Organelle movement in melanophores
Effects of *Panax ginseng*, ginsenosides and quercetin

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Cover: Bright field microscopy picture taken by the author, showing dispersed melanophores from Xenopus laevis

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“If there is effort, there is always accomplishment”

Jigoro Kano (1860-1938)
Organelle movement in melanophores

Effects of *Panax ginseng*, ginsenosides and quercetin

**Therese L Eriksson**

**Akademisk avhandling**

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Abstract

Panax ginseng is a traditional herb that has been used for over 2000 years to promote health and longevity. Active components of ginseng include ginsenosides, polysaccharides, flavonoids, polyacetylenes, peptides, vitamins, phenols and enzymes, of which the ginsenosides are considered to be the major bioactive constituents. Although widely used, the exact mechanisms of ginseng and its compounds remain unclear. In this thesis we use melanophores from Xenopus laevis to investigate the effects of Panax ginseng extract G115 and its constituents on organelle transport and signalling. Due to coordinated bidirectional movement of their pigmented granules (melanosomes), in response to defined chemical signals, melanophores are capable of fast colour changes and provide a great model for the study of intracellular transport. The movement is regulated by alterations in cyclic adenosine 3′:5′-monophosphate (cAMP) concentration, where a high or low level induce anterograde (dispersion) or retrograde (aggregation) transport respectively, resulting in a dark or light cell. Here we demonstrate that Panax ginseng and its constituents ginsenoside Rc and Rd and flavonoid quercetin induce a concentration-dependent anterograde transport of melanosomes. The effect of ginseng is shown to be independent of cAMP changes and protein kinase A activation. Upon incubation of melanophores with a combination of Rc or Rd and quercetin, a synergistic increase in anterograde movement was seen, indicating cooperation between the ginsenoside and flavonoid parts of ginseng. Protein kinase C (PKC) inhibitor Myristoylated EGF-R Fragment 651-658 decreased the anterograde movement stimulated by ginseng and ginsenoside Rc and Rd. Moreover, ginseng, but not ginsenosides or quercetin, stimulated an activation of 44/42-mitogen activated protein kinase (MAPK), previously shown to be involved in both aggregation and dispersion of melanosomes. PKC-inhibition did not affect the MAPK-activation, suggesting a role for PKC in the ginseng- and ginsenoside-induced dispersion but not as an upstream activator of MAPK.
Sammanfattning

*Panax ginseng* är ett av de vanligaste naturläkemedlen i världen och används traditionellt för att öka kroppens uthållighet, motståndskraft och styrka. Ginseng är ett komplext ämne bestående av ett antal olika substanser, inklusive ginsenosider, flavonoider, vitaminer och enzymer, av vilka de steroidlika ginsenosiderna anses vara de mest aktiva beståndsdelarna. Flavonoider (som finns i till exempel frukt och grönsaker) och ginseng har genom forskning visat sig motverka bland annat hjärt-och kärlsjukdomar, diabetes, cancer och demens. Trots den omfattande användningen är dock mekanismen för hur ginseng verkar fortfarande oklar. I den här studien har vi använt pigmentinnehållande celler, melanoforer, från afrikansk klogroda för att undersöka effekterna av *Panax ginseng* på pigment-transport och dess maskineri. Melanoforer har förmågan att snabbt ändra färg genom samordnad förflyttning av pigmentkorn fram och tillbaka i cellen, och utgör en utmärkt modell för studier av intracellulär transport. Förflyttningen regleras av förändringar i halten av cykliskt adenosin-monofosfat (cAMP) i cellen, där en hög eller låg koncentration medför spridning av pigment över hela cellen (dispergering) eller en ansamling i mitten (aggregering), vilket resulterar i mörka respektive ljusa celler. Här visar vi att *Panax ginseng*, ginsenosiderna Rc och Rd samt flavonoiden quercetin stimulerar en dispergering av pigmentkornen. När melanoforerna inkuberades med en kombination av ginsenosid Rc eller Rd och quercetin, kunde en synergistisk ökning av dispergeringen ses, vilket tyder på en samverkan mellan ginsenosid- och flavonoiddelarna av ginseng. Ett protein som tidigare visats vara viktigt för pigmenttransporten är mitogen-aktiverat protein kinas (MAPK), och här visar vi att också melanoforer stimulerade med ginseng, men dock inte med ginsenosider eller quercetin, innehåller aktiverat MAPK. Genom att blockera enzymet protein kinas C (PKC) (känd aktivator av dispergering), minskade den ginseng- och ginsenosid-inducerade dispergeringen, medan aktiveringen av MAPK inte påverkades alls. Detta pekar på en roll för PKC i pigment-transporten men inte som en aktivator av MAPK.
List of papers


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenylate cyclase</td>
</tr>
<tr>
<td>A_f</td>
<td>final absorbance</td>
</tr>
<tr>
<td>A_i</td>
<td>initial absorbance</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine 3’:5’-monophosphate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DIC</td>
<td>dynein intermediate chain</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET-3</td>
<td>endothelin-3</td>
</tr>
<tr>
<td>G_i/o</td>
<td>pertussis toxin sensitive inhibitory G-protein</td>
</tr>
<tr>
<td>GIRK</td>
<td>G-protein-coupled inwardly rectifying K^+ channel</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanine nucleotide binding regulatory protein</td>
</tr>
<tr>
<td>G_s</td>
<td>stimulatory G-protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HOGl</td>
<td>high osmolarity glycerol MAPK</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IP_3</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>c-Jun N-terminal kinase/stress-activated protein kinase</td>
</tr>
<tr>
<td>KAP</td>
<td>kinesin-associated protein</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>M-EGF</td>
<td>PKC-inhibitor Myristoylated EGFR-fragment (651-658)</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Enzyme/Chemical Name</td>
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<tr>
<td>--------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>MSH</td>
<td>alpha-melanocyte-stimulating hormone</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PI3-K</td>
<td>phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PPD</td>
<td>protopanaxadiol</td>
</tr>
<tr>
<td>PPT</td>
<td>protopanaxatriol</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
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1. Introduction

1.1 Pigment cells

Pigment cells in the skin are responsible for the diverse and beautiful coloration of animals. The slow long-lasting change of colour, called morphological colour change, in mammals and birds is a result of an altered pigment cell number and/or pigment content in the cells. The pigment (melanin) is synthesized and stored in organelles (melanosomes) within melanocytes and then transferred into surrounding epidermal keratinocytes, feathers or hair (deOliveira et al., 1996; Nery and Castrucci, 1997; Barral and Seabra, 2004). However, the main task of lower vertebrate pigment cells is to bi-directionally transport pigment organelles between the cell centre and periphery, resulting in a rapid colour change. This, so called physiological colour change, is usually a direct response to environmental stimuli, e.g. light intensity, background colour or a changed social context (Nery and Castrucci, 1997; Nascimento et al., 2003). Six types of pigment cells, called chromatophores, are known to be involved in the coloration of poikilothermal vertebrates: black or brown melanophores, yellow xanthophores, red erythrophores, white leucophores, metallic or iridescent iridophores and blue cyanophores (Fujii, 2000; Barral and Seabra, 2004). Pigment is stored in organelles, termed melanosomes, xanthosomes, erythrosomes, leucosomes and cyanosomes respectively, within the chromatophore (Fujii, 2000). In contrast, iridophores are typically non-dentritic and contain stacks of transparent light-reflecting thin crystals of guanine (platelets) instead of coloured organelles (Fujii, 2000).

A change of colour often results from a combined response of multiple pigment cells existing together in well-organized units in the dermis. The most important is the dermal chromatophore unit, which consists of three layers of cells. Xanthophores and erythrophores in the top layer contain carotenoid and pteridine pigments absorbing short wave-light. The second layer consists of reflecting iridophores and the basal
layer of melanophores absorbing light across the whole spectrum (Fujii, 2000; Grether et al., 2004). When the reflecting platelets of the iridophores are dispersed throughout the cytoplasm all light are reflected and the skin appears silver, yellow, orange or red depending on the type and quantity of pigment present in the xanthophores. Stacking of platelets result in a blue or yellow-green-blue colour in the absence or presence of xanthophores respectively (Grether et al., 2004). Aggregation of melanosomes to the cell centre leads to skin lightening while distribution throughout the cytoplasm (dispersion) causes a darkening (Fujii, 2000).

The amphibian dermal melanophores are dispersed when unstimulated and their dendritic processes are arranged radially or basketlike with arms directed upward wrapping around adjacent iridophores. Upon dispersion melanosomes are transported into these processes covering the reflecting iridophores and making the animal appear dark (Fig 1). On the contrary, during lightening of the melanophores, melanosomes move to a position around the nucleus leaving visible xanthophore and iridophore layers (Fig 1) (Bagnara, 1986; Sugden et al., 2004). Melanophores are large in size with a diameter ranging from 10-100 μm and contain several thousands of melanosomes, membrane-bound lysosome-related organelles sharing features with both lysosomes and secretory granules (Schliwa, 1986; Dell'Angelica et al., 2000; Nascimento et al., 2003). Melanosomes are around 0.5 nm in diameter and spherical in
form. The black/brown pigment melanin is a highly polymerized compound synthesized in melanosomes via conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPA to DOPA-quinone, both reactions catalyzed by tyrosinase (monophenol oxygen reductase) (Schliwa, 1986; deOliveira et al., 1996). Besides melanin, melanosomes also contain enzymes, lipids and proteins, of which some are melanosome-specific and some are shared with other organelles (Simon et al., 2008).

Melanophores are derived from a pluripotent neural crest cell population. In the developing embryo, starting between stage 24 and 30, melanoblasts migrate from the dorsal side of the neural tube to their final positions in the integument (Bagnara, 1986; Tomlinson et al., 2009). The fast physiological colour change, due to translocation of pigment organelles, is regulated by various hormones and neurotransmitters (e.g. melatonin, alpha-melanocyte-stimulating hormone (MSH) and catecholamines) acting on transmembrane receptors on the cell surface. Melanosomes are transported bi-directionally along microtubules and actin filaments, attached to specific molecular motors (Nery and Castrucci, 1997).

1.2 Melanosome transport

All eukaryotic cells rely on a functioning intracellular transport for vital cellular processes as secretion, endocytosis, cell division, neuronal signalling and intracellular trafficking (Sugden et al., 2004; Semenova et al., 2009). Molecular motors use the energy of ATP hydrolysis for the movement of organelles, complexes and particles along microtubules and actin filaments. Three classes of motors are involved in the intracellular cargo transport: kinesin and dynein moving toward plus- and minus-ends of microtubules respectively and myosin which move along actin filaments toward their plus-ends. The cargo are transported along both types of cytoskeleton tracks and several types of motors are attached to the cargo simultaneously (Fig. 2) (Karcher et al., 2002; Bruno et al., 2008; Semenova et al., 2009).
1.2.1 Cytoskeleton tracks

Microtubules and actin filaments are polar structures and the orientation of their plus- and minus-ends determines the direction of vesicle movement (DePina and Langford, 1999). *Xenopus laevis* melanophores are a commonly used model system for the study of intracellular transport. Their pigment organelles are large and easily monitored by light microscopy or absorbance measurements. Also, during the bi-directional movement, melanosomes undergo regular pauses and reversals making the transport in frog melanophores similar to organelle movement in other cell types (Nascimento et al., 2003). As in most cells, melanophore microtubules are oriented with the minus ends located at the nucleus and their plus ends toward the periphery near the plasma membrane (Gundersen and Cook, 1999; Nascimento et al., 2003). Microtubules are dynamic polymers that are nucleated at the microtubule organizing centre which, in addition to plus- and minus-end motors, have been shown to be capable of microtubule

![Fig 2. Distribution of microtubules and actin filaments in a Xenopus laevis melanophore. Retrograde transport of melanosomes (aggregation) is driven by the molecular motor dynein along microtubules and anterograde movements (dispersion) by kinesin II and myosin Va along both microtubules and actin filaments. The actin filaments trap melanosomes at the cell periphery, assuring an even distribution of pigment throughout the cell.](image-url)
structure assembly (DePina and Langford, 1999; Nascimento et al., 2003). In melanophores, melanosome aggregation and dispersion are achieved by minus- and plus-end directed movement respectively (Fig. 2) (Gross et al., 2002). In contrast to microtubule organization, actin filaments are randomly oriented in a meshwork close to the plasma membrane. The filaments consist of globular subunits arranged head-to-tail into double helical polymers, giving the structure polarity (Pollard and Borisy, 2003). Actin filaments are required for complete melanosome dispersion but also for maintenance of the dispersed state (Fig 2) (Tuma and Gelfand, 1999).

1.2.2 Kinesin and dynein
The structure of molecular motors consists of two parts: a motor domain that reversibly bind to the cytoskeleton converting chemical energy into motion and the tail which is attached to the cargo. The divergent tail domains allow the motor to bind various types of organelles and particles via interactions with receptor proteins (Karcher et al., 2002). Xenopus melanophore plus-end directed movement has been shown to be driven by kinesin-II, a heterotrimeric kinesin of the kinesin superfamily which is found in many species. It is formed by two homologous motor subunits of 85 and 95 kD and a non-motor subunit of 115 kD, called kinesin-associated protein (KAP), which is thought to mediate cargo binding (Fig 3A) (Tuma et al., 1998; Tuma

![Fig 3. Schematic figures of (A) kinesin II, (B) dynein and (C) the dynactin complex.](image-url)
Cytoplasmic dynein is responsible for the aggregation of pigment organelles in melanophores and consist of two heavy chains containing the motor domains as well as various intermediate, light intermediate and light chains (Fig 3B) (Nilsson and Wallin, 1997; Karcher et al., 2002). Dynein has been shown to interact with many of its cargos, including pigment organelles, via dynactin (Karcher et al., 2002; Deacon et al., 2003). Dynactin is a multisubunit complex formed by at least 10 polypeptides, of which p150\textsuperscript{Glued} and p50 (dynamitin) is the best characterized ones (Fig 3C) (Deacon et al., 2003). Both microtubules and the intermediate chain of dynein (DIC) interact directly with p150\textsuperscript{Glued}, and phosphorylation near the microtubule-binding domain and of DIC have been demonstrated to regulate these interactions (Deacon et al., 2003). In addition Deacon et al (2003) demonstrated that the p150\textsuperscript{Glued} subunit of dynactin interact with the KAP subunit of kinesin II, thus serving as a receptor for kinesin II during dispersion of *Xenopus* melanosomes. The same region of p150\textsuperscript{Glued} was shown to be involved in binding both dynein and kinesin but did not bind DIC and KAP at the same time, indicating coordination of the microtubule retrograde and anterograde movement of organelles (Deacon et al., 2003).

### 1.2.3 Myosin Va

The actin-based motor required for melanosome dispersion is myosin Va, a member of the unconventional class V myosin family which is found in many different organisms (Rogers and Gelfand, 1998; Fukuda et al., 2002). The head domain of the myosin V heavy chain, containing the motor activity, is followed by the neck domain and ends with the divergent tail, capable of binding a diverse range of substrates (Fig 4) (Cheney, 1998; Woolner and Bement, 2009). Myosin Va binds to melanosomes through a complex of melanophilin and Rab27a, a small GTP-binding Ras-like GTPase (Fig 4) (Wu et al., 2002; Nascimento et al., 2003). Rab27a first binds to the melanosome and then recruits melanophilin, who’s N-terminal interact with the GTP-bound Rab27a. Subsequently myosin Va, requiring binding of both Rab27a and...
melanophilin to the melanosome, is able to interact with the C-terminal portion of melanophilin (Fukuda et al., 2002; Wu et al., 2002).

![Diagram](image)

**Fig 4.** Myosin Va binds to melanosomes through a complex of GTP-bound Rab27a and melanophilin. Rab27a binds to the melanosome first and then recruits melanophilin. Myosin Va requires the binding of both Rab27a and melanophilin before being able to interact with melanophilin.

**1.2.4 Coordination**

The interaction of microtubules and actin filaments include the binding of several motors to the same cargo at the same time, and movement of organelles along both types of cytoskeleton tracks. Studies have demonstrated that the microtubule motor activity is coordinated while the microtubule and actin-based transport are competitive (Gross et al., 2002). During aggregation the myosin Va dependent transport is down-regulated and unable to compete with the microtubule motors that are attached to the organelle. Though, melanosomes connected with actin filaments only, can still move and they do so until encountering a microtubule. This results in a transfer from actin to microtubules and the organelle are transported toward the cell centre (Gross et al., 2002). During dispersion, pigment organelles are transported along microtubules and then switched onto actin filaments, resulting in removal of the organelles from microtubule tracks and an even distribution of pigment throughout the cytoplasm.
However, melanosomes are simultaneously attached to both microtubules and actin, leading to a constant competition between the systems. The switch from microtubule to actin-based transport mainly occurs during dynein-driven movements, which ends the minus-directed runs and results in a net anterograde transport (dispersion) (Gross et al., 2002). In contrast, microtubule motors are not competitive but instead coordinated to prevent simultaneous activity. An advantage of using two transport systems is that melanosomes are less likely to become stuck (Gross et al., 2002). Bruno et al (2008) recently showed that the reversions observed during pigment organelle transport are triggered by collisions with obstacles in the cytoplasm, leading to detachment of the organelle from the microtubule, free movement in the cytoplasm and a possibility for motors of opposite polarity to attach to the track. The regulatory mechanisms of this coordinated transport are mainly unknown.

Though, Kashina et al (2004) demonstrated that protein kinase A (PKA) was associated with two different complexes on the melanosomes: a kinesin II- and myosin Va-containing dispersion complex and a dynein-containing aggregation complex. They showed that removal of PKA from the pigment organelles inhibited aggregation but not dispersion, and that removal of the PKA regulatory subunit (RIIα) caused disruption of the dynein-containing complex. RIIα was also shown to bind to the melanosomes through A-Kinase anchoring proteins (AKAPs), which scaffold PKA and bring it close to its substrates. Recently Semenova et al (2009) indentified moesin as the AKAP in the dispersion-complex. Kashina et al (2004) suggested a model for the arrangement of the motor and signalling complexes as follows: Dynein or kinesin II associates with the organelle via p150Glued which in turn are bound to the melanosome through the dynactin complex and through binding to the RIIα-AKAP complex. Upon appropriate signals the interaction of RIIα with AKAP and of p150Glued with dynactin is inhibited, leading to detachment of p150Glued with bound dynein from the melanosomes. Kinesin II would still be bound to the organelle through the interaction with myosin Va, which is attached to the melanosome via melanophilin and Rab27a (see Kashina et al (2004) for illustration).
In conclusion, aggregation of melanosomes require transport by cytoplasmic dynein along microtubules and during dispersion the microtubule motor kinesin II are responsible for long-range outward movement and the actin dependent myosin Va for the shorter random transport and trapping of organelles at the cell periphery (Tuma and Gelfand, 1999).

1.3 Intracellular signalling

The bidirectional transport of pigment organelles in melanophores is a well regulated process. Hence, these cells have been extensively used for the study of organelle transport regulation and signalling. The movement of melanosomes are regulated by the intracellular concentration of cyclic adenosine 3’:5’-monophosphate (cAMP) in the melanophore, where increased levels induce dispersion and low levels trigger aggregation (Fig 5) (Andersson et al., 1984; Nery and Castrucci, 1997).

1.3.1 Aggregation
Aggregation is stimulated by the binding of the pineal gland hormone melatonin to its G_{i/o}-protein coupled membrane receptor, resulting in activation of the α-subunit (White et al., 1987; Nery and Castrucci, 1997). In turn, G_{i/o} inhibits adenylate cyclase (AC), leading to decreased levels of cAMP and protein kinase A (PKA) inactivation.

**Fig 5.** cAMP increase and decrease induce anterograde (dispersion) and retrograde (aggregation) transport of pigment organelles, resulting in dark and light cells respectively
Subsequent activation of protein phosphatase 2A (PP2A) results in dephosphorylation of still unknown proteins required for dynein-mediated organelle transport toward the cell centre (Fig 6) (Reilein et al., 1998). However a 53kD protein have been demonstrated to be phosphorylated during dispersion (de Graan et al., 1985). Consequently dephosphorylation of the same protein could be involved in aggregation. The melatonin receptor is a high-affinity G-protein-coupled receptor (GPCR), called Mel1c, which (as all GPCRs) consists of seven hydrophobic α-helical transmembrane domains, one N-terminal extracellular domain, three extracellular loops, three intracellular loops and one C-terminal intracellular domain. Three different subtypes of the melatonin receptor have been cloned: Mel1c which is expressed in amphibians and birds only, while Mel1a and Mel1b are found in many vertebrates including chicken, frog, fish and mammals (Ebisawa et al., 1994; Reppert et al., 1996; Sugden et al., 1997; Chan et al., 2002).

1.3.2 Dispersion

Alpha-melanocyte-stimulating hormone (MSH), a tridecapeptide produced in the pituitary gland, brain or skin, has been shown to be involved in pigment cell regulation in all vertebrates studied so far (deOliveira et al., 1996). Dispersion is triggered by a rise in cAMP, caused by activation of AC via MSH stimulation of its G_s-protein coupled receptor. The following activation of PKA in turn induces phosphorylations of unknown proteins, regulating motor activities and allowing organelle transport by kinesin II and myosin Va to the cell periphery (Fig 6) (Nery and Castrucci, 1997). Dispersion has also been demonstrated to be stimulated by G_q activation of phospholipase C (PLC), which activate protein kinase C (PKC) via stimulation of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Phosphorylations by PKC induce microtubule and actin filament transport (Fig 6) (Graminski et al., 1993; Nery and Castrucci, 1997; Reilein et al., 1998). Binding of endothelin-3 (ET-3) to the endogenous G-protein-coupled ETc receptor, later coupled to the PLC-pathway, has been shown to induce dispersion (Karne et al., 1993; McClintock et al., 1996). Also, anterograde transport via PLC and DAG has been demonstrated upon stimulation of murine bombesine and substance P receptors in melanophores (Graminski et al.,
1993). The activation of the bombesin receptor was not followed by an intracellular cAMP increase, showing that dispersion can be induced without cAMP participation (Graminski et al., 1993). Reilein et al (1998) demonstrated that basal PKA activity is needed for the dispersed state of resting melanophores and that PKC could partially disperse melanosomes. However, in the presence of basic PKA activity, PKC could induce full dispersion. Although mammals do not display any physiological colour change, the same signalling pathways are used for morphological changes, since melanin-synthesis in melanocytes are dependent on both PKA and PKC phosphorylations (Nery and Castrucci, 1997).

1.3.3 Phosphorylation and MAPK signalling
Phosphorylations have also been shown to be involved in Xenopus melanophore aggregation. Karlsson et al (2000) demonstrated that a 280kD protein was tyrosine phosphorylated during melatonin-induced aggregation. Both melanosome aggregation

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**Fig 6.** Signalling pathways for aggregation and dispersion in Xenopus melanophores.
and phosphorylation of the 280kD protein was decreased upon treatment of the melanophores with the tyrosine kinase inhibitor genistein (Karlsson et al., 2000). In addition, melatonin-treated melanophores showed an increased transient activation of extracellular signal-regulated kinase (ERK 1/2), a member of the mitogen activated protein kinase (MAPK) family (Andersson et al., 2003). ERK has also been shown to be activated in melanophores by both melatonin- and MSH-stimulation and this activation was accompanied by an increase in the number of moving melanosomes (Deacon et al., 2005). The myosin Va-dependent dispersion was not affected upon treatment of the melanophores with a MEK-inhibitor (an upstream kinase of ERK) (Deacon et al., 2005).

MAP kinases are an evolutionarily conserved family of serine/threonine kinases that are involved in the transduction of signals regulating cell-differentiation, -growth, -division and apoptosis (Luttrell and Luttrell, 2003). Three major classes have been identified in mammals: the ERK, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38/high osmolarity glycerol (HOG1) MAP kinases. ERK activity is stimulated by various signalling pathways including receptor tyrosine kinases, proto-oncogenes and GPCRs, whereas JNK/SAPK and p38/HOG1 are activated by environmental and hormonal stress. GPCR activation of MAPKs can be initiated by PKA, PKC and intracellular calcium, cross-talking between GPCRs and

![MAPK cascades](image)

**Fig 7.** MAPK cascades.
receptor tyrosine kinases or by direct interaction with β-arrestins and elements from the MAP kinase cascade. Regulation of MAP kinases is exerted through a series of parallel phosphorylation cascades, where each cascade consists of three kinases that phosphorylate the subsequent downstream component (Fig 7). The first component of the ERK pathway, Raf (a MAP kinase kinase kinase), phosphorylates the dual function threonine/tyrosine kinases MEK 1 and MEK 2 (MAP kinase kinases), which in turn activate ERK 1 and 2 (MAPK 44/42). The constituents of the other two MAP kinase pathways are not characterized but the cascades are similar to ERK with activation of a MAP kinase kinase kinase, followed by phosphorylation of MAP kinase kinase and JNK/SAPK or p38/HOG1. Upon activation the three MAP kinases phosphorylates a range of different membrane-, cytoplasmic-, nuclear- and cytoskeletal substrates (Houslay and Kolch, 2000; Luttrell and Luttrell, 2003).

1.4 Melanophores as model

A functioning intracellular transport is important for all eukaryotic cells. Since the start of using of pigment cells for the study of organelle transport in the 60’s, extensive research has been done investigating intracellular movement and signalling as well as motor protein function and regulation (Tuma and Gelfand, 1999). *Xenopus* melanophores provide an excellent model as: 1) They are specialized in rapid bi-directional movements of pigment granules between cell centre and periphery, in response to defined signals. 2) The intracellular transport of melanosomes is reversible and also includes regular pauses and reversals resembling organelle transport in other cell types. 3) Melanosome movement is clearly visible by bright field microscopy and can easily be monitored and measured. 4) Transfection with exogenous DNA, expressing e.g. known or orphan GPCRs, tyrosine-kinase-coupled receptors and cytokine receptors allow easy characterization of receptors, ligands and their signalling pathways. 5) The heavy density of melanin and a constant large number of cells make it possible to isolate pure fractions of melanosomes for the study of organelle motility in vitro. (Tuma and Gelfand, 1999; Nascimento et al., 2003; Sugden et al., 2004;
Aspengren et al., 2009). Although, in spite of all research, there is still many questions regarding the mechanisms that regulate microtubule motors and the coordination of the plus- and minus-end directed microtubule transport.

In the past melanophores has been used as biosensors for the detection of catecholamines in blood plasma (Elwing et al., 1990), pertussis toxin in saliva samples (Karlsson et al., 1991) and chemical contaminants in water (Iuga et al., 2009). They have been used for studies of ligand actions on endogenous receptors (Potenza and Lerner, 1992) and for the expression and investigation of many different exogenous GPCRs that act via AC or PLC/PKC signalling pathways. Melanophores possess seven endogenous GPCRs and more than 100 GPCRs have been expressed and functionally characterized using the melanophore assay (Jayawickreme and Kost, 1997; Gatlin et al., 2001), including human β₂ adrenergic receptors (Potenza et al., 1992), human D₂ and D₃ dopamine Gₛ-coupled receptors (McClintock et al., 1993; Lerner, 1994; Potenza et al., 1994), murine substance P and bombesin receptors (Graminski et al., 1993; McClintock et al., 1993), human opioid receptor 3 (Karlsson et al., 2002) and pituitary adenylate cyclase (PAC1) receptors (Pereira et al., 2002). Melanophores cultured in a 96-well microtiter plate format allow easy and rapid evaluation of agonist/antagonist-receptor interactions, as changes in melanosome distribution can be seen within 5-30 minutes and detected by absorbance measurements, melanosome tracking or bright field microscopy (Potenza et al., 1992; Jayawickreme and Kost, 1997).

1.5 Panax ginseng

The genus name Panax (Araliaceae family) was given by the Russian botanist C.A. Meyer in 1842 and is derived from the Greek words “pan” (all) and ”akos” (cure/healing), based on the use of the plant in China as a panacea (cure for all illnesses) (Chang et al., 2003; Radad et al., 2006; Choi, 2008). Ginseng refers to the Chinese word “rensheng” meaning human, since the root of ginseng resembles the
human body (Radad et al., 2006). 11 species have been identified, of which *Panax ginseng* (Asian ginseng) from China and Korea is the most widely used (Gillis, 1997; Chang et al., 2003). Other species include *P. quinquefolius* (American ginseng) grown in Canada and United States, and less frequently encountered *P. japonicus* (Japan) and *P. notoginseng* from China (Gillis, 1997; Radad et al., 2006).

Ginseng is a perennial self-pollinating plant with a white fleshy root (Choi, 2008). As the wild plant is nearly extinct, ginseng is now extensively cultivated in China and Korea (Chang et al., 2003). The roots are harvested between 4-6 years of age and prepared in three different ways. Fresh ginseng is from plants less than 4 years old and can be consumed fresh. White ginseng are derived from air-dried roots of 4-6 year old plants and red ginseng from steamed and dried plants of at least 6 years of age (Chang et al., 2003; Zhou et al., 2004).

The Chinese nature of ginseng first appeared in the Jia-Gu-Wen (Oracle bone script) between 1600-1100 BC and more detailed descriptions of its efficacy and medical applications can be found in a herbal compendium, “Shen-Nong-Ben-Cao-Jing”, published around 200 A.D. (Chang et al., 2003; Chen et al., 2008). In Asia, ginseng is traditionally used as an adaptogen, an agent that increases the body’s resistance to physical, chemical and biological stress. It is believed to enhance physical capacity, prevent aging, promote health, restore homeostasis and increase alertness and resistance to disease (Gillis, 1997; Kiefer and Pantuso, 2003; Zhou et al., 2004; Radad et al., 2006; Choi, 2008). Since the 1970s, modern science and technology have made progress in determining the pharmacological properties of ginseng. Although, the exact mechanisms behind the efficacy are still unknown. However, effects of ginseng have been demonstrated in the central nervous, cardiovascular and immune systems, on neuroendocrine function, carbohydrate and lipid metabolism, to enhance liver function, improve sexual function and relieve pain. In addition, ginseng have been ascribed anti-oxidant, anti-diabetic and anti-cancer actions (Friedl et al., 2001; Chang et al., 2002; Nam et al., 2005; Xiang et al., 2008).
More specific, research of the ginseng actions on psychological function showed an improved cognitive performance, including better reaction, attention and abstract thinking (Kiefer and Pantuso, 2003). Effects on the immune system included higher natural killer cell activity, enhanced phagocytosis, chemotaxis and an increased lymphocyte count (Kiefer and Pantuso, 2003). The ability of ginseng to promote NO release has been suggested to underlie its vasodilation and anti-oxidant properties (Gillis, 1997). Ginseng also has various other effects on the cardiovascular system, such as inhibition of angiotensin-converting enzyme (ACE) activity, reduction of catecholamine concentration in circulation (associated with stress), increased levels of HDL, and a decreased lipid peroxidation and platelet adhesiveness (Zhou et al., 2004; Persson et al., 2006a). Several studies have shown a wide range of ginseng actions in the CNS, where increased cell survival, extension of neurite growth, rescuing of neurons from death and protection against neurotoxic effects are included (Radad et al., 2006). Moreover, ginseng are able to modulate neurotransmission, e.g. modifying the release and re-uptake of acetylcholine and increasing dopamine and norepinephrine concentration, possibly explaining the positive effects of ginseng on memory and cognitive performance (Radad et al., 2006). One study showed that the lipophilic fraction of Panax ginseng induced neuron differentiation, neurite growth and promoted neuron survival via PKC-dependent signalling pathways (Mizumaki et al., 2002). Kim et al (2009) demonstrated that ginseng, in combination with differentiation-inducing agents, synergistically stimulated differentiation of HL-60 leukemia cells into mono- or granulocytes via PKC- and ERK-mediated signalling.

About 200 substances have been isolated and characterized from Panax ginseng. The active constituents include ginsenosides, polysaccharides, flavonoids, polyacetylenes, peptides, amino acids, fatty acids and vitamins, of which the ginsenosides are considered to be the main bioactive components (Attele et al., 1999; Chang et al., 2002; Chang et al., 2003; Zhou et al., 2004; Qian et al., 2009).
1.5.1 Ginsenosides

Ginsenosides are triterpene saponins, unique to the Panax species, that consist of a four-ring steroid-like skeleton with sugar-moieties attached (Fig 8) (Attele et al., 1999; Radad et al., 2006). More than 80 ginsenosides have been identified in the Panax taxa and 38 can be found in Panax ginseng (Chen et al., 2008; Choi, 2008). Their different biological activities depend on the type, position and number of sugar moieties attached and based on this chemical structure they are classified into 4 categories: protopanaxadiols (PPD; e.g. Rb1, Rb2; Rc; Rd), protopanaxatriols (PPT; e.g. Re, Rf, Rg1), oleanolic acid (non-steroidal, e.g. Ro) and ocotillol (e.g. majonoside R2) types (Zhu et al., 2004; Radad et al., 2006; Chen et al., 2008). The content of ginsenosides in a ginseng-preparation may vary depending on the species, age and part of the plant.

![Molecular structures of the ginsenosides investigated. PPD, protopanaxadiol; PPT, protopanaxatriol; Glc, glucopyranoside; Ara(p), arabinopyranoside; Ara(f), arabinofuranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucoside ring that links the two carbohydrates.](image-url)
extracted, as well as the season of harvest, extraction method and sample preparation (Gillis, 1997; Kennedy and Scholey, 2003). Conflicting results in the literature concerning the mechanisms of ginseng may to a certain extent be due to differences in the ginsenoside content of ginseng samples (Gillis, 1997). Hence, use of a standardized extract or individual ginsenosides is important to achieve replicable scientific research. The most commonly used ginseng extract is G115©, which is standardized to contain 4% ginsenosides (Soldati and Sticher, 1980; Gillis, 1997).

The skeleton of steroid hormones allows them to intercalate into the hydrophobic bilayer of plasma membranes. Since the ginsenoside structure are similar to steroids, ginsenosides also have the ability to interact with plasma membranes, resulting in altered membrane fluidity and function and a cellular response (Attele et al., 1999). With these properties, ginsenosides may alter membrane dynamics and modify the activity of receptors, ion channels and enzymes (Attele et al., 1999). Ginsenoside Rf have e.g. been shown to alter the activity of membrane-bound G-proteins (Attele et al., 1999) and ginsenoside Rg3 to decrease the membrane fluidity and consequently block drug efflux (Kwon et al., 2008). In addition, enzymes that are sensitive to alterations in membrane fluidity and function, such as PKC, could be modulated by ginsenosides through a change of membrane properties (Attele et al., 1999).

Ginsenoside effects on cAMP, PLC/PKC and MAPK signalling pathways have been shown by a number of studies. E.g. Choi et al and Jeong et al (2001; 2003a; 2004) demonstrated that a mixture of ginsenosides enhanced the Ca^{2+}-activated Cl^{-} current and inhibited the G-protein-coupled inwardly rectifying K^{+} (GIRK) channel activity in Xenopus oocytes via activation of G_{a_{q/11}}, PLC and PKC. Also, the PPD type ginsenoside Rh2 stimulated differentiation of human leukemia (HL-60) cells into granulocytes, which was shown to be accompanied by an increase in PKC-activity (Kim et al., 1998). ERK1/2 and MEK was demonstrated to be suppressed and inactivated by ginsenosides in 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated mouse skin and irradiation-treated rat epithelial cells respectively (Surh et al., 2002; Tamura et al., 2008). Also, upregulation of ERK1/2 in acute-renal-failure rats has been
shown to be enhanced by ginsenoside pre-treatment, and ginsenoside Rg1 to induce an increase in ERK1/2 phosphorylation in PC12 cells and synaptosomes (Hu et al., 2008; Zhang et al., 2009). Xue et al (2006) showed that neurotransmitter release stimulated by ginsenoside Rb1 was transducted via PKA activation. Moreover, ginsenoside Rg1 in combination with a cAMP analog, synergistically induced an enhancement of glucocorticoid receptor-mediated transcription in rat hepatoma cells (Chung et al., 1998).

1.5.2 Flavonoids and other constituents

Besides ginsenosides, *Panax ginseng* also contains polysaccharides, flavonoids, polyacetylenes, peptides, amino acids, fatty acids and vitamins. Flavonoids are the most common group of polyphenolic compounds in the human diet and are present in all photosynthesising cells (Boots et al., 2008; Murakami et al., 2008; Spencer, 2009). More than 5000 different flavonoids have been identified and they can be found in fruits, vegetables, nuts, cereals and plant-derived beverages such as tea and wine (Boots et al., 2008; Spencer, 2009). Flavonoids consist of a three-ring structure with two aromatic (A and B, Fig 9) and one heterocyclic ring (C, Fig 9). It is the variations in the chemistry of the heterocyclic ring and the patterns of hydroxylation and their substitutions that divide the flavonoids into various subclasses including: flavones – found in parsley and celery, isoflavones – found in soy products, flavonols – found in onions, leeks and broccoli, flavanols – found in green tea, red wine and chocolate, flavanones – found in citrus fruit and tomatoes, anthocyanidins – found in red wine and chalcones – found in rooibos (Bramati et al., 2002; Boots et al., 2008; Murakami et al., 2008; Spencer, 2009). Beneficial effects of flavonoids have been proposed in cardiovascular disease, neurodegenerative disorders and cancer (Spencer et al., 2004; Williams et al., 2004; Persson et al., 2006b). Their cellular effects are possibly mediated by the flavonoid anti-oxidant activity, such as scavenging of reactive species and influence on the intracellular redox state, and by modulation of intracellular signalling (Spencer et al., 2004; Spencer, 2009). Flavonoids are suggested to interact with specific proteins, receptors and kinases (e.g. phosphoinositide 3-kinase (PI3-K), Akt/protein kinase B (PKB), tyrosine kinases, PKC, MAPK pathways, phosphatases
and transcription factors) in several protein kinase and lipid kinase signalling pathways. This would result in an altered phosphorylation pattern and an effect on cellular function (Spencer et al., 2004; Williams et al., 2004; Spencer, 2009). Flavonoid effects in the brain, such as improvement in cognition and learning, could be due to protection of neurons via interactions with neuronal signalling pathways as well as to stimulation of neurogenesis, neuronal function and regeneration (Spencer, 2009). Their ability to bind to the ATP-binding pockets of many different proteins, e.g. PKA and PKC, could explain the numerous proteins reported to be potential targets of flavonoid actions (Williams et al., 2004).

One of the few specific flavonoids reported as a constituent of *Panax ginseng* is quercetin, the main flavonol present in our diet (Fig 9) (Ali et al., 2007; Boots et al., 2008). Quercetin, found in e.g. apples, onions and green tea, have demonstrated antioxidant, anti-proliferative, anti-inflammatory, anti-fibrotic, anti-coagulative, antibacterial and anti-hypertensive properties (Williams et al., 2004; Boots et al., 2008; Murakami et al., 2008; Persson et al., 2008). Moreover, quercetin has been shown to suppress JNK and ERK activation and to induce cell death in cancer cell lines (Williams et al., 2004; Boots et al., 2008; Murakami et al., 2008). Also, the flavanol

![Molecular structure of the flavonol quercetin.](image)
has been reported to affect hormone receptor function and signal transduction receptors such as the epidermal growth factor receptor (EGFR) (Boots et al., 2008).

Polyacetylenes have shown diverse actions such as anti-cancer and anti-oxidative effects, to inhibit platelet function, promote neurite outgrowth and improve memory deficit (Choi, 2008; Choi et al., 2008). The polyacetylenic compound (9R, 10S)-epoxyheptadecan-4,6-diyn-3-one (EHD) isolated from Panax ginseng was able to inhibit the Na⁺ current in rat neurons, which could be associated with the pain-relieving effects of ginseng (Choi et al., 2008). Panaxynol, isolated from Panax notoginseng, has been demonstrated to both inhibit MAPK in rat aortic vascular smooth muscle cells and activate cAMP-dependent protein kinase and MAPK in rat PC12 cells (Wang et al., 2006; Jiang et al., 2008). Two other polyacetylenes from ginseng, panaxydol and panaxytriol, was shown to affect PI3-kinase and PKC in rat and human cells (Halim et al., 2008; Hai et al., 2009a; Hai et al., 2009b).

A polysaccharide fraction isolated from Panax ginseng showed anti-septicaemic activity by stimulation of macrophages and to increase the NO production and inducible nitric oxide synthase (iNOS) synthesis in macrophages (Friedl et al., 2001; Lim et al., 2002). Ginsan, a polysaccharide extracted from Panax ginseng, have been ascribed radioprotective effects and shown to activate multiple immunomodulatory effectors as well as to reduce inflammatory responses via expression of phospho-JNK1/2, phospho-p38MAPK and NF-κB (Lee et al., 1997; Ahn et al., 2006; Ivanova et al., 2006).
2. Aims

The aims of the present study were:

♦ To study the effects of *Panax ginseng* on pigment organelle transport using *Xenopus laevis* melanophores as a model system.

♦ To study the influence of *Panax ginseng* on PKA, PKC and MAPK signalling pathways, which regulate pigment organelle movement in melanophores.

♦ To investigate potential effects of different constituents of *Panax ginseng* on pigment organelle transport and signalling.
3. Methods

3.1 Cell culture

Previously, melanophores from *Xenopus laevis* (African clawed frog) were collected from 20 *Xenopus* tadpoles between stages 30-35, rinsed, tritured and the resulting suspension of cells plated onto culture dishes and incubated at 27°C. The medium was changed twice a week and after 1-2 months the large colonies of melanophores visible in the primary cultures was isolated by percoll density-gradient centrifugation (Daniolos et al., 1990). The propagation of the fibroblast and melanophore cell lines was modified from protocols described by Daniolos et al and McClintock and Lerner (1990; 1997). Media from the fibroblasts, containing unknown growth factors needed for melanophores to proliferate, were collected through a 0.22 μm sterile filter twice a week and added to melanophores. Once a week fibroblast were split by trypsination, followed by removal of the resulting suspension and a phosphate buffered saline (PBS) rinse, before adding new conditioned frog-media to the flasks. Melanophores were split once a week at a one-fifth to one-sixth ratio. Both cell lines were incubated at 27°C in flasks with closed lids. For long-time storage cells were kept at 20°C to minimize cell division. Melanophores were cultured at 27°C for 2 weeks before used in new experiments. *Xenopus laevis* fibroblasts and melanophores were generous gifts from Michael R. Lerner, Arena Pharmaceuticals Inc (San Diego, CA, USA).

3.2 Chemicals

Cell culture reagents were from Invitrogen (Carlsbad, CA, USA). Melatonin, α-melanocyte stimulating hormone (MSH), endothelin-3 (ET3), H-89, saponin, dimethyl sulfoxide (DMSO) and quercetin and were from Sigma-Aldrich (St. Louis, MO, USA). PKC inhibitor Myristoylated EGF-R Fragment (651-658) (M-EGF), latrunculin A and
PD98059 were purchased from Calbiochem (La Jolla, CA, USA) and ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 from Extra Synthese (Genay, France). Inderal® (propranolol hydrochloride) was from AstraZeneca (Södertälje, Sweden) and *Panax ginseng* extract G115® (ginseng) a kind gift from Pharmaton S.A., Switzerland. Rabbit phospho-p44/42 MAPK (ERK1/2) and rabbit p44/42 MAPK were obtained from Cell Signaling Technology (Beverly, MA, USA). Secondary horseradish peroxidase conjugated donkey anti-rabbit antibody was from Amersham Pharmacia Biotech (Uppsala, Sweden) (paper I) and Cell Signaling Technology (paper II). Melatonin and ET3 were prepared from a stock solution in ethanol. MSH, H-89, latrunculin A, quercetin and ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, and Rg1 were prepared from stock solutions in DMSO and ginseng extract G115, M-EGF and saponin from stock solutions in phosphate buffered saline (PBS). *Panax ginseng* stock solution was prepared by dissolving 0.5 g G115 extract in 5 ml of PBS, considered as 100 mg/ml. The solution was incubated in a water bath at 60°C for 60 minutes, passed through a 0.22 μm sterile filter and stored at 4°C for a maximum of 2 weeks. Stock solutions of all drugs, except quercetin and H89 which were kept at 4 ºC, were stored in aliquots at −20°C.

### 3.3 Microtiter plate assays

Microtiter plate assays were modified from previously described protocols (Potenza et al., 1992). *Xenopus* melanophores were seeded at a concentration of 20 000–40 000 cells per well in flat-bottomed 96-well microtiter plates and cultured in 27°C for 2–3 days to reach confluence, before changing to serum-free 70% Leibovitz L15 media 4–24 hours prior to experiments. For dispersion assays melanophores were treated with 1 nM melatonin for 30 minutes in darkness to induce melanosome aggregation, before addition of drugs. Absorbance was measured at 650 nM using a microplate reader, before (Ai) and after (Af) stimulation with drugs. Final absorbance (Af) was measured every 5 minutes for 1 hour using kinetic mode. Dispersion was calculated as $1 - 10^{\text{Ai-Af}}$ (Potenza and Lerner, 1992). Negative control cells were incubated with L15 medium.
or DMSO. Individual experiments were performed in duplicate, repeated three or four times and normalized to positive control cells.

### 3.4 Western blot

Confluent melanophores growing in 6-well plates were incubated 4-24 hours with 70% L15 medium in 27°C before addition of drugs. Control cells were incubated with L15 medium or DMSO. The stimulation was stopped by removal of the reagent medium and addition of ice-cold 70% PBS. Melanophores were rinsed 3 times in PBS, lysed in ice-cold lysis-buffer and scraped off the wells using a cell scraper. Cell-suspensions were kept for 30 minutes at 4°C on an orbital shaker and centrifuged at 10 000xg at 4°C for 10 minutes. The protein content of the supernatant was determined using a BCA Protein Assay Kit. 40 μg protein per sample was heated at 95°C for 5 minutes followed by separation on SDS-poly-acrylamide gels. Proteins were transferred over night to polyvinylidene diflouride (PVDF) membranes using a Mini Trans-Blot Electrophoretic Transfer Cell. The membranes were blocked for 60 minutes in room temperature with 5% (w/v) dry milk in 0.1% (v/v) Tween20 in PBS to minimize unspecific binding. First and second antibodies were incubated with the membranes for one hour each. Between incubations membranes were rinsed 3x5 minutes in 0.1% Tween20 in PBS. Analysis was done using ECL western blotting detection reagents in an imaging detection device coupled to a CCD-camera. To measure specific signals, individual background to each band was subtracted.

### 3.5 cAMP immunoassay

Cyclic AMP was measured using a EIA direct cAMP enzyme immunoassay kit (Assay Design Inc, Ann Arbor, MI, USA). *Xenopus* melanophores were grown to confluence in 6-well plates and incubated 4-24 hours with 70% L15 medium in 27°C before addition of drugs. Positive control cells were stimulated with MSH and negative
control cells with L15 medium only. Stimulations were stopped by washing the cells with cold PBS followed by addition of ice-cold 95% ethanol. Plates were stored in -70°C until used. After thawing, cells were lysed by HCl treatment, passing through a 200 μl pipette and a syringe with a 27 ¾ needle. Cell lysates were centrifuged for 10 minutes at room temperature and protein content in the supernatants determined by a BCA Protein Assay Kit. For the measurements of cAMP, 50 μg of protein was used for sample analysis, performed according to the kit instructions. In short, a polyclonal antibody to cAMP was used to bind cAMP in the standard or sample in a competitive way. Excess reagents were removed and substrate added. After 1 hour of incubation the enzyme reaction was stopped and the resulting yellow colour measured on a microplate reader at 405 nM. The concentration of cAMP was calculated from the measured optical density.

3.6 Statistics

All results are expressed as means ± standard error of the mean (SEM). For cAMP assays in paper I, data were normalized to the cAMP concentration in the presence of MSH, expressed as 100%. For western blot results in paper I and II, protein bands were quantified in arbitrary units and data normalized to positive control, set to 100%. When multiple groups were compared, statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test. In paper II the EC50 values for the ginseng-induced dispersion and concentration-dependent phosphorylation of MAPK was calculated using nonlinear regression. Data were analyzed using GraphPad Prism™ (GraphPad Software, San Diego, CA, USA).
4. Results and discussion

*Panax ginseng* has been among the most popular botanic products in the world for a long time. Originally the character and application of ginseng was described by Oriental medical science theory. Since the 1970s modern scientific technology has made progress in determining the pharmacological properties of ginseng. However, research is only in its initial stages and despite being used for centuries, the mechanisms of ginseng and especially its non-ginsenoside constituents are mainly unknown. The actions of ginseng include effects on central nervous system, neuroprotective effects, anticancer, anti-aging and immunomodulation effects (Nicholson et al., 1991; Kennedy and Scholey, 2003; Choi, 2008; Xiang et al., 2008). In this study we examine the effects of *Panax ginseng*, ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1) and flavonoid quercetin on pigment organelle transport as well as on PKA, PKC and MAPK signalling pathways.

### 4.1 Paper I – Pigment organelle transport induced by *Panax ginseng*

Due to their fast change in colour (movement of pigment granules) upon intracellular cAMP changes, melanophores from the African clawed frog *Xenopus laevis* are exceptional as a cell-based system for the study of substances acting on G-protein and protein kinase signalling pathways. In paper I we showed that *Panax ginseng* (ginseng) induced a concentration-dependent anterograde transport (dispersion) of melanosomes (paper I, Fig 1). MSH is known to induce dispersion via AC, a rise in cAMP and activation of PKA (Abe et al., 1969; Cozzi and Rollag, 1992; Tuma et al., 1998; Gross et al., 2002; Skold et al., 2002). In melanophores treated with ginseng there was no rise in cAMP level and no difference in dispersion could be seen upon pre-incubation with the PKA-inhibitor H-89 (paper I, Fig 2-3), indicating that the common pathway for anterograde transport was not activated. The concentration of H-
89 used here is in the higher range used for inhibition of PKA in melanophores (40-150 μM) (McClintock et al., 1996; Nilsson et al., 2001; Teh and Sugden, 2001; Deacon et al., 2005). Still, H-89 only slightly decreased the MSH-induced dispersion (27% decrease at 30 minutes), making the interpretation of the ginseng results difficult. Also dispersion induced by ET3, known to signal via PLC/PKC without elevating cAMP (Graminski et al., 1993; Karne et al., 1993; McClintock et al., 1996; Reilein et al., 1998), was attenuated by H-89 showing possible unspecific binding. Lack of specificity for H89 has been shown before. McClintock et al (1996) demonstrated that H89 blocked the responses of substance P and bombesin (known to specifically couple via PLC) in melanophores, and suggested that since H89 is an ATP-competitive inhibitor, it is plausible that H89 also bind to the ATP-binding site of PKC. Moreover, H89 has been shown to inhibit PKC (Ki=31.7 μM) in an in vitro assay (Chijiwa et al., 1990). On the other hand, Reilein et al (1998) suggested that a

![Fig 10. Inhibition of MSH-induced dispersion by PKA-inhibitor H89 (A) and H89 in combination with PKC-inhibitor M-EGF (B). Melanophores were pre-aggregated with melatonin before incubation with H89 at 80 μM (A), M-EGF at 5 μM (B) or the combination of the two inhibitors (B) for 30 minutes, followed by stimulation with MSH at indicated concentrations for 30 minutes (A) or at 100 nM for indicated time in B. Results are presented as ± SEM, n=2.](image)
basal PKA-activity is needed to achieve full dispersion via activation of the PLC/PKC pathway. In that case, inhibition of PKA by H89 would result in a decrease of the ET-3-induced dispersion.

In a preliminary experiment, melanophores were pre-incubated with H-89 followed by stimulation with MSH from $1 \times 10^{-12}$ to $1 \times 10^{-6}$ M, and at a concentration of $1 \times 10^{-10}$ maximum dispersion and in the same time close to full inhibition (88%) by H-89 was demonstrated (Fig 10A, n=2). At concentrations of $1 \times 10^{-9}$ and higher only 20% of the dispersion was inhibited, suggesting that MSH at higher levels activates a second pathway for dispersion (Fig 10A). Experiments with melanophores treated with the PKC-inhibitor M-EGF, showed a 46% inhibition of the ginseng-induced dispersion at 50 μM of the inhibitor, compared to 100 and 89% inhibition of the MSH- and ET-3 stimulated cells respectively, at 20 μM of M-EGF (paper I, Fig 4). This indicates a possible role for PKC in the ginseng and MSH-induced anterograde transport. Next, we treated melanophores with a combination of the PKA- and PKC-inhibitors H-89 and M-EGF before MSH stimulation and in correspondence with previous results, the combination showed a synergistic inhibition of the MSH-induced dispersion (Fig 10B, n=2). Incubation with H-89 (Δ) and M-EGF (○) resulted in 12 and 28% inhibition of the MSH-induced dispersion at 30 minutes respectively, compared to an 88% decrease.

Fig 11. PKC-inhibitor M-EGF did not affect the melanosome transport in itself. Melanophores were pre-incubated with melatonin before stimulation with MSH, L15 or M-EGF for 60 minutes. MSH, 100 nM for 60 minutes; L15, medium only for 60 minutes; M-EGF, PKC-inhibitor Myristoylated EGF-R Fragment (651-658) at 20 μM for 60 minutes; ns, not significant p> 0.05. n=8.
in dispersion when the two inhibitors were combined (♦) (Fig 10B). Consequently ginseng may induce dispersion via both a direct activation of PKA (without a cAMP increase) and stimulation of the PKC-pathway.

However the fact that 20 μM of the PKC-inhibitor blocked 90-100% of the ET-3 and MSH effect and nothing of the ginseng-induced dispersion, strongly suggest the participation of other signalling components in the actions of ginseng. It is also possible that ginseng exert its effect/s downstream PKC, affecting still unknown proteins. M-EGF was shown not to affect the pigment organelle anterograde transport in itself (Fig 11). Though, there was a slight tendency toward aggregation upon incubation with M-EGF (Fig 11). A possible explanation could be that by inhibiting the PKC-dispersing pathway, aggregation would eventually be favoured (PKC is needed to maintain dispersion). The same thing can be seen by PKA-inhibition. Sugden and Rowe (1992) showed that direct inhibition of PKA resulted in a slow aggregation of melanosomes. M-EGF is an active-site directed PKC-inhibitor (α, β and γ isoforms) that generates a covalent linkage, disulfide bond, between itself and a cysteine-residue in the active site of the enzyme, leading to irreversible inhibition of PKC (Ward et al., 1995). A cysteine-residue is also present in the active-site of PKA, suggesting possible unspecific binding of M-EGF to PKA, which could maybe explain the decreased MSH-induced dispersion. Unspecificity of kinase-inhibitors is a problem and our results here should be confirmed by further methods, e.g. by expression of recombinant inhibitors of PKA and PKC; by showing that the effect of the inhibitor is abolished upon expression of a drug-resistant mutant of the kinase; or by demonstrating phosphorylation of downstream substrate/s.

An explanation to the ginseng effect on melanosome movement could be that the myosin Va-dependent transport along actin filaments is enough for dispersion to occur in spite of protein kinase A and C inhibition. An actin filament disrupting drug, latrunculin A, was shown to decrease the dispersion induced by ginseng, ET-3 and MSH by 60-70%, showing involvement of actin filaments is important also for the dispersion stimulated by ginseng and ET-3 (paper I, Fig 5).
Another kinase which has been shown to be activated during bi-directional transport of melanosomes is MAPK (ERK 1/2) (Andersson et al., 2003; Deacon et al., 2005). Here, ginseng and ET-3 stimulated an activation of 44/42-MAPK (paper I, Fig 6 A-B). The ET-3-induced MAPK-phosphorylation was significantly decreased after pre-treatment with PKC-inhibitor M-EGF (paper I, Fig 6 C). There was a tendency to a decrease also in the ginseng and MSH-induced dispersion, though not significant. Hence PKC seems to be an upstream activator of MAPK upon ET-3 treatment and possibly, in combination with other unknown components, also in ginseng and MSH stimulated cells. To investigate whether MAPK activation was important for the anterograde movement of melanosomes, we pre-incubated melanophores with the MEK-inhibitor PD98059 significantly inhibits the ET-3-induced dispersion and there is a tendency to inhibition of the MSH- and ginseng-induced dispersion (A, n=8). Preliminary experiments show inhibition of the MSH-, ET-3- and ginseng-induced activation of 44/42 MAPK upon pre-incubation with PD98059 (B, n=2). Melanophores were pre-aggregated with melatonin before incubation with PD98059 at 100 μM for 30 minutes, followed by stimulation with MSH at 100 nM, ginseng at 10 mg/ml and ET-3 at 1 μM for indicated time (A) and for 5 minutes (B). Results were calculated as change in absorbance (1-10^Ai-Af) (A) or presented in AU ± SEM (B) and normalized to control cells stimulated with MSH for 30 minutes (A) or ginseng at 10 mg/ml (B). PD98 and PD, PD98059; Gin, ginseng, Control, L15 medium; ns, not significant p>0.05, ***p<0.001; AU, arbitrary units.
PD98059 prior to ginseng-, ET-3- and MSH-stimulation and measured the change in absorbance. After 20 minutes there was a small but significant inhibition (33%) of the ET-3 induced dispersion, but no significant alterations of the dispersion induced by ginseng and MSH, although there was a tendency toward a decreased dispersion (Fig 12A). PD98059 did not affect the point at which the MSH-, ginseng- and ET-3-stimulated dispersion reached their peaks. In paper II we show that a combination of ginsenoside Rd and quercetin induce an 82% dispersion (paper II, Fig 2B) but no activation of MAPK (paper II, Fig 6A and B). These results together indicate that an early activation of MAPK may be important to later reach full dispersion, but does not affect how fast dispersion reaches its maximum. Preliminary experiments demonstrated that PD98059 was effective as a MEK-inhibitor, since the MSH-, ginseng- and ET-3-induced activation of MAPK was reduced by 83, 53 and 91% respectively (Fig 12B). Hence, in spite of the 50-90% inhibition of MAPK activation detected in the western blot assay, the results from the dispersion-assay indicate that there is still enough activated MAPK for dispersion to occur. On the contrary, another explanation could be that even though MAPK is activated, it may not be part of the signalling pathway for dispersion.

In this paper we showed that the increased anterograde transport of melanosomes upon ginseng-treatment was not mediated via an elevation of cAMP and only partially transducted via PKC. Ginseng and ET-3 stimulated an activation of MAPK, where the effect of ET-3, but not ginseng, was significantly reduced by PKC-inhibition, showing a role for PKC upstream MAPK. Since the ginseng-induced activation of MAPK was only slightly decreased by PKC-inhibitor M-EGF, other still unknown signalling components must be involved. MAPK was activated upon stimulation with ginseng, ET-3 and MSH and may be important to accomplish full dispersion, although its function in anterograde movement is still uncertain.
4.2 Paper II – Effects of ginsenosides and quercetin on pigment organelle transport

In paper II we continued the research on *Panax ginseng* by studying the effects of seven of its major ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1) and the flavonoid quercetin on melanophore pigment organelle transport and signalling. The bioactive constituents of ginseng include ginsenosides, flavonoids, polysaccharides, polyacetylenes, peptides, vitamins, phenols and enzymes, of which the ginsenosides are considered to be the main pharmacologically active elements.

We found that two of the ginsenosides, Rc and Rd, induced a significant increase in the anterograde transport of melanosomes (paper II, Fig 1). Rc and Rd along with Rb1 and Rb2 all belong to the protopanaxadiol ginsenoside group, in contrast to the protopanaxatriols Re, Rf and Rg1, suggesting that the differences in the number and sort of attached sugar moieties and hydroxyl groups are crucial for their effects on

![Graph](image)

**Fig 13.** The sum of the dispersion induced by Rc or Rd and quercetin alone, compared with the dispersion induced by their combination. Melanophores were pre-aggregated with melatonin before incubation with Rc or Rd at 50 μM and/or quercetin at 50 or 100 μM for 15 minutes. Negative control cells were incubated with DMSO (Control) and positive control cells with ginseng (Gin) at 10 mg/ml and MSH at 100 nM (not shown in graph). Results were calculated as change in absorbance (1-10^(A1-A0)) and normalized to control cells stimulated with MSH for 30 minutes and presented as ± SEM (n=8); ns, not significant p>0.05, *p<0.05, **p<0.01,***p<0.001. Q50, quercetin at 50 μM; Q100, quercetin at 100 μM.
organelle movement. However, the increase was only 35-40% of the ginseng-induced dispersion, suggesting the involvement of other constituents in the pigment organelle movement. The concentration of Rc and Rd in the extract and in the isolated fractions were about the same: 0.07 and 0.03 mg/ml for Rc and Rd respectively in the extract (Soldati and Sticher, 1980) compared to 0.06 mg/ml for Rc and Rd in the fractions.

Although flavonoids are identified components of ginseng there is not much known about which specific flavonoids that are included. Though, since the flavonol quercetin has been shown to be a part of the ginseng root (Dong et al., 2003; Rahman and Punja, 2005; Ali et al., 2007) we incubated melanophores with quercetin and measured the altered absorbance. Quercetin was found to induce a concentration-dependent increase in dispersion, with significant elevation of anterograde movement at 50 and 100 μM (20 and 37% respectively) (paper II, Fig 2A). 37% is far from a 100% dispersion, although when ginsenoside Rc or Rd and quercetin were combined and incubated with melanophores, a synergistic increase in dispersion was seen (e.g. 82% dispersion with Rd and quercetin at 100 μM) (paper II, Fig 2C). The combination of Rc or Rd and quercetin were significantly separated from the control, Rc or Rd alone and quercetin alone (paper II, Fig 2C). In figure 13 the sum of the dispersion induced by Rc or Rd and quercetin alone is compared with the dispersion induced by their combination. Figure 14 show the dispersion induced by ginsenoside and quercetin alone and the combination of the two substances over time. For Rc (Fig 14A-B) or Rd (Fig 14C-D) together with quercetin, the peak was reached at 20-25 minutes of stimulation, for Rc or Rd alone at 15 minutes and for quercetin alone at 55 (50 μM) or 25 minutes (100 μM) (Fig 14). This effect suggests that the ginsenoside and flavonoid parts of the ginseng extract act in synergy to induce anterograde movement of pigment organelles, seen in melanophores stimulated with the ginseng extract.

Further examination of ginsenosides was concentrated on PKC signalling, since the ginseng extract in paper I was shown not to increase the cAMP level and not to be affected by PKA-inhibition. Melanophores pre-treated with PKC-inhibitor M-EGF showed a decreased dispersion upon incubation with ginsenoside Rc and Rd (from 20
and 35% to 9 and 6 % respectively) (paper II, Fig 3). Also, we demonstrated that ginseng at concentrations of 0.5 and 1 mg/ml (compared to 10 mg/ml used in paper I) induced an increased anterograde movement (56 and 68% respectively) which to a great extent was reduced by pre-incubation with PKC-inhibitor M-EGF at 20 μM (11 and 23 % dispersion respectively with inhibitor present) (paper II, Fig 4). The fact that ginsenosides are known to be able to modulate PKC-activity (Kim et al., 1998; Jeong et al., 2004) together with our results here, indicate that PKC is one part of the

Fig 14. A combination of Rc (A-B) or Rd (C-D) and quercetin at 50 or 100 μM induces a synergistic increase in dispersion. Melanophores were pre-aggregated with melatonin before incubation with Rc or Rd at 50 μM and/or quercetin at 50 or 100 μM for indicated time. Negative control cells were incubated with DMSO and positive control cells with ginseng at 10 mg/ml (Gin) and MSH at 100 nM. Results were calculated as change in absorbance (1-10 Ai-Af) and normalized to control cells stimulated with MSH for 30 minutes (not shown in graphs) and presented as ± SEM, n=8. Q50, quercetin at 50 μM; Q100, quercetin at 100 μM.
dispersion signalling pathway stimulated by ginseng and ginsenosides. In addition, quercetin has previously been shown to directly activate e.g. PKA and PKC by binding to their ATP-binding sites (Spencer et al., 2004; Williams et al., 2004), showing a possible pathway for the flavonoid part of ginseng to cause dispersion. The synergistic effect seen when combining ginsenosides Rc or Rd and quercetin could be due to simultaneous activation of both PKA and PKC pathways, where ginsenosides affect PKC and quercetin PKA (and possibly also PKC). Further studies are needed to investigate the exact mechanisms of the ginseng constituents. E.g. measurements of

![Image of Western Blot and Graph]

**Fig 15.** Preliminary experiments show that a combination of seven ginsenosides (GS) (A) or a combination of Rc or Rd and quercetin (B) does not affect 44/42-MAPK phosphorylation. After pre-aggregation with melatonin, melanophores were stimulated with ginseng at 10 mg/ml, ginsenosides at 50 μM or ginsenoside Rc or Rd at 50 μM in combination with quercetin at 100 μM for 5 minutes, followed by lysis and analysis by western blot as described under methods. Data were normalized to ginseng at 10 mg/ml, set to 100%. Results are presented as AU ± SEM (B) (A, n=1; B, n=2). Gin, ginseng; GS, ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1; Q100, quercetin at 100 μM; AU, arbitrary units.
cAMP and studies of effects on AC, phosphodiesterase (PDE), PLC, IP₃ and DAG (using inhibitors, antibodies and competitive radioactive assays) upon ginsenoside and/or quercetin stimulation would, in combination with studies of dominant negative mutants of PKA and PKC, indicate whether their effects are direct actions on PKA and/or PKC or not.

In paper I we showed a transient activation (with a peak at 5 minutes and declining between 10 and 45 minutes) of 44/42-MAPK when stimulating melanophores with ginseng at 10 mg/ml. Here the ginseng-induced phosphorylation of 44/42 MAPK was found to be concentration-dependent with the highest degree of activation at 3 mg/ml (paper II, Fig 5). Moreover, melanophores pre-treated with PKC-inhibitor M-EGF prior to stimulation with ginseng at 0.5 and 1 mg/ml showed no decrease in MAPK activation (paper II, Fig 5). Together with results from paper I demonstrating only a slight effect of the PKC-inhibitor on the activation of MAPK by ginseng at 10 mg/ml, this indicate that PKC has no part in the signalling pathway for MAPK phosphorylation stimulated by the ginseng extract. The seven ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1) and quercetin did not induce an activation of MAPK (paper II, Fig 6). In addition, preliminary results showed no MAPK activation upon stimulation of melanophores with the combination of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 (Fig 15A) or the combination of Rc or Rd and quercetin (Fig 15B). It appears that neither the ginsenoside nor quercetin constituents of the ginseng extract are responsible for the activation of MAPK seen upon ginseng stimulation of melanophores. The ginseng component panaxynol, a polyacetylene, has been shown to induce differentiation in PC12 cells via mediation of cAMP and MAPK (Wang et al., 2006) and could thus be the part of the ginseng extract inducing MAPK activation in melanophores.

In summary we showed that ginsenosides Rc and Rd and the flavonoid quercetin induced an anterograde transport of pigment organelles, which was synergistically increased when melanophores were stimulated with the combination of Rc or Rd and quercetin. The ginseng and ginsenoside effect on dispersion were demonstrated to be
partially transduced via PKC. Also, ginseng, but not ginsenosides or quercetin, induced a concentration-dependent activation of MAPK, which was not dependent on PKC.

4.3 Effects on membrane fluidity, receptors and intracellular signalling

Different effects of ginseng and its components on cAMP, MAPK and PLC/PKC have been showed in other cell systems (see section 1.5 *Panax ginseng*). Our results here, using the melanophores, showed an increased organelle transport, possible involvement of PKC, activation of MAPK, but no increase in cAMP upon ginseng, ginsenoside and quercetin treatment, further adding information to the complex actions of *Panax ginseng*. The fact that quercetin are able to affect hormone receptor function (e.g. the EGFR receptor) and signal transduction (Boots et al., 2008), suggest that the quercetin-induced dispersion of melanosomes could be conducted via interaction with the MSH or ET-3 receptors on the melanophore membrane. Also, the interactions of quercetin with the ATP-binding-sites of several enzymes (Spencer et al., 2004; Williams et al., 2004), including PKA and PKC, propose further studies on the effects of PKA- and PKC-inhibitors on quercetin-induced organelle transport. Moreover, additional research on the polyacetylene constituents of ginseng is needed. The polyacetylenes panaxynol, panaxydol and panaxytriol have been shown to affect MAPK, PI3-kinase and PKC in other cells (Wang et al., 2006; Halim et al., 2008; Hai et al., 2009a; Hai et al., 2009b). Hence, in melanophores, the ginseng-induced activation of MAPK and maybe also a PKC-dependent dispersion could be due, in part or solely, to the polyacetylene constituent/s.

The ginseng modulation of neurotransmission, such as increased transport and release of neurotransmitter vesicles, release and re-uptake of acetylcholine and increased dopamine and norepinephrine levels could explain believed effects of ginseng on better memory and cognitive performance (Kiefer and Pantuso, 2003; Radad et al., 2006). However, little is known about the signalling pathways leading to the ginseng
actions on neurotransmitter release. Since melanophores are derived from the neural crest and specialized in organelle transport (regulated by known chemical signals), the effects of ginseng on melanophores could indicate which signalling pathways that may be responsible for the ginseng-modulation of neurotransmitter availability. Several characteristics are shared between melanophores and neuronal cells, including neuronal or hormonal regulated organelle transport via binding to GPCRs and bi-directional transport of organelles along both actin filaments and microtubules (Aspengren et al., 2007). It is also speculated that myosin Va has a role in synaptic vesicle trafficking, since the synaptic vesicle protein synaptophysin is a binding-partner for myosin Va (Karcher et al., 2002). In paper I and II we showed an increased organelle transport (independent of cAMP and partly dependent on PKC) and phosphorylation of MAPK upon ginsenoside and ginseng treatment of melanophores. Consistent with our results, Xue et al (2006) showed that ginsenoside Rb1 stimulated neurotransmitter release in PC12 cells by activation of PKA, followed by phosphorylation of synaptic vesicles and increased release of glutamate.

Since ginsenosides are membrane-permeable it is not clear whether they exert their effects on intracellular signalling from outside and/or within the cells (Attele et al., 1999). A few studies report ginsenoside interactions with different membrane-bound receptors. Choi et al (2001) demonstrated that the ginsenoside-induced Ca\(^{2+}\)-activated Cl\(^-\)current was mediated through actions on G\(_{q/11}\)-protein-coupled receptors at the extracellular side of the cells. Moreover, ginsenoside Re have been shown to interact with an estrogens-receptor (Nakaya et al., 2007), ginsenoside Rg3 to inhibit 5-HT3A receptor channels (Lee et al., 2007), Rg2 to inhibit acetylcholine- (ACh) induced inward current through effects at nicotinic ACh receptors (Choi et al., 2002) and Rc to enhance GABA-stimulated inward peak current via actions at the GABA(A) receptor (Choi et al., 2003b). Melanophores possess several endogenous membrane-bound GPCRs that are known to stimulate dispersion via AC, a rise in cAMP concentration and activation PKA: the MSH, serotonin, vasoactive intestinal peptide (VIP), oxytocin and calcitonin gene-related peptide β (β-CGRP) receptors (McClintock et al., 1996; Jayawickreme and Kost, 1997). Hence, it is possible that ginseng induce dispersion in
melanophores via interactions with one or more of these receptors. However, such a scenario is not likely since no increased cAMP-level was detected in ginseng-treated melanophores. In that sense, an effect on the endogenous ET-3 receptor with following activation of PLC/PKC and an increased organelle movement, would be more possible.

Ginsenosides are amphiphilic and have the ability to insert themselves into plasmamembranes and interact with membrane phospholipids, leading to altered membrane dynamics and modification of receptors, ion-channels and enzymes (Attele et al., 1999). Membrane-associated enzymes, such as PKC, are sensitive to changes in membrane fluidity and function (Attele et al., 1999). Some studies have shown effects of ginsenosides on membrane properties. Ginsenoside Rf was demonstrated to alter the activity of membrane-bound G-proteins, although it was not known whether Rf bound to the GPCR or directly modulated G-protein activity (Attele et al., 1999). Kwon et al (2008) showed that ginsenoside Rg3 decreased membrane fluidity and subsequently blocked drug efflux. Tamoxifen is another compound much like ginsenosides, highly lipophilic with a structure similar to cholesterol and an ability to incorporate into membranes (Mandlekar and Kong, 2001). It has been demonstrated to stabilize membranes by decreasing fluidity and to modulate signalling proteins such as PKC (Mandlekar and Kong, 2001). A possible explanation to the ginseng intracellular effects in melanophores could be that ginseng affects PKC indirectly by altering membrane properties. An incorporation of ginsenoside into the membrane, leading to stabilization and decreased fluidity, could release proteins attached to the membrane, e.g. PLC and PKC, followed by phosphorylation of downstream proteins and resulting in organelle anterograde movement. The effects of ginsenosides or other constituents of the ginseng extract on melanophores could also be due to interactions with the endogenous ETc-Gq-coupled receptor (known to stimulate dispersion of melanosomes through PLC/PKC), either outside on receptor or intracellular on the G-protein. Ginsenoside action on the MSH Gs-protein-coupled receptor is more unlikely, since there was no increase in cAMP concentration in melanophores stimulated with the ginseng extract. Given that we saw no or incomplete effects of PKA and PKC
inhibitors, and that a disrupted actin filament network reduced dispersion by 60-70%, another possibility is that ginseng exert its effect/s downstream the protein kinases, directly affecting actin filaments and their associated proteins. Also, further investigations are required of the ginseng-effect on melanophores treated with a combination of PKA- and PKC-inhibitors.

There are many studies on the pharmacological mechanisms of ginseng, ginsenosides and flavonoids, although the main part are in vitro studies and consequently their actual effects in vivo and whether they can reach their intended sites of action or not, is largely unknown. Also, given the fact that the metabolism of flavonoids include a large number of processes and that the composition of ginseng is highly complex, investigations of their bioavailability are very difficult, and only a few studies on ginseng, ginsenoside and flavonoid absorption and metabolism have been performed in humans (Walle, 2004; Lee et al., 2009).
5. Conclusions

Final conclusions on the effects of *Panax ginseng* and its constituents ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and flavonoid quercetin on pigment organelle transport in melanophores were as follows.

♦ *Panax ginseng* induced a concentration-dependent anterograde transport of melanosomes. The increased dispersion was not mediated via a cAMP rise or PKA activation and only partially transducted via PKC.

♦ Ginsenosides Rc and Rd and flavonoid quercetin stimulated an increased dispersion of melanosomes. When Rc or Rd and quercetin were combined, a synergistic increase in anterograde transport of melanosomes was demonstrated, showing that the ginsenoside and flavonoid parts of ginseng are capable of cooperating to stimulate full dispersion. The actions of Rc and Rd may partially be transducted via PKC.

♦ *Panax ginseng*, but not ginsenosides and quercetin, induced a transient concentration-dependent activation of 44/42 MAPK, previously shown to be involved in both aggregation and dispersion of melanophore pigment organelles. The slight, but not significant, decrease in the MSH- and ginseng-induced MAPK activation upon pre-treatment with a PKC-inhibitor, indicate the involvement of other signalling components. However, in the case of ET-3 (known to induce dispersion via PLC/PKC) the activation of MAPK was significantly decreased upon PKC-inhibition, showing that PKC has a role upstream MAPK in the case of ET-3.

♦ The constituent/s of *Panax ginseng* inducing an activation of MAPK is/are still unknown.
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7. References


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