and cyclin D1 in human endothelial cells [8]. Recent results suggest that this may be attributed to the delphinidin inhibition of phosphorylation of VEGF-receptor [16].

Hou et al. [12] found that delphinidin blocks TPA-induced ERK and JNK phosphorylation suggesting that delphinidin also modulate kinases in the PKC/Ras pathway. On the other hand, Martin et al. [30] have found that delphinidin inhibit cell proliferation through a transient activation of ERK. cAMP and PKA activates Rap1/B-Raf in PC12 cells, which in turn phosphorylate MEK1/2 and activates ERK1/2. In glioma cells however, cAMP and PKA inhibit ERK and Akt in a Rap1 dependent pathway [33], clearly indicating that the cAMP/PKA mediated modulation of MAPK is cell specific. Nardini et al. [22] have shown that a polyphenol-rich extract from Pine bark inhibits PKC and PKA. Quercetin and genistein, a flavonoid and isoflavonoid, respectively, have been used extensively as protein kinase inhibitors. In accordance with studies by Cochet et al. [4], quercetin did not inhibit PKA activity measured by luciferase^{PKA} (data not shown). Taken together, there is a clear picture emerging in which dietary polyphenols exert modulatory activities on important kinase mediated signaling pathways. There is mounting evidence that polyphenols reach cells in target tissues in sufficient concentrations to gain biological activity and a recent meta-analysis by Manach et al. [18] concluded that anthocyanins reach concentration in the 100 nM range in plasma after ingestion of polyphenol-rich food items.

In pharmacological industry, enormous resources are put into screening for compounds that can modulate the activity of kinases as therapeutic targets. It has been suggested that PKA could be an interesting drug target in memory disorders [3] and for controlling fluid secretion in the upper airways in cystic fibrosis [34]. We have set up a screening system that allows testing of a vast number of potential PKA modulators in vitro. The method described here introduces for the first time the possibility to measure effects of physiologically relevant metabolism of dietary and pharmaceutical compound on PKA activity. This proof-of-concept study suggests that the modifications of luciferase by specific phosphorylation sites may serve as a general tool for screening of dietary and pharmacological protein kinase modulators. The importance of this is underscored by the recent finding by Malik et al. [17] that a diet containing pomegranate fruit extracts inhibits tumor growth in mice with transplanted human prostate cancer cells. Recent development of a mouse model transgenic for luciferase^{PKA} may provide evidence regarding their activity in a complete physiological setting in vivo [20].

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Conflict of interest None of the authors has conflicts of interest.

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