The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation

Lina Thors
Abstract

Anandamide (arachidonoyl ethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG) exert most of their actions by binding to cannabinoid receptors. The effects of the endocannabinoids are short-lived due to rapid cellular accumulation and metabolism, for AEA, primarily by the enzymes fatty acid amide hydrolase (FAAH). This has led to the hypothesis that by inhibition of the cellular processing of AEA, beneficial effects in conditions such as pain and inflammation can be enhanced. The overall aim of the present thesis has been to examine the mechanisms involved in the cellular processing of AEA and how they can be influenced pharmacologically by both synthetic natural compounds.

Liposomes, artificial membranes, were used in paper I to study the membrane retention of AEA. The AEA retention mimicked the early properties of AEA accumulation, such as temperature-dependency and saturability.

In paper II, FAAH was blocked by a selective inhibitor, URB597, and reduced the accumulation of AEA into RBL2H3 basophilic leukaemia cells by approximately half. Treating intact cells with the tyrosine kinase inhibitor genistein, an isoflavone found in soy plants and known to disrupt caveolae-related endocytosis, reduced the AEA accumulation by half, but in combination with URB597 no further decrease was seen. Further on, the effects of genistein upon uptake were secondary to inhibition of FAAH. The ability to inhibit the accumulation and metabolism of AEA was shared by several flavonoids (shown in paper III). In paper IV, the isoflavone biochanin A and URB597 had effects in vivo, in a model of persistent pain, effects decreased by the cannabinoid receptor 1 antagonist AM251.

In paper VI, the cellular processing of the endocannabinoid metabolites following degradation was examined, a mechanism poorly understood. It was found that nitric oxide (NO) donors significantly increased the retention of tritium in cell membranes following incubation with either tritiated AEA or 2-AG. Further experiments revealed that the effect of NO donors mainly involves the arachidonate part of the molecules. Inhibition of FAAH completely reduced the effect of NO donors in cells with a large FAAH component, indicating that the effects were downstream of the enzyme.

These results suggest that the cellular processing of endocannabinoids can be affected in a manner of different ways by pharmacological manipulation in vitro and that naturally occurring flavonoid compounds can interact with the endocannabinoid system.

Key words: endocannabinoids, anandamide, fatty acid amide hydrolase, flavonoids, cellular processing, pain
The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation

## Table of Contents

**ORIGINAL PAPERS** ................................................................. 6

**ABBREVIATIONS** ............................................................... 7

**INTRODUCTION** .................................................................. 8

- **The cannabinoid system** ................................................. 8
  - Cannabinoid receptors and synthetic ligands ..................... 9
  - Other targets for endocannabinoids ................................. 13
  - Endocannabinoids and their synthesis ............................ 13
  - Cellular accumulation of AEA ....................................... 16
  - Endocytosis ..................................................................... 17
  - AEA and endocytosis ...................................................... 19
  - Metabolism of AEA .......................................................... 21
  - Other pathways for AEA metabolism ............................... 23
  - Recycling of AEA metabolites ........................................ 24

- **Biosynthesis, uptake and metabolism of 2-AG and PEA** ...... 24

- **Flavonoids** ....................................................................... 25

- **Pain** .................................................................................. 27
  - Cannabinoids and pain .................................................... 27
  - Flavonoids and pain ........................................................ 29

**AIMS OF THE THESIS** ........................................................ 31

**METHODOLOGICAL CONSIDERATIONS** .......................... 32

- **Liposomes (Paper I)** .......................................................... 32
- **Cell culture (Papers II, III, IV, V, VI)** .............................. 34
- **Assay of [3H]AEA uptake (Paper II, III, IV)** .................. 35
  - Assay conditions throughout accumulation of AEA - a construction of artefacts or helpful tools? .......................... 35
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASSAY OF FAAH (PAPER II, III, IV, V, VI)</td>
<td>39</td>
</tr>
<tr>
<td>ASSAY OF MEMBRANE TRITIUM ACCUMULATION (PAPER I, IV, V, VI)</td>
<td>42</td>
</tr>
<tr>
<td>ANIMALS (PAPER V)</td>
<td>44</td>
</tr>
<tr>
<td>IMMUNOCYTOCHEMISTRY FOR PHOSPHO-ERK (PAPER V)</td>
<td>44</td>
</tr>
<tr>
<td><strong>RESULTS AND DISCUSSION</strong></td>
<td>46</td>
</tr>
<tr>
<td>AEA RETENTION IN LIPOSOMES (PAPER I)</td>
<td>46</td>
</tr>
<tr>
<td>ACCUMULATION OF AEA IN ADHERENT CELLS (PAPER II, III, IV)</td>
<td>47</td>
</tr>
<tr>
<td>INHIBITION OF FAAH BY FLAVONES AND ISOFLAVONES (PAPER II, III, IV, V, VI)</td>
<td>52</td>
</tr>
<tr>
<td>MEMBRANE TRITIUM ACCUMULATION (PAPER IV, V, VI)</td>
<td>55</td>
</tr>
<tr>
<td>PERK STAINING (PAPER V)</td>
<td>57</td>
</tr>
<tr>
<td><strong>CONCLUSIONS</strong></td>
<td>59</td>
</tr>
<tr>
<td><strong>POPULÄRVETENSKAPLIG SAMMANFATTNING</strong></td>
<td>60</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>62</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>64</td>
</tr>
</tbody>
</table>
Original papers

The present thesis is based on the following publications and manuscripts, which will be referred to in the text by heir Roman numerals.

   Manuscript

   Br J Pharmacol. 150:951-60.

III. Thors L, Eriksson J, Fowler CJ. (2007) Inhibition of the cellular uptake of anandamide by genistein and its analogue daidzein in cells with different levels of fatty acid amide hydrolase-driven uptake. 

IV. Thors L, Belghiti M, Fowler CJ. (2008) Inhibition of fatty acid amide hydrolase by kaempferol and related naturally occurring flavonoids. 
   Br J Pharmacol. 155, 244–52

   Manuscript

VI. Thors L, Fowler CJ. Effect of nitric oxide donors on membrane tritium accumulation of endocannabinoids and related endogenous lipids. 
   Manuscript

Reprints of the published papers were made with permission from the respective publisher.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEA</td>
<td>arachidonoyl ethanolamide (anandamide)</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonoyl glycerol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cav</td>
<td>caveolin</td>
</tr>
<tr>
<td>CB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>cannabinoid receptor 1</td>
</tr>
<tr>
<td>CB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>cannabinoid receptor 2</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>LOX</td>
<td>lipoxygenase</td>
</tr>
<tr>
<td>MCD</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>MGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>NAPE</td>
<td>N-acetyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acyl phosphatidylethanolamine phospholipase D</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PEA</td>
<td>palmitoyl ethanolamide</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>THC</td>
<td>Δ⁹-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid type 1</td>
</tr>
<tr>
<td>URB597</td>
<td>FAAH inhibitor</td>
</tr>
</tbody>
</table>
The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation

**Introduction**

**The cannabinoid system**

For thousands of years, cannabis has been used for both medicinal and social purposes. It is obtained from the herb *Cannabis sativa* and the name of the plant reflects its ancient use. Eastern cultures have used marijuana as medicine for centuries; the Chinese emperor Huang Ti advised taking Cannabis for the relief of cramps and pain as early as 2600 BC, compared to the Western world that accepted the medicinal use of the herb first in the nineteenth century. Working in India, the Irish army surgeon Sir William O'Shaughnessy (1809–1889) made the first scientific study of cannabis that led to an expansion of the medicinal use of cannabis in Britain in the mid-nineteenth century (for a review of the history of cannabis, see Mechoulam, 1986).

During the 1960s and 1970s cannabis also became associated with the escalation of the drug culture and the recreational use of *Cannabis sativa* preparations, and indeed, marijuana is still one of the most common illegal drugs of abuse in the world (Degenhardt et al., 2008). In the late 1990s the use of cannabis for medical conditions got attention based on several reports of symptomatic relief from a variety of medical conditions, such as pain, but a report commissioned by the House of Lords in the U.K. concluded that further research was needed (The House of Lords, 1998).

Initially, the term “cannabinoid” was used to indicate a structure similarity with Δ⁹-tetrahydrocannabinol (THC), the principal psychoactive component of *Cannabis sativa*, although the definition has been extended to include compounds that interact with cannabinoid receptors (see below). Cannabis contains over 60 cannabinoids, of which THC and cannabidiol are well studied (Hanus et al., 1987). A fixed ratio combination of THC and cannabidiol have been evaluated and resulted in the drug Sativex® for treatment of neuropathic pain in multiple sclerosis patients. Clinical trials with Sativex® have found that the drug had a short-term efficacy against
central pain associated with multiple sclerosis (Rog et al., 2005; Svendsen et al., 2004; Wade et al., 2004; Zajicek et al., 2003). Since April 2006, Sativex® is approved for use in Canada and is also available in the UK and Spain to individual patients the clinics consider may benefit. THC (dronabinol) and nabilone, a synthetic cannabinoid based on THC, are available in the US and UK for treatment of the chemotherapy-induced nausea and vomiting in cancer patients and for appetite stimulation in HIV/AIDS-infected patients (Dejesus et al., 2007; Ware et al., 2008). The psychotropic side-effects of these compounds have limited usefulness in cannabis-based drugs, although a recent review reported that short-term use of cannabinoids for medical purposes elicited mild adverse effects (Wang et al., 2008).

**Cannabinoid receptors and synthetic ligands**

In 1964, THC was isolated (Gaoni et al., 1964), a discovery that paved the way for the discovery that the plant cannabinoid acted through at least two types of receptors. The first cannabinoid binding site in the rat brain was identified in 1988 and later cloned in 1990, subsequently named cannabinoid receptor 1 (CB₁) (Devane et al., 1988; Matsuda et al., 1990). The second receptor, cannabinoid receptor 2 (CB₂), was discovered shortly after (Munro et al., 1993).

The two Gᵢ/o protein-coupled cannabinoid receptors, CB₁ and CB₂, have a diverse distribution throughout the body. CB₁ is mainly located in several brain regions, predominantly on neuronal presynaptic terminals but also on postsynaptic structures and glial cells (Katona et al., 1999; Mackie, 2005; Rodriguez et al., 2001) but expression of the CB₁ has also been found peripherally in sympathetic nerves (Ishac et al., 1996), adipocytes (Cota et al., 2003), liver (Osei-Hyiaman et al., 2005), skeletal muscles (Cavuoto et al., 2007) and prostate epithelial cells (Ruiz-Llorente et al., 2003). The distribution of CB₂ is predominantly on immune cells derived from macrophages, activated microglia and in some neurons in the brain (Galiegue et al., 1995; Van Sickle et al., 2005).
The activated CB₁ and CB₂ initiate a variety of intracellular signalling mechanisms, including inhibition of adenylyl cyclase (Childers et al., 1993; Howlett, 1984) and activation of mitogen activated protein kinases (Bouaboula et al., 1995; Derkinderen et al., 2003). Regulation of ion currents, as a result of inhibition of voltage gated Ca²⁺ channels and activation of inward rectifying potassium channels, are also a signal transduction mechanism for CB₁ (Caulfield & Brown, 1992; Mackie & Hille, 1992; Mackie et al., 1995) whilst cannabinoid-induced apoptosis through ceramide accumulation have been reported for CB₂ (Galve-Roperh et al., 2000; Sanchez et al., 2001). In retrograde signalling in the central nervous system (CNS), the established role of CB₁ has mainly been controlling the release of several neurotransmitters, such as glutamate (see figure 1 in the section below) (Lovinger, 2008).

Generally most of the cannabinoid agonists show similar affinity for both receptors but developing CB₂ specific agonist would be of therapeutic interest due to the immuno-modulatory properties of CB₂ without achieving the psychoactive effects subsequent to CB₁ activation. A number of ligands selective of the cannabinoid receptors have been described. Some of the more commonly used compounds are summarized in table 1 below.
### Table 1. Commonly used agonists and inverse agonists/antagonists of CB receptors.

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Receptor affinity</th>
<th>Examples of effect <em>in vivo</em></th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-selective</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| THC                 | $K_i^{CB_1} = 25$ nM  
 $K_i^{CB_2} = 35$ nM | Produces the “tetrad” of behaviour effects (analgesia, hypomotility, catalepsy, hypothermia) due to activation of central CB$_1$. | (Little *et al.*, 1988) |
| HU210               | $K_i^{CB_1} = 0.25$ nM  
 $K_i^{CB_2} = 0.4$ nM | Reduces the axonal damage and the AMPA-induced excitotoxicity in a chronic mice model of multiple sclerosis through activation of both cannabinoid receptors. | (Docagne *et al.*, 2007; McPartland *et al.*, 2007) |
| **CB$_1$-selective**|                  |                                                                                             |                                     |
| ACEA                | $K_i^{CB_1} = 1.4$ nM  
 $K_i^{CB_2} = 3.1$ µM | Attenuates mechanical allodynia and hyperalgesia, and decreases mechanically-evoked responses of Aδ-nociceptors during inflammation. Effects blocked by co-admin of AM251 but not with AM630. | (Hillard *et al.*, 1999; Potenzieri *et al.*, 2008) |
| **CB$_2$-selective**|                  |                                                                                             |                                     |
| JWH133              | $K_i^{CB_1} = 677$ nM  
 $K_i^{CB_2} = 3.4$ nM | Reduces the enhanced gastrointestinal contractile response in lipopolysaccharide treated rats. Reversed by AM630. | (Duncan *et al.*, 2008; Huffman *et al.*, 1999) |
| HU308               | $K_i^{CB_1} > 10$ µM  
 $K_i^{CB_2} = 23$ nM | Decreases the levels of inflammatory mediators and the extent of liver damage in a mouse model of hepatic ischemia/reperfusion injury. | (Hanus *et al.*, 1999; Rajesh *et al.*, 2007) |
### Inverse agonists/Antagonists

<table>
<thead>
<tr>
<th>Inverse agonists/Antagonists</th>
<th>Receptor affinity</th>
<th>Examples of effect in vivo</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CB₁-selective</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SR141716</strong> (Rimonabant®)</td>
<td>( K_i \text{CB}_1 = 11.8 \text{ nM} ) ( K_i \text{CB}_2 &gt; 10 \text{ µM} )</td>
<td>Reduces development of atherosclerosis and decreases production of inflammatory mediators in low-density lipoprotein receptor-deficient mice without modulating the total serum cholesterol content. Approved by the European Commission for clinical use in 2006 (Acomplia®), for management of obesity, but suspended in 2008 due to the increased risk of severe psychiatric problems.</td>
<td>(Dol-Gleizes et al., 2008; Felder et al., 1998; Le Foll et al., 2008; Rinaldi-Carmona et al., 1994; Van Gaal et al., 2008)</td>
</tr>
<tr>
<td><strong>AM251</strong></td>
<td>( K_i \text{CB}_1 = 7.5 \text{ nM} ) ( K_i \text{CB}_2 = 2.3 \text{ µM} )</td>
<td>Increases the mean blood pressure acutely and affects the systolic function in rats with liver cirrhosis. Not seen in control rats.</td>
<td>(Batkai et al., 2007a; Lan et al., 1999)</td>
</tr>
<tr>
<td><strong>CB₂-selective</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SR144528</strong></td>
<td>( K_i \text{CB}_1 &gt; 10 \text{ µM} ) ( K_i \text{CB}_2 = 5.6 \text{ nM} )</td>
<td>Decreases the ( \mu )-opioid receptor activity by antagonising the binding of noladin ether to the ( \text{CB}_2 ) receptor. Have off-target effects on the PPARα pathway.</td>
<td>(LoVerme et al., 2006; Paldyova et al., 2008; Ross et al., 1999)</td>
</tr>
<tr>
<td><strong>AM630</strong></td>
<td>( K_i \text{CB}_1 = 5.2 \text{ µM} ) ( K_i \text{CB}_2 = 31.2 \text{ nM} )</td>
<td>Prevents bone loss in wild-type mice but not in ( \text{CB}_2^{-/-} ) mice.</td>
<td>(Idris et al., 2008; Ross et al., 1999)</td>
</tr>
</tbody>
</table>
Other targets for endocannabinoids

Endocannabinoids, and indeed, synthetic cannabinoids do not only interact with CB₁ and CB₂, and a number of other targets, both receptors and systems have been suggested to be involved in the physiological and pharmacological effects of the cannabinoids (Fowler, 2008). The GPR₅₅ receptor was identified in the late 90s and subsequently led to the association with cannabinoids (for review see Ross, 2009). In addition, a “non-CB₁, non-CB₂” receptor has been postulated (Jarai et al., 1999; Mackie & Stella, 2006). None of the novel receptors has yet formally been classified as cannabinoid receptors (Alexander et al., 2008).

Among the non-cannabinoid targets recent findings have shown a connection between the peroxisome proliferator-activated receptors (PPARs), a group of nuclear receptor proteins involved in regulation of cellular differentiation, development and metabolism, and the cannabinoids (Bouaboula et al., 2005; Gasperi et al., 2007; Kozak et al., 2002b). Conversely, ligands of PPARs can interact with the endocannabinoid system (Lenman & Fowler, 2007).

Several other proteins have also been reported as targets of AEA; examples are the ability of AEA to act as an agonist at the transient receptor potential vanilloid type 1 (TRPV1) (Zygmunt et al., 1999) and as an inhibitor of the outward K⁺-channel TASK-1 (Maingret et al., 2001)

Endocannabinoids and their synthesis

In early 1990s it was found that mammalian tissues could synthesize and release agonists to the cannabinoid receptors, which led to the discovery of the first endogenous cannabinoids (endocannabinoids). The most well studied endocannabinoids are anandamide (arachidonylethanolamine, AEA) and 2-arachidonyl glycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995).
The identified endocannabinoid agonists are derivates of long-chain polyunsaturated fatty acids, particularly arachidonic acid, demonstrating varying selectivity for the two cannabinoid receptors and other targets (McAllister & Glass, 2002). Both AEA and 2-AG play a dominant role in the cannabinoid signalling, even though 2-AG function with a greater efficacy to both subtypes (Gonsiorek et al., 2000). But also other bioactive lipids, fatty acids with carbon chains of varying length and degrees of saturation, with cannabimimetic activity have been discovered. Noladin ether, for example, binds to both CB receptors (Hanus et al., 2001).

AEA and 2-AG are both synthesized following hydrolysis of lipid precursors, but by entirely different pathways. The concentration of AEA is generally low under normal conditions and produced “on demand” in contrast to 2-AG with a more abundant concentration in most tissues (Di Marzo et al., 1994; Schmid, 2000; Sugiura et al., 2002). Activation of metabotropic glutamate receptors (mGluR) or neuronal depolarisation elevates the level of intracellular Ca$^{2+}$ that trigger the biosynthesis of AEA (see figure 1 in the section below). Formation of N-acyl ethanolamines, the group of bioactive compounds that comprises AEA, including membrane phospholipids transformed in a two step enzymatic reaction. The first step consists of formation of N-acylphosphatidyl ethanolamines (NAPE) by transferring a fatty acyl chain from glycerophospholipids to the amino group of phosphatidylethanolamine. In the following step, the NAPE-selective phospholipase D (NAPE-PLD) catalyses the hydrolysis of NAPE to produce both AEA and phosphatidic acid (Di Marzo et al., 1994; Okamoto et al., 2004). The ability of NAPE-PLD$^{-/-}$ mice to convert NAPE to AEA indicates the presence of alternative pathways for synthesis of AEA; conversion of NAPE by phospholipase A2, transformation of NAPE through the intermediate phospho-AEA followed by dephosphorylation of protein tyrosine phosphatise N22 to AEA and as a third possibility, alteration of NAPE by lysophospholipase D (Leung et al., 2006; Liu et al., 2006; Sun et al., 2004). The synthesis of 2-AG is described in a later section.
Figure 1. Retrograde signalling and regulation of cannabinoid synthesis.

Synthesis of endocannabinoids occurs following release of excitatory neurotransmitters and their leakage outside the synaptic region. The action of mGluR leads to release of intracellular Ca\(^{2+}\) and opening of voltage-dependent Ca\(^{2+}\) channels. An elevated level of Ca\(^{2+}\) activates the enzymes responsible for endocannabinoid synthesis from lipid precursors. AEA and/or 2-AG is released and following retrograde diffusion in the synaptic cleft, presynaptic CB\(_1\) is activated. The receptor activation results in inhibited Ca\(^{2+}\) channels and/or activated K\(^+\) channels. The decreased intracellular Ca\(^{2+}\) level then prevents neurotransmitter release from the presynaptic terminal.
Cellular accumulation of AEA

After synthesis and release of AEA into the extracellular space, AEA is effectively removed by cellular uptake followed by enzymatic metabolism (see figure 2).

Figure 2. Cellular accumulation and hydrolysis of AEA. Research has led to several postulated models of AEA transport. (A) Passive diffusion of AEA across the plasma membrane driven by an inward concentration gradient maintained by FAAH. (B) AEA is accumulated by caveolae-dependent endocytosis. (C) Transport of AEA by a specific carrier protein over the plasma membrane. (D) Sequestering of AEA within the cell by a lipid compartment or protein carrier. (E) Following the cellular uptake, AEA is hydrolysed to ethanolamine and arachidonic acid by the enzyme mainly responsible for AEA metabolism, FAAH. AMT= anandamide membrane transporter.
The uptake of AEA into cells, prior to hydrolysis consists of several characteristics that, at first sight, are consistent with facilitated diffusion; time dependency, saturability and temperature dependency, without dependence of ATP or sodium. The fact that the uptake can be inhibited by compounds with structural similarities to AEA is also consistent with a designated transport mechanism (Beltramo et al., 1997; Di Marzo et al., 1994; Hillard et al., 1997). The concept of an AEA transport protein is, however, questioned and other mechanisms that explain the temperature dependency and saturability have been suggested (Bojesen & Hansen, 2006; Thors & Fowler, 2006b).

**Endocytosis**

Transport over the cellular membrane for a large variety of molecules occurs through a basic process described as endocytosis. The diversity of molecules; receptors, viruses, hormones and nutrients included, and the many internalization pathways used for the specific cargo is significant for numerous cell signalling events (Botta et al., 2008; Byers et al., 2008; Chen & Zhuang, 2008; Chlon et al., 2008; Wu et al., 2008). Generally pinocytosis and phagocytosis are the two major forms of endocytosis (Conner & Schmid, 2003). Phagocytosis occurs in cells such as macrophages, monocytes and neutrophils, all specialized phagocytes involved in the immune system, in contrast to pinocytosis that are present in most mammalian cell types.

Reports have suggested that a segregation of lipids is initiating the bud arrangement which results in curving of the membrane and subsequently pinching by, for example, dynamin (Merrifield et al., 2002; Shajahan et al., 2004a; Shpetner & Vallee, 1989; Vallee et al., 1993). In general, the intracellular vesicle is fused into an early endosome. Within the compartment, the molecules are carefully sorted and further transported to different subcellular organelles (Millman et al., 2008; Park et al., 2004; Parton et al., 1992; Prekeris et al., 1999).
Caveolae, a form of lipid rafts sharing the detergent-resistant properties, are invaginations in the plasma membrane identified by the presence of the caveolin (Cav) family, consisting of three proteins. The caveolar membrane fraction also consists of sphingolipids, cholesterol, signalling molecules and glycosyl phosphatidylinositol-anchored proteins, and a variety of molecules, ranging from lipids to pathogens are transported by this path (see figure 3) (Henley et al., 1998; Murata et al., 1995; Oh et al., 1998; Simons & Ikonen, 1997; Yamada, 1955).

Figure 3. Caveolin-dependent endocytosis. Caveolin is synthesized in the endoplasmic reticulum and phosphorylated by an unknown protein kinase to avoid further polymerization. Following transport to the Golgi apparatus, caveolin is dephosphorylated which enables the transport to the plasma membrane and clustering to cholesterol. Deformation of the plasma membrane in association with the actin skeleton allows the internalization of e.g. fatty acids. After pinching off from the plasma membrane the caveolae vesicles fuse with either the sorting endosome or the early endosome, the caveosome. The sorting endosome or caveosome acts as a primarily sorting station where the fate of the cargo is decided.
Phosphorylation of Cav-1, mediated by the Src kinase, is associated with loss of plasma membrane caveolae, a process which generates increased endocytic activity and which is required for caveolae internalization by cholesterol and glycosphingolipids (Glenney, 1989; Ko et al., 1998; Shajahan et al., 2004b; Sharma et al., 2004). Tyrosine kinase inhibitors block the caveolae-dependent endocytosis and such compound are often used to identify raft-dependent endocytosis (Kojic et al., 2007; McFarland et al., 2004). Down-regulation of Src kinases reduces the phosphorylation required for dynamin association with Cav-1 and results in decreased uptake (Shajahan et al., 2004a).

**AEA and endocytosis**

There is evidence that endocytic pathways may contribute to the cellular accumulation of AEA. AEA is capable of associating with membrane compartments and N-acyl ethanolamines can form complexes with cholesterol, an important component in lipid rafts, and other extracellular proteins. The cellular membrane domains involved may regulate AEA uptake, cannabinoid receptor signalling and synthesis (Barnett-Norris et al., 2005; McFarland et al., 2006; Ramakrishnan et al., 2002; Rimmerman et al., 2008; Stremmel et al., 2001).

Depletion of cholesterol by methyl-β-cyclodextrin (MCD) reduced the uptake of AEA and without affecting FAAH (Bari et al., 2005). AEA has also been reported to inhibit cell proliferation through a lipid raft dependent mechanism, possibly by the accumulation of ceramide and recruitment of Fas and Fas ligands into the lipid rafts, a process that was disrupted by MCD and filipin, which cross-links and binds intracellular cholesterol (DeMorrow et al., 2007). The CB₁ activity is associated with the specific membrane domains and treatment of MCD alters the signalling and distribution of CB₁ (Bari et al., 2005; Bari et al., 2008; Sarnataro et al., 2005).

In our hands, cholesterol depletion or sequestering by MCD and filipin treatments reduced the uptake of AEA into RBL2H3 cells *in vitro* (Thors &
Fowler, 2006a). However, the cell viability was significantly reduced (see figure 4) and complicated the interpretation of our uptake data.

Figure 4. Cell viability of RBL2H3 cells after pretreatment of URB597, MCD and filipin for 30 min following incubation of 100 nM $[^{3}H]$AEA for a further 4 min. Cells were stained by trypan blue following completed $[^{3}H]$AEA uptake assay. No effect on the AEA uptake was observed at the lowest concentration of MCD and filipin respectively. (Thors & Fowler, 2006a) n=3. *Significance versus the corresponding vehicle was determined by Dunnett's Multiple Comparison Test following significant one-way ANOVA for repeated measures.

In the first paper implicating caveolae-dependent endocytosis, McFarland et al. (2004) reported that, genistein, N-ethylmaleimide and the combination of nystatin and progesterone reduced the uptake of AEA by approximately half into cells. In addition, fluorescent AEA and immunofluorescently labelled flotillin-1 and CAV-1 established that AEA was co-localized with the endocytotic markers. Within the caveolin-rich membrane, both the arachidonate and ethanolamide part of AEA was identified but only small amounts of intact AEA was observed, measured as accumulation of tritium in the cellular membrane. Inhibition of FAAH decreased the tritium accumulated, suggesting that FAAH is required for enrichment of AEA metabolites. On the other hand, FAAH did not co-localize with CAV-1. In a subsequent study using modified cells, the authors found that the AEA...
uptake pathway was dependent on the endocytosis protein dynamin 2 (McFarland et al., 2008).

**Metabolism of AEA**

Following reuptake, AEA is primarily inactivated through FAAH-catalysed hydrolysis of the amide bond to arachidonic acid and ethanolamine (Cravatt et al., 1996; Deutsch & Chin, 1993; Ueda et al., 1995a). FAAH has a wide substrate selectivity and catalyses the hydrolysis of many fatty acid amides and monoacyl glycerols, including both palmitoylethanolamide (PEA) and 2-AG (Cravatt et al., 1996; Fowler et al., 2001; Goparaju et al., 1998; Schmid et al., 1985). FAAH−/− mice demonstrated a 50-100 fold decrease in hydrolysis of AEA, significant increase in AEA brain levels and up-regulated AEA signalling due to the alteration of the endocannabinoid tone. The behaviour effects induced by AEA were found to be CB₁-mediated and among other things, exhibited a reduced inflammatory response in a model of paw oedema, but the animals did not show behaviour effects of a general activation of CB₁ in the brain (Batkai et al., 2007b; Cravatt et al., 2001; Moreira et al., 2008; Wise et al., 2008; Wise et al., 2007)

The high FAAH expression in the CNS is similar in both humans and rats, but there are general differences in the distribution in peripheral tissue, e.g. FAAH is not detected in skeletal muscle in rats (Cravatt et al., 1996; Giang & Cravatt, 1997). In the brain, the distribution of FAAH and CB₁ are complementary, CB₁ is principally presynaptically located in contrast to the postsynaptic FAAH (Egertova et al., 1998). The enzyme is mainly located to subcellular membranes; established in the smooth endoplasmic reticulum and outer membrane of the mitochondria, but also found on nerve dendrites, frequently co-localized with postsynaptic CB₁ and in adiposomes (Egertova et al., 1998; Gulyas et al., 2004; Oddi et al., 2008).

FAAH belongs to the family of amidase proteins containing the signature region rich in serine, glycine and alanine residues common among more than 80 amidases (Cravatt et al., 1996; Giang & Cravatt, 1997; Goparaju et
al., 1999). A second FAAH enzyme has recently been described; FAAH-2 shared a 20% sequence homology with the original enzyme but with different distribution, inhibitor sensitivity and substrate selectivity. FAAH-2 is identified in primates but is not in mice and rats (Wei et al., 2006).

The first FAAH inhibitors were non-selective compounds such as phenylmethylsulphonyl fluoride and compounds structurally related to the substrates of FAAH such as the ketones, e.g. arachidonyltrifluoromethylketone, and fluorophosphonates, e.g. methoxy arachidonyl fluorophosphonate (Boger et al., 1999; Deutsch & Chin, 1993; Deutsch et al., 1997; Koutek et al., 1994). More recent and more FAAH-selective compounds include the α-ketoheterocyclic inhibitors, such as OL-135, lacking the structure similarity of FAAH substrates, but have exhibited in vivo effects in both mice and rats (Boger et al., 2000; Chang et al., 2006; Naidu et al., 2007). The carbamate-type inhibitor URB597 is frequently used as a fairly selective inhibitor of FAAH with a low nanomolar IC\textsubscript{50} value (Kathuria et al., 2003). In rodent models, URB597 increases the AEA level and cause an effect in many different fields including antinociceptive attenuation, reduction of inflammation, antidepressant-like activity, anxiety and reduction of elevated blood pressure (Adamczyk et al., 2008; Batkai et al., 2004; Fegley et al., 2005; Holt et al., 2005; Jayamanne et al., 2006; Kathuria et al., 2003). In recent years, several pharmaceutical companies have developed FAAH inhibitors, the leading compounds are in phase I and early phase II clinical trials against pain.

In addition to the FAAH-selective inhibitors, compounds that primarily act upon other targets have also been reported to affect FAAH. These include the non-steroidal anti-inflammatory drugs, such as indomethacin and ibuprofen, and the anaesthetic compound propofol (Fowler et al., 1997; Paria et al., 1996; Patel et al., 2003).
Other pathways for AEA metabolism

Several other routes are involved in the AEA metabolisms concurrently with FAAH (see figure 5).

Figure 5. The main degrading pathways and products for AEA. The main degrading pathways revealed in vitro, however, the in vivo significance requests further investigations. EA= ethanolamine; AA= arachidonic acid; PG-EA= prostaglandin ethanolamide; HPETE= hydroperoxyeicosatetraenoic acid; HETE= hydroxyeicosatetraenoic acid; EET= epoxyeicosatrienoic acid

AEA are oxidized by cyclooxygenase 2 (COX-2) to a variety of prostaglandin ethanolamides (PG-EAs) (Kozak et al., 2002a; Yu et al., 1997). Less attention attained but nevertheless involved in the degradation of AEA are the lipoxygenases (LOX), and in comparison to the COX-2 metabolites the LOX derivates are capable of binding to cannabinoid receptors to some extent and to inhibit FAAH, depending on the metabolite investigated (Massi et al., 2008; Ueda et al., 1995b; van der Stelt et al., 2002).

Cytochrome P450 oxygenases are known to act on arachidonic acid to produce the epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), but also to metabolise AEA. In both mouse and human
The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation

tissue, metabolism of AEA by the P450 resulted in a number of oxygenated products (Bornheim et al., 1993; Snider et al., 2007; Snider et al., 2008). The connection between the P450 and cannabinoid system has recently been extended by a novel group of P450 epoxygenase-dependent metabolites of arachidonic acid, with properties of endocannabinoids, that are capable of activating both CB₁ and CB₂ (Chen et al., 2008).

Recycling of AEA metabolites

Recycling of anandamide has lately been investigated in vitro, the production of new AEA molecules derived from supplementary anandamide or arachidonic acid in RBL2H3 cells was established within the plasma membrane, together with previous findings that metabolites of AEA is establish in the cellular membrane following hydrolysis (McFarland et al., 2004; Placzek et al., 2008).

Biosynthesis, uptake and metabolism of 2-AG and PEA

2-AG belongs to the group of monoacyl glycerols, synthesized by utilizing entirely different biosynthetic enzymes and precursors than AEA and through more than one pathway, but similarly produced “on demand” following the enhancement of intracellular Ca²⁺ concentrations (Ben-Shabat et al., 1998; Bisogno et al., 2003; Bisogno et al., 1999; Di Marzo et al., 1999).

In contrast to the fairly well characterized uptake of AEA, the cellular accumulation of 2-AG is very poorly understood. The accumulation of 2-AG share many characteristics with the AEA uptake, such as apparent time- and temperature-dependency, and a membrane transporter has been suggested albeit not proven (Bari et al., 2006; Bisogno et al., 2001; Hajos et al., 2004). However, in contrast to AEA, the 2-AG accumulation is not dependent upon its subsequent metabolism (Beltramo & Piomelli, 2000; Fowler & Ghafouri, 2008).

2-AG is mainly metabolised by the monoacylglycerol lipase enzyme (MGL) to produce the metabolites glycerol and AA, although FAAH also metabolises
this endocannabinoid (Di Marzo et al., 1999; Goparaju et al., 1999; Goparaju et al., 1998; Saario et al., 2004). In addition, 2-AG is a substrate for COX and LOX, to provide metabolites that have biological activity (Guindon & Hohmann, 2008; Hu et al., 2008; Kozak et al., 2002b; Kozak et al., 2000; Moody et al., 2001).

PEA is an endogenous fatty acid amide homologue of anandamide with primarily anti-inflammatory and antinociceptive effects (Costa et al., 2008; Genovese et al., 2008). PEA has no direct effect upon cannabinoid receptors but activates the PPARα pathway to produce analgesic effects (Capasso et al., 2001; Jhaveri et al., 2008; LoVerme et al., 2006). The synthesis of PEA seems to be similar as for AEA, although an alternative two-step process has been proposed (Natarajan et al., 1984; Okamoto et al., 2004).

Very little is known about the cellular uptake of PEA, and the small number of published studies are contradictory. No accumulation of tritiated PEA was reported using the same conditions as for uptake of [3H]AEA by cortical neuron cultures (Piomelli et al., 1999). However, uptake of PEA have been demonstrated in two different cell lines where approximately half of the uptake was due to an apparently temperature-dependent process and the remaining accumulation almost certainly by a passive diffusion process without involvement of FAAH (Jacobsson & Fowler, 2001). In addition, the ability of anandamide accumulation inhibitors to block the uptake of PEA have recently been published (Hillard et al., 2007).

The two major enzymes responsible for metabolism of PEA are FAAH and a second hydrolytic enzyme referred to as PEA-preferring acid amidase. PEA-preferring acid amidase was identified by the dissimilar pH-profile compared to FAAH (Cravatt et al., 1996; Ueda et al., 2001).

**Flavonoids**

Flavonoids are a class of plant metabolites which possess a wide range of biological activities including antioxidant, anti-inflammatory and anti-
cancer activity (Fimognari et al., 2008; Jackman et al., 2007; Khan et al., 2008; Rahman et al., 2006; Ravindranath et al., 2004; Syed et al., 2008). With a wide distribution, ubiquitously in green plants, and a relatively low toxicity compared to other active plant compounds, they have been postulated to provide a variety of health benefits (Bravo, 1998). A normal human diet contains an estimate of 1-2 g of flavonoids daily (de Vries et al., 1997).

The chemical synthesis of plant pigments, flavonoids included has been well investigated. Flavonoids are mainly derived from the amino acid phenylalanine and are divided in numerous groups including isoflavones, flavonoles and catechines (Croft, 1998; Hassig et al., 1999; Schroder & Schroder, 1990). Natural variations; hydroxylation, methylation, acetylation and glycosylation can account for 300 000 members and consequently, flavonoids are one the largest groups of natural products identified (see figure 6). The great structural diversity results in a large variation in profile from one compound to another (Havsteen, 2002).

Figure 6. Molecular structure of the backbone structures of flavonoids and isoflavonoids. The flavonoid backbone consists of a benzene ring condensed with a six-member ring. The position of the benzenoid substituent divides the flavonoid class into (A) flavones (2-position) and (B) isoflavones (3-position).

These compounds usually exist in a pro-form in the plant, modified to limit their biological activity or present in a concentrated form to facilitate sequestering. This allows them to be made available rapidly in times of stress (Graham, 1991). Flavonoids are capable of inhibiting several enzymes, including hydrolases, oxidoreductases and kinases, and the list is
continuously being updated (Kong et al., 2001; Kyo et al., 1998; Li et al., 1997).

The low accumulation by the liver and the direct excretion of the decomposition products with the urine are major disadvantages in the use of flavonoids for medical applications (Calias et al., 1996; Calnan, 1972; Hong et al., 2001; Saladino et al., 2008). Flavonoids have also been shown to complicate drug metabolism (Cermak, 2008; Conney et al., 1980).

The isoflavone genistein and related compounds, including the structural analogue daidzein, possess the capability of binding to the estrogen β receptor, but with a much lower affinity than the sex hormone estradiol, and because of their ability to exert estrogenic activity, are referred to as a phytoestrogens (Hsieh et al., 1998; Miksicek, 1995). Genistein and daidzein is found in soybeans and soy-based foods, and have been of interest as chemoprotective agents for a variety of human diseases and cancers based on epidemiological evidence of reduced cancer rates in populations with a high intake of soy. However, concerns have been raised of potential unfavourable effects due to the activity of the isoflavones on estrogenic receptors and other targets (Goldwyn et al., 2000; Sirtori, 2001). In addition to the estrogenic properties, the compounds produces other beneficial effects, such as inhibition of tyrosine kinase by genistein, a property not shared by daidzein (Akiyama et al., 1987).

**Pain**

**Cannabinoids and pain**

Cannabinoids may affect several sites and exert actions given that cannainoid receptors are found in all neuroanatomical nociceptive pathways and in descending supraspinal pain modulation via the periaqueductal gray and rostral ventromedial medulla (Jayamanne et al., 2006; Katona & Freund, 2008). The first evidence for cannabinoid-mediated analgesia was provided by the pharmacologist W.E. Dixon, demonstrating that dogs does
not respond to the pin prick test after inhalation of cannabis smoke (Dixon, 1899). Further work has predominantly included electrophysiological and neurochemical tests as systemic administration of cannabinoids can produce motor effects in animals that complicated the data analysis where motor responses to pain stimuli are used (Walker & Hohmann, 2005). The formalin test is a commonly used model of inflammatory pain in mice and rats. Injection of formalin into the hind paw of the animal generates a biphasic pattern, an initial phase by acute activation of C and Aδ fibers (5–10 min) and a second phase involving inflammatory reaction in the periphery, CNS sensitization and activation of afferent nociceptors (60–90 min) (Coderre & Melzack, 1992; Patrignani et al., 2005; Puig & Sorkin, 1996; Tjolsen et al., 1992).

Using the formalin model, the antinociceptive properties of cannabinoids have been demonstrated. Systemic administration of synthetic cannabinoid receptor agonists, such as WIN 55,212-2, CP55,940 and HU210, all resulted in suppressed pain behaviour and decreased the stimulus-evoked Fos protein expression induced by formalin (Finn et al., 2003; Guindon & Hohmann, 2008; Tsou et al., 1996).

AEA suppresses formalin pain when administrated intraplantarly, an effect blocked by the cannabinoid receptor antagonists SR141716A and SR144528. Co-administration of AEA and PEA suggested for a CB₁- and CB₂-mediated antinociceptive mechanism (Calignano et al., 1998). However, the effect of PEA have been shown to be mediated by PPARα (LoVerme et al., 2006). The effect of CB₁ and CB₂, in both phases of the formalin test, has also been shown using a combination of AEA and the COX-inhibitor ibuprofen (Guindon & Hohmann, 2008). The involvement of the CB₂ has further been demonstrated. Systemic administration of SR144528 resulted in hyperalgesia and CB₂ receptor agonists, such as HU308, reduced the response in late phase of the formalin test (Beltramo et al., 2006; Devane et al., 1992).
The levels of endocannabinoids following inflammation induced by formalin are evidently inconsistent. The AEA, 2-AG and PEA levels in the paw of rats have been reported to be unaltered and, in contrast, decreased 1 hour after intraplantar injection of formalin (Beaulieu et al., 2000; Maione et al., 2006). In periaqueductal gray-controlled analgesia, elevated levels of AEA in the periaqueductal gray-extracellular fluid have been demonstrated after formalin injection into the hind paw (Walker & Hohmann, 2005). Systemic administration of the FAAH and AEA uptake inhibitor LY2318912 elevated the brain AEA levels and reduced the nociceptive behaviour in the formalin paw-licking model without producing motor function disabilities (Moore et al., 2005). Blocking the FAAH activity by intra-peliaqueductal gray injections of URB597 caused elevated levels of AEA and 2-AG in the ventrolateral periaqueductal gray and either diminished or amplified thermal nociception caused by formalin via TRPV1 or CB₁ (Maione et al., 2006). Mice lacking FAAH revealed an increased AEA level in the brain simultaneously with reduced response pain sensation to formalin (Cravatt et al., 2001). How or, more precisely, where cannabinoids is involved in the pain mechanism is under discussion. In the FAAH⁻/⁻ mice the reduced pain behavior was reversed by the CB₁ located in the brain (Cravatt et al., 2001). However, recently the cannabinoids were reported to mediate analgesia via CB₁ on nociceptive neurons localized in the peripheral nervous system (Agarwal et al., 2007).

**Flavonoids and pain**

The use of complementary and alternative medicine has increased over the years among both adults and children; one study indicated that 40% of Americans use complementary and alternative medicine for treatment of various pain conditions (Eisenberg et al., 1998; Morton et al., 2002; Patel et al., 2007). However, the knowledge of the relationship between dietary compounds and pain is rather poor.

Human intestinal bacteria metabolize the β-glycoside, genistin and daidzin, upon ingestion to form the biological active forms, genistein and daidzein
(Nielsen & Williamson, 2007). The pain-suppressing properties of a soy diet have been shown in both animal models and in clinical studies, however, the exact process(es) involved are not clear (Bryant et al., 2005; Komatsu et al., 2008; Shir et al., 1998; Sun et al., 2005; Yehuda & Carasso, 1987). However, genistein and daidzein have been found to decrease the nociceptive excitability in neurons through a non-specific inhibition of voltage-dependent sodium channels (Batkai et al., 2004). In a model of neuropathic pain, genistein associated to estrogenic receptors produced an anti-allodynic and anti-hyperalgesic result, an effect prevented by a specific estrogenic receptor antagonist (Valsecchi et al., 2008).

The antinociceptive properties of extracts isolated from a range of plants and pure flavonoids, such as quercetin, have to some extent been investigated in the formalin model. A significant inhibition of both phases of nociception have been reported, through several suggested mechanisms, such as linked to either LOX or COX via the arachidonic acid cascade, opioid receptors and serotonin receptors (Ahmadiani et al., 2000; Delporte et al., 2007; Filho et al., 2008; Rajendran et al., 2000; Rinaldi-Carmona et al., 1994).
Aims of the thesis

The cannabinoid system plays many roles in the body. Several potent and selective cannabinoid agonists and antagonists, as well as inhibitors of endocannabinoid metabolism, have been synthesized and characterized for their therapeutic potential. However, the mechanism(s) responsible for the AEA uptake remain controversial, although a potentially attractive target for development of new pharmaceuticals. The general aims of the study have been to shed the light on the mechanisms of the cellular processing of the endocannabinoid AEA utilizing a pharmacological approach. The specific aims of the publications and manuscripts discussed in this thesis are:

**Paper I:** To characterize the AEA retention by synthetic liposomes as a model of membrane accumulation of AEA, in the absence of any proteins.

**Paper II:** To examine the compounds used to demonstrate the involvement of endocytosis on the cellular uptake of AEA (genistein and a combination of nystatin and progesterone).

**Paper III:** To investigate further whether the effects produced by genistein upon the cellular accumulation of AEA in cells primarily is due to inhibition of FAAH or through inhibition of endocytosis.

**Paper IV:** To study the ability of the naturally occurring flavonoids to inhibit the activity of FAAH.

**Paper V:** To evaluate the isoflavones biochanin A and formononetin as inhibitors of FAAH, and to study the effects of biochanin A in vivo relating to peripheral inhibition of FAAH.

**Paper VI:** To investigate the involvement of NO donors in the cellular processing of anandamide metabolites and related endogenous lipids following metabolism by FAAH.
Methodological considerations

Studies included in this thesis mainly consist of in vitro models for characterization of paths involved in the cellular processing of endocannabinoids. The breakdown of multi-step processes into individual components is ideal for understanding the function of a particular mechanism. On the other hand, in vivo models allow elucidation of the importance of a single process in a complex biological system. The details of each method are given in the original papers and so in this section the methodologies are shortly described and subsequently discussed.

In paper V, some experiments were performed by other laboratories; the behavioural effects of biochanin A and the radioligand binding experiments were undertaken at the Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia, USA and the School of Medical Sciences, University of Aberdeen, Aberdeen, Scotland, UK, respectively. The transfections of COS7 cells for expression of human FAAH were carried out at the Department of Chemistry and Skaggs Institute for Chemical Biology, La Jolla, California, USA.

Liposomes (Paper I)

Liposomes were first described by Alec D Bangham and colleague in the early sixties at the Animal Physiology Institute in Cambridge, England. The images from the new electron microscope served as the first real evidence for the cell membrane being a bilayer lipid structure due to the resemblance of negatively stained phospholipids to a plasma membrane (Bangham & Horne, 1964).

A liposome is a vesicle, consisting of the same material as a cell membrane and the properties can vary depending on the lipid composition; however, the same preparation method can be used for all lipid vesicles regardless of composition. Membranes are usually made of phospholipids, which have hydrophilic (polar) heads and hydrophobic tails, resulting in an amphilic
molecule. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment. Conversely, a second layer of heads faces inside the cell, attracted by the water inside the cell. The combined structure forms a bilayer as their hydrophobic tails facing each other (Disalvo et al., 2008; Epand, 2007; Muller et al., 2008).

When cell membrane phospholipids are disrupted, they can reassemble themselves into vesicles either as bilayers or monolayers, a condition used when preparing liposomes for studying cell membrane properties in vitro (Mozafari, 2005). However, liposomes are also an accepted and commercially available strategy to formulate pharmaceuticals for stable administration (Soussan et al., 2009).

Unilamellar vesicles are prepared from multilamellar vesicles (MLVs), the large circular structures formed when amphiphilic lipids are hydrated. Mainly two types of unilamellar vesicles are possible to obtain; "small unilamellar vesicles" (SUVs), usually prepared by sonication, or "large unilamellar vesicles" (LUVs), prepared by a variety of methods including extrusion. SUV are typically smaller in diameter while LUV range from 100-200 nm or larger and are stable on storage, however, SUV will spontaneously fuse when falling below the phase transition temperature of the lipid forming the vesicle (Mozafari, 2005).

In our study, liposomes were prepared by the extrusion method. Briefly, desired quantities of lipids (20 mg/ml) were dissolved in chloroform in a round-bottom flask (for total lipids see the original article). A lipid film was formed by slowly removing the organic solvent by rotary evaporation under reduced pressure for 10 min and thereafter under high vacuum at room temperature over night. The lipid film was re-suspended in buffer, equilibrated by vortexing for 1 hour in room temperature to obtain MLVs. The MLV suspension was subjected to 5 freeze–thaw cycles, involving quenching in -80 °C, followed by immersion in 37 °C water bath. The lipid solution were then stored at -80 °C until the day of the experiment.
On the day of the experiment, LUVs were produced by extrusion 15 times through polycarbonate membranes with pore size of 100 nm on a LiposoFast Extruder at room temperature. The extruded liposomes were immediately used after preparation.

The risk of contamination of proteins was avoided due to use of syntetic lipids, compared to preparations of cell membrane lipids. For future studies, adding potential transport proteins such as fatty acid binding proteins, esterases for fluorescent substrates and other proteins, for example FAAH and COX-2 for enzymatic activity, allows characterizing of the importance of individual constituents of AEA uptake.

**Cell culture (Papers II, III, IV, V, VI)**

RBL2H3 rat basophilic leukaemia cells, C6 rat glioma cells, P19 mouse embryonic carcinoma cells, b.End5 mouse endothelial cells, 3T3-L1 mouse fibroblast cells, PC3 human prostate adenocarcinoma cells and R3327 AT-1 rat prostate carcinoma cells were used. All cells were grown in 75cm² culturing flasks at 37°C with 5% CO₂ in humidified atmospheric pressure.

Cellular accumulation and metabolism of AEA has been characterized in a number of central nervous system-derived cell lines and primary cultures of cerebellar granule cells (Battista et al., 2002; Beltramo et al., 1997; Deutsch & Chin, 1993; Di Marzo et al., 1994; Jacobsson & Fowler, 2001). An underlying assumption of many studies has been that all cells accumulate AEA by the same mechanism; however, inconsistencies between laboratories suggest that this may not be the case. It has been shown that cells that accumulate AEA for signalling purposes may utilize different mechanisms than cells scavenging arachidonic acid (Hillard & Jarrahian, 2005).

C6 cells are widely used in neurobiological research and commonly utilized when investigating the cellular accumulation of AEA. The accumulation of AEA in C6 cells is well characterized allowing comparison of findings between laboratories (Deutsch et al., 2001; Ligresti et al., 2004). RBL2H3
cells were the first cell type with peripheral origin investigated for anandamide uptake and possesses the biochemical components required for endocannabinoid synthesis, receptor signalling, uptake, and metabolism (Bisogno et al., 1997; Rakhshan et al., 2000). Inhibition of FAAH has resulted in a reduced rate of AEA uptake in some cell lines but not in others, in some cases despite of the enzyme expression (Ruiz-Llorente et al., 2004). Comparing cells with various activity of the enzyme, when studying the uptake of AEA, is consequently important.

**Assay of $[^3\text{H}]$AEA uptake (Paper II, III, IV)**

The $[^3\text{H}]$AEA accumulation assay with adherent cells were developed by Rakhshan et al. (2000) and later modified by Sandberg et al. (2005). Briefly, cells were plated in 24 well plates and incubated over night. Following incubation, cells were washed with warm assay buffer (Krebs–Ringer HEPES (KRH) buffer (120mM NaCl, 4.7mM KCl, 2.2mM CaCl$_2$, 10mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), 0.12mM KH$_2$PO$_4$, 0.12mM MgSO$_4$ in MilliQ deionised water, pH 7.4) and preincubated with selected compounds or vehicle in prior of addition of AEA, labelled in the arachidonate part of the molecule. To stop the reaction plates were placed on ice and washed three times with ice-cold assay buffer containing 1% bovine serum albumin (BSA) to terminate the reaction, and finally NaOH was added and the plates were incubated at 75°C to solubilise cells. Following transfer of aliquots to scintillation vials, the tritium content was assayed by liquid scintillation spectroscopy with quench correction.

**Assay conditions throughout accumulation of AEA- a construction of artefacts or helpful tools?**

The conditions in AEA uptake assays are frequently debated and may be a confounding factor for interpretation of the data. The lipophilic nature of both AEA and the putative transport inhibitors cause several difficulties for the researcher to take into consideration.
• Use of BSA in the assay

In all assays, we have used BSA to stabilize the AEA in the buffer, however, this is of controversy among researchers (Glaser et al., 2005b). The addition of BSA to the transport media was reported to completely prevent all observable uptake of AEA, although, may reflect the decreased non-specific binding of AEA (Di Marzo et al., 1994; Karlsson et al., 2004). In paper III, we investigated the sensitivity of fatty-acid free BSA on the AEA uptake in 3T3-L1 cells. An increased concentration of BSA revealed a reduction in cellular accumulation of AEA; however, the uptake was undoubtedly greater than the accumulation seen for wells alone at all concentrations.

Under the conditions used in our studies, less than 10% of the added concentration is readily accumulated by cells and is not saturable. The reduced amount of the added concentration of AEA by BSA, would therefore not impact on the outcome (Thors & Fowler, 2006b). The concentration of free AEA can be calculated using a formula specified for the interaction of AEA with fatty acid-free BSA at a given temperature (Bojesen & Hansen, 2003). The concentration of 15 µM of BSA has been used throughout all experiments.

• Temperature-dependent uptake

Two different assay temperatures, in general 4°C and 37°C, can modify the level of energy-dependency for classical uptake system (Berk & Stump, 1999). However, we have previously found that the temperature-dependent uptake is due to the concentration of AEA available for uptake rather than the assay temperature (Thors & Fowler, 2006b). In addition, (Kaczocha et al., 2006) verified that the calculated unbound AEA concentrations at 0 °C were lower than those at 37°C. Consistent with our findings, the temperature-dependency of Kd affected the amount of AEA available for uptake.
Temperature-dependent effects unrelated to substrate availability can be demonstrated at longer incubation times (Thors & Fowler, 2006b). At incubation times > 1 min a temperature-dependent component of the uptake is present, although, this may reflect the involvement of an intracellular event (Hillard & Jarrahian, 2003).

- **Incubation times**

To determine transport of fatty acids in cells, short incubation times has in general been used (Cupp et al., 2004; Kampf et al., 2006; Schaffer, 2002). The uptake of AEA is a very fast process, but investigations concerning AEA uptake have generally been using long incubation times (Bojesen & Hansen, 2005; Dickason-Chesterfield et al., 2006; Hillard et al., 2007; Ortar et al., 2008). However, the methodology has started to change (Kaczocha et al., 2006).

At short incubation times, ≤1 min, the saturability of AEA uptake has been questioned (Kaczocha et al., 2006; Ligresti et al., 2004; Sandberg & Fowler, 2005; Thors & Fowler, 2006b). The saturated transport of hydrophobic compounds has been explained by the existence of an unstirred layer around cells, causing a significant resistance to the accumulation of AEA (Bojesen & Hansen, 2006).

In contrast, at longer incubation times an apparent saturability is seen. Given that the concentration of AEA at equilibrium is far greater within cells than the concentration in the extracellular media, one possibility is that the saturability imply for an intracellular redistribution of AEA. Binding of AEA to a shuttle protein, such as cholesterol or a larger lipid compartment, would exhibit saturability in uptake assays at longer incubation times (Hillard & Jarrahian, 2003; Oddi et al., 2008; Stremmel et al., 2001).

The involvement of FAAH differs considerably between incubation times used, probably due to the large initial influx of AEA seen before reaching equilibrium. The uptake is significantly dependent of FAAH in cells with
high expression of the enzyme, such as RBL2H3 cells, using ≥ 1 min but evidently independent at time points lower than 1 min (Kaczocha et al., 2006).

- Vehicle concentration

The lipophilic nature of the substrates and inhibitors used raises the question of the solubility of lipids in the solvent used. However, the amount of vehicle (DMSO, EtOH or ACN) was found to affect the quantity of AEA accumulated (see figure 7) and was therefore kept at lowest possible concentration in all assays. Unless otherwise stated, the concentration never exceeded 0.6% in the assay.

![Figure 7](image.png)

Figure 7. Effect of vehicle concentration (EtOH) upon AEA uptake by adherent C6 cells and wells alone (Thors & Fowler, unpublished results).

- Coating of wells

The 3T3-L1 cells used in paper III had adhesion problems to plastic and to avoid causing cell detachment in the numerous washing steps included in the uptake assay, the 24 well plates were coated with either fibronectin, vitronectin, a fibronectin-like protein polymer or 2 % BSA in prior of adding cells. In our hands, the coating did not affect the uptake; nevertheless, fibronectin is involved in many processes of the cell, such as stimulating
TRPV1 translocation, a receptor known to interact with AEA (Jeske et al., 2009; Zygmunt et al., 1999).

So in conclusion, the standard uptake assays only measure the amount of tritium accumulated and are therefore an effective tool for studying known inhibitors or to quickly evaluate novel compounds. However, in order to study specific processes, such as enzyme activity, in detail other methods need to be used.

**Assay of FAAH (Paper II, III, IV, V, VI)**

The main degrading enzyme of AEA, FAAH, is member of the serine hydrolase family which catalyzes the conversion of AEA to arachidonic acid and ethanolamine. A variety of methods, such as high-performance liquid chromatography, thin-layer chromatography and fluorescence-based screening kits, have been used to investigate the activity of FAAH (Glaser et al., 2005a; Huang et al., 2007; Maccarrone et al., 1999). The assay used in this thesis was originally described by Omeir et al. (1995), utilizing chloroform:methanol extraction for separation of the water-soluble ethanolamine from lipophilic AEA and arachidonic acid. In the present form, activated charcoal has been used to separate substrates from products (Boldrup et al., 2004).

The homogenates used were from adult Wistar or Sprague–Dawley rats. The frozen brains (minus cerebella) were thawed and homogenized and thereafter centrifuged at 4 °C. After resuspension in buffer, recentrifugation and a second resuspension in buffer, the pellets were incubated at 37 °C in order to hydrolyse all endogenous FAAH substrates. Following recentrifugation, the pellets were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and frozen at -80 °C in aliquots until used.

For cell homogenates, cells were cultured in culturing flasks and after two washes with ice-cold phosphate-buffered saline (PBS), cells were collected using a rubber policeman. On ice, cells were resuspended in cold PBS
centrifuged and resuspended in 10mM Tris buffer and stored at -80°C in aliquots. The protein content of all homogenates was determined using bovine serum albumin as standard (Harrington, 1990).

In the FAAH activity assay, as previously described by Boldrup et al. (2004), [3H]AEA labelled in the ethanolamine part of the molecule was incubated with the enzyme substrate (homogenates from either intact cells or rat brains as described above) following pretreatment of selected compounds. Tubes were then centrifuged subsequent to addition of an active charcoal mixture to stop the enzyme reaction. Aliquots of the aqueous phase was transferred into scintillation vials and counted with scintillation spectroscopy with quench correction. The lipophilic AEA molecule remains bound to the charcoal, whereas the hydrophilic [3H]EA product formed is transferred to the scintillation vials. Blanks were in all experiments tubes with buffer in place of homogenates.

For experiments using intact cells, [3H]AEA hydrolysis was measured as described by Paylor et al. (2006). Cells were plated in culture medium and allowed to attach to the wells overnight. On the day of the experiment wells were initially washed with warm assay buffer, following the preincubation time of 10 min, the [3H]AEA labelled in the ethanolamine part was added to a final assay concentration of 100 nM. After incubation for 20 min, the culture plate was placed on ice, methanol added and cells were then collected by using a rubber policeman. Aliquots were transferred to glass tubes, chloroform added and tubes were vortexed twice. Following centrifugation, aliquots of the aqueous phase were determined for tritium content by liquid scintillation spectroscopy with quench correction. Blank values were obtained from wells on the same culture plates that had not been seeded with cells.

The final concentration of solvent in the experiments using intact cells did not exceed 0.5 %. In experiments carried out with homogenates, the concentration were higher (5 %), however, in previous studies this
concentration did not affect the rate of hydrolysis of AEA (Ghafouri et al., 2004).

As discussed in the introduction, FAAH is not the only enzyme involved in breakdown of AEA. However, oxidation of AEA by COX or LOX generates lipophilic products and therefore not obtained in the water phase quantified for tritium (Patrignani et al., 2005; Ueda et al., 1995b). In addition, COX inhibitors that do not have built-in FAAH inhibitory properties, were unsuccessful of reducing the AEA hydrolysis in brain homogenates (Holt & Fowler, 2003). The major contribution of FAAH in metabolising AEA have been shown in studies were mice lacking FAAH demonstrated a 50-100 times reduced rate of AEA hydrolyzation (Cravatt et al., 2001).

The observed activity of FAAH and potency of inhibitors observed in the assay used is highly dependent upon pH. The main degrading enzyme FAAH has the best possible activity at ~pH 9 (Holt et al., 2001; Omeir et al., 1995; Ueda et al., 1995a; Ueda et al., 1999). Recently a second “amidase signature” enzyme was identified, FAAH-2, with a maximum rate at pH 8. However, FAAH-1 was much more active in metabolising AEA but both shared similar inhibitor sensitivity profiles. Furthermore, FAAH-2 is not expressed in rodents (Wei et al., 2006). An acidic enzyme, NAAA, with a pH-optimum of 5 is also known to hydrolyze AEA but lacks sensitivity to FAAH inhibitors, such as PMSF (Ueda et al., 1999). The inhibitors of FAAH have also been found to be responsive to the assay pH. URB597 was found to be more effective at pH 8 than at pH 6 in both rat brain homogenates and in intact C6 cells (Paylor et al., 2006). The assay pH of 7.4 has been used throughout all experiments to resemble the environment in the human body.

The use of charcoal to separate the metabolites of AEA avoids the exposure of potentially hazardous organic solvents, such as chloroform, and provides a simple method suitable for screening of new inhibitors of FAAH (Wilson et al., 2003).
Assay of membrane tritium accumulation (Paper I, IV, V, VI)

Cells were pelleted by centrifugation and resuspended in cell culture medium and preincubated with either test compounds or vehicle prior to addition of radiolabelled molecules in culture medium. Subsequent to incubation, cells were washed with ice-cold medium following centrifugation using a microcentrifuge. Aliquots of the cell suspension were placed in 96-well plates and the radioactivity retained by the cell membranes was determined by filtration through polyethylenimine-coated filters using a cell harvester. Rupture of cells was caused by a 30 second period of washing with deionized water and the accumulation of tritium was defined as the radioactivity recovered on the filter papers by liquid scintillation spectroscopy with quench correction. The retention of radioactivity by the filters alone was determined by performing assays without cells. All experiments were carried out in absence of BSA.

The products of FAAH-catalyzed AEA hydrolysis have been reported to be enriched in the lipid raft regions of the cell membrane. Inhibition of FAAH by AM404 and MAFP reduced the amount of tritium localized in the CAV-1-rich membrane fraction of RBL2H3 cells (McFarland et al., 2004).

Measuring the accumulation of tritium in cell membrane fractions after incubation of intact cells in suspension with radiolabelled molecules can be used in two ways, namely 1) a method of examining the pathways involved in the cellular processing of the AEA metabolites and 2) a simple indirect way of measuring the FAAH activity in the cell type used. Consistent with our study, an enrichment of tritium in the lipid raft membrane fraction following incubation of [3H]AEA were not observed in HeLa cells lacking FAAH activity, however, in HeLa cells transfected with the FAAH cDNA, an accumulation of tritium similarly as seen in RBL-2H3 cells, were detected (McFarland et al., 2004).
One potential drawback of the method is the fact that in our experiments cells were kept in suspension throughout the whole experiment, which is not ideal for adherent cells. However, no effect upon the cell viability was seen (see figure 8).

![Figure 8. Cell viability of RBL2H3 cells after completed membrane tritium accumulation assay (Thors & Fowler, unpublished results).](image)

In addition, the ability of Jurkat cells, a suspension cell line, to accumulate [3H]AEA was examined. However, Jurkat cells demonstrated a modest capability of accumulation without any effect of pretreatment of URB597 (results not shown). These results may be explained by the low expression of FAAH in these cells (Aguado et al., 2005).
Animals (Paper V)

For the in vivo experiments, male C57B/6 mice (7-8 weeks, 20-25 g) were used and maintained at the animal facility, Umeå University, on a 12 h light–dark cycle. The mice were fed with standard food and water ad libitum. Animals were kept at least a week at the animal facility before experiments and for one hour in the laboratory room to become acclimatized. All experiments were approved by the local ethical committee at Umeå University. Initial experiments were performed at the Washington University Pain Center, St Louis, USA under supervision of Professor Robert W. Gereau IV.

Immunocytochemistry for phospho-ERK (Paper V)

The mechanisms underlying inflammatory pain have been extensively studied, and evidence suggests that inflammation sensitizes peripheral afferents and dorsal horn neurons (Ji, 2004). Phosphorylation of ERK has proved to play a critical role in dorsal horn neurons in various pain models (Choi et al., 2005; Ji et al., 1999; Karim et al., 2001; Pang et al., 2008; Tang et al., 2007). The present experimental model were therefore selected for studying the involvement of endocannabinoids, by treatment of FAAH inhibitors, in inflammatory pain mediated by sustained ERK activation in dorsal horn neurons.

Mice were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg). Ten minutes (determined from time course of ERK activation) after 3% subcutaneous formalin injection into the hindpaw, mice were perfused transcardially with warm saline (37°C, 0.9% NaCl), followed by ice-cold 4% paraformaldehyde (PFA) solution. L4–S1 lumbar spinal cord sections were dissected out and post-fixed at 4°C with PFA, followed by cryoprotection at 4°C in 30% sucrose for 2 days. Tissue sections were embedded in OCT embedding medium and coronal sections (30 µM) were cut using a cryostat, and sections were kept in PBS for immunocytochemistry.
Sections were incubated at 4°C for 72 hr in anti-phospho-p44/42 ERK primary antibody (1:500 dilution), followed by incubation in a secondary biotinylated anti-rabbit IgG antibody for 2h (1:100) and finally stained with 3,39-diaminobenzidine tetrahydrochloride (DAB) solution (0.025% DAB). Sections were mounted onto superfrost glass slides, coverslipped and observed for the pERK staining.

This model provides a robust tool for investigating the ability of novel compounds upon persistent pain. One concern of this study may be the reality that the animals are under anesthesia during the entire experiment. The impact of pentobarbital on pERK expression has been considered in a few studies and the results have been rather contradictory (Ji et al., 2002; Springell et al., 2005; Takamura et al., 2008). However, in our study was the pERK staining clearly detectably although to a slightly lesser extent on the ipsilateral side of the injection than seen in previous studies using the same model (Karim et al., 2001).

Using fluorescent labelled secondary antibodies, the involvement of several possible mechanisms to explain the altered expression of pERK may be determined.
Results and discussion

AEA retention in liposomes (Paper I)

Unravelling the nature of the cellular AEA transport has been a matter of debate ever since the identification of this endogenous neurotransmitter. A general agreement is that AEA uptake occurs by an ATP-independent process; however, the exact uptake mechanism at the plasma membrane remains unknown. There are several characteristics of cellular AEA accumulation that have been demonstrated repeatedly and can be considered as well supported; the AEA uptake exhibits saturation, temperature-dependency and is inhibited by a variety of compounds, many of them structural analogs to AEA (Di Marzo et al., 1994; Hillard et al., 1997; Maccarrone et al., 2000; Rakhshan et al., 2000). These characteristics are consistent with the hypothesis that AEA movement across cellular plasma membranes involves its interaction with a saturable cellular component. However, other explanations for these effects have been supported (Hillard & Jarrahian, 2000; Kaczocha et al., 2006), and a key issue in establishing the extent to which the uptake properties require a membrane protein transport. To investigate this further, we applied a simple model, liposomes made by commercially available lipids, to study the retention of AEA. The initial experiments consisted of characterizing the established properties of AEA accumulation in this cell-free system.

The retention of AEA was found to be time-dependent using liposomes with a composition of POPC and POPG (9:1 w/w) and in a preparation containing POPC, POPG and cholesterol (8:1:1 w/w) without any difference between the two preparations. However, there are data implicating lipid rafts, regions enriched in cholesterol, in the cellular uptake of AEA. In fact, both cholesterol depleting agents and endocytic inhibitors have been reported to reduce the accumulation of AEA in RBL2H3 cells (Bari et al., 2005; McFarland et al., 2004). In addition, the ability of AEA and related N-acylethanolamines to form complexes with cholesterol has been reported (Di Pasquale et al., 2009; Ramakrishnan et al., 2002). On the other hand, the
specificity of the compounds used has been questioned and the actual ratio of cholesterol in intact cells may also be a dilemma (Spector & Yorek, 1985).

The temperature-dependency and saturability of the AEA retention was investigated in experiments undertaken in 4 ºC and 20 ºC. Similar to experiments performed in RBL2H3 cells with a short incubation time, a saturable and non-saturable component was seen (Kaczocha et al., 2006; Ligresti et al., 2004). At concentration ≤ 100 nM the retention was saturable whereas above the accumulation was linear. There was also marked temperature dependence. The pharmacological sensitivity to VDM11, AM404 and URB597 was also tested. However, none of the compounds had a significant effect upon the retention of AEA.

The results obtained in our study, revealed that liposomes are a straightforward and applicable model of investigating the AEA retentation to membranes. Given that the composition of the liposomes can be extensively manipulated, the model has wide-ranging possibilities.

**Accumulation of AEA in adherent cells (Paper II, III, IV)**

A number of AEA uptake inhibitors have been developed over the years, the majority with reported effects in both in vitro and in vivo. However, the mechanism(s) of action still remain a matter of debate. One difficulty of this matter is that the inhibitors of AEA accumulation have been characterized individually with just a few reports comparing several inhibitors in a single study. An additional issue worth pointing out is that many studies use a single timepoint of AEA incubation, in general a long incubation time (≥ 4 min) that may rather reflect an intracellular event in view of the fact that the membrane translocation of AEA is very rapid (Bojesen & Hansen, 2005).

In this thesis, our goal was to characterize the AEA uptake in several cell lines combining disrupting agents of the various suggested mechanisms involved. In addition, we wanted to investigate the possibility that the
different processes contributes to the AEA accumulation under separate time frames and assay temperatures.

The cell lines have primarily been selected for their different endocannabinoid system properties, such as FAAH activity, rather than for their origin. The RBL2H3 rat basophilic leukemia cells are used in papers reported here, given that the uptake in these cells are well characterized by different laboratories and therefore allow comparison of results (Bisogno et al., 1997; Jacobsson & Fowler, 2001; Rakhshan et al., 2000). The FAAH activity of the RBL2H3 cells, 3T3-L1 mouse fibroblast cells, R3327 AT-1 rat prostate cells, PC3 human prostate cancer cells was examined in paper III and rendered a possibility of investigating the specificity of FAAH-inhibition of the compounds used. The 3T3-L1 cells were also selected due to the high caveolae content as caveola is known to be important in endocytic processes (Sowa et al., 2001; Westermann et al., 2005). The C6 rat glioma cells, commonly used in AEA uptake assays, and P19 mouse embryonic carcinoma cells were also used.

A confounding factor in AEA uptake assays is the ability of AEA to bind to the plastic wells (Karlsson et al., 2004). We have considered this this by performing assays in absence of cells and excluded inhibitors obtaining an effect on the retention of AEA into wells alone, since this impact upon the interpretation of data in cells.

The hypothesis that FAAH regulates the transfer of AEA across the cell membrane by contributing to the concentration gradient that drive AEA from the outside to the inside of the cell is well supported, although not seen in all cells (Day et al., 2001; Deutsch et al., 2001; Glaser et al., 2003; Kaczocha et al., 2006). URB597, a selective FAAH inhibitor, was selected to
determine the involvement of FAAH in all cell lines used (see figure 9).

Figure 9. Effect of URB597 on [³H]AEA uptake in different cell lines. Pretreatment of URB597 (0.1 µM) for 10 min following incubation with 100 nM [³H]AEA for a further 10 min.

At incubation times ≥ 4 min, URB597 (≥ 1 nM) significantly decreased the AEA uptake in the cell lines with measureable FAAH activity (RBL2H3, C6, P19, AT-1), whereas in cells with a low FAAH activity (PC3, 3T3-L1) URB597 had no effect at any concentration tested. At the 1 min time point, the P19 cells was unaffected by pretreatment of URB597, compared to the RBL2H3 cells and C6 cells where concentrations above 0.1 µM was significant. However, the time-dependent accumulation of AEA was unaffected even in the presence of URB597 in all three cell lines (RBL2H3, C6, P19). URB597 did not affect the retention of wells alone. The time-dependent component of AEA uptake may reflect that AEA passes the plasma membrane very rapidly, a process not effected by specific inhibitors, and at longer incubation times the effects seen is owing to intracellular events (Hillard & Jarrahian, 2000). Although inhibition of FAAH contributes to a large decrease in AEA uptake, a residual uptake still remains (~50%) and possibly may other processes be involved as AEA accumulation also is present in FAAH⁻/⁻ mice (Ortega-Gutierrez et al., 2004).
As previously reported by McFarland *et al.* (2004) preincubation of cells with genistein, N-ethylmaleimide or a combination of progesterone and nystatin decreased the AEA uptake by approximately half. Taken together with the reduced AEA accumulation with an 18 °C temperature block, the authors implicated a caveolae-dependent endocytic process of AEA uptake. We re-evaluated this hypothesis by analysing the compounds used by McFarland *et al.* individually and in combination with URB597.

The combination of nystatin and progesterone reduced the retention of AEA into wells by roughly the same quantity as seen for cells (in percentage) and thereby was excluded for further investigation. Genistein (100 µM, as used by McFArland *et al.*) produced a ~50% decrease in AEA uptake in RBL2H3 cells without effect on wells alone. In fact, a preincubation time of 30 min with concentrations of genistein ≥ 5 µM had, in our hands, a significant effect on the cellular accumulation of AEA in both RBL2H3 and P19 cells at all AEA incubation times assessed. The combination of genistein (10-100 µM) and URB597 (0.1-1 µM) did not decrease the AEA accumulation to any further extent than seen for the compounds individually in RBL2H3, C6 and P19 cells. Utilizing the assay of FAAH, genistein was found to be an effective inhibitor of FAAH with a $K_i$ value of 2.8 µM.

The other treatments used by McFarland *et al.* was not examined as N-ethylmaleimide previously has previously been established as a FAAH inhibitor and the temperature-dependency of AEA uptake have been attributed to the reduced amount of AEA available for uptake (Schmid *et al.*, 1985; Thors & Fowler, 2006b).

In paper III, the ability of genistein to inhibit AEA uptake was further examined. Using 3T3-L1 and PC cells, with a low FAAH-activity, and daidzein, a structure analogue to genistein without the ability of inhibiting tyrosine kinases, the accurate mechanism of which genistein is acting on AEA uptake was determined. The effect of genistein were found to be related to its effects upon FAAH rather then tyrosine kinases because pretreatment
of 3T3-L1 cells with genistein or URB597 was without effect upon the AEA accumulation at any concentration tested; in comparison to the RBL2H3 cells were both compounds produced a significant effect. The effect of daidzein mimicked that of genistein and daidzein was also found to be an effective inhibitor of FAAH in both preparations from RBL2H3 cells and intact cells. Kinetic experiments revealed a $K_i$ value of 1.7 µM.

Adding all together, the effects of genistein, daidzein and URB597 on AEA uptake was compared in three cell lines (RBL2H3, PC3 and AT-1). As expected, the level of inhibition was clearly dependent upon the FAAH-activity of the cell line used. In AT-1 and RBL2H3 cells, all three compounds had a significant effect with similar patterns, whereas in the PC3 cells no effect was seen. However, a similar residual activity of AEA accumulation was detected in all three cell lines after treatment. None of the compounds affected the retention into wells alone.

The ability of three flavones to disrupt AEA uptake, due to their ability of inhibit FAAH, was also examined. RBL2H3 and PC3 cells were pretreated with either 7-OH-Flavone (10 µM), 3,7-DiOH-flavone (30 µM) or kaempferol (100 µM) for 10 min in prior of incubation of AEA for 5 min. All substances significantly inhibited the uptake of AEA in RBL2H3 cells (see figure 10), but not in the PC3 cells. The uptake inhibitor OMDM-1 was also tested but, as previously reported, it affected the retention of AEA by wells alone (Fowler et al., 2004).
In conclusion, the present study supports the role of FAAH in the uptake of AEA into cells with a marked activity of the enzyme. Moreover, there is no evidence for an endocytic process to be involved in the process of AEA accumulation. We have also examined dynasore, a reversible dynamin-specific inhibitor and consequently an inhibitor of endocytosis (Macia et al., 2006), but dynasore was without effect at the concentration tested (80 µM) (Thors & Fowler, unpublished results). No matter what, further detective work is required to give the accurate explanation to the case of AEA uptake.

**Inhibition of FAAH by flavones and isoflavones (Paper II, III, IV, V, VI)**

The finding of genistein och daidzein being potent inhibitors of FAAH raised the possibility that flavonoids in general may function as inhibitors of FAAH. Flavonoids are one of the largest groups of substances found in nature,
primarily recognized for their phytoestrogen actions and antioxidant properties, have been suggested to have potential use in treatment of disease such as cancer (Helferich et al., 2008; Li-Weber, 2009; Yang et al., 2009). Consequently, we started to screen flavonoids to discover novel FAAH inhibitors. It should be pointed out, however, that flavonoids are involved in many biological processes when used in micromolar concentrations and a number of pathways share common characteristics with the endocannabinoid system (Cortes et al., 2007; Kuang et al., 2009; Liang et al., 2001; Pagano et al., 2007). Thus, useful when developing new potent and more specific FAAH inhibitors.

In this thesis, the inhibition of FAAH by flavonoids has been studied with several sources of the enzyme, such as intact cells, rat brain homogenates and preparations from mice and rat livers. This has resulted in a widespread knowledge of the inhibitors, for example the potency in the CNS in relation to the periphery and whether the effect varies between species. However, the same procedure was used throughout all experiments, by separating the radiolabelled ethanolamine part of AEA from arachidonic acid and unmetabolised AEA, the activity of FAAH was determined.

In paper IV, we tested the ability of 20 flavonoids and related compounds to inhibit FAAH. The structure-activity relationship of these compounds was determined by analysing the requirement of the hydroxyl substituents for inhibition. 7OH-flavone and 3,7DiOH-flavone were the most potent inhibitors considered in this study. The mutual property of these two compounds was the hydroxyl group located at position 7 of the benzopyran-4-one ring. However, most substances possessed this hydroxyl group although were ineffective as FAAH inhibitors. Additionally, supplementary hydroxyl groups on the benzopyran-4-one ring resulted in a decreased effect. The effect of flavonoids on the hydrolysis of AEA in intact RBL2H3 cells was comparable to the results achieved in the rat brain homogenates described above.
In the selection of isoflavones in paper V, evaluated for the ability to inhibit FAAH, two compounds were identified. Biochanin A and formononetin, together with the previously established genistein and daidzein, were both potent inhibitors of FAAH in the low micromolar range. In contrast to daidzein and formononetin, biochanin A and genistein had consistent inhibitory potency between the species.

The substances identified as potent inhibitors of FAAH are all found in nature, and a certainly significant issue is whether the concentrations required can be reached after dietary ingestion. The two flavonoids ascribed as the most potent FAAH inhibitors, 7OH-flavone and 3,7DiOH-flavone, are both limited by the distribution to very restricted areas in the world and consequently nor likely to be ingested in normal diet. Biochanin A and formononetin are found in red clover extracts and have been isolated and tested for their effectiveness in treating a variety of conditions (Katz, 2002; Low Dog, 2005). Although isolated isoflavone products are very different from the whole herb, the treatment of conditions associated with for instance menopause has been promising due to the estrogenic properties of the compounds (Geller & Studee, 2005). At high doses, plasma concentrations corresponding to ~170 and ~40 nM of biochanin A and formononetin respectively have been observed (Howes et al., 2002). These concentrations are, however, not adequate for inhibition of FAAH. Genistein and daidzein are both found in the soy bean and the plasma concentration of genistein and daidzein measured in humans after dietary ingestion differs greatly between studies (Nielsen & Williamson, 2007). In Asian individuals where a soy-rich diet in common, a relatively high plasma concentration has been established of both daidzein and genistein (~2-4 µM), but considering the concentration required for a decreased FAAH activity in human recombinant cells, these plasma concentrations are unlikely to cause any modification in FAAH activity in a human being (Morton et al., 2002).
Membrane tritium accumulation (Paper IV, V, VI)

The recycling of AEA metabolites is a poorly understood process although it may be important in the biosynthesis of new AEA molecules. The AEA metabolites are suggested to be rapidly enriched in the caveolae of the cell membrane, a process strictly regulated by FAAH. Further on, synthesis of AEA may be derived from exogenous arachidonic acid or AEA. Agents that disrupt the organization of caveolae and stimulation of cells by ionomycin resulted in a decreased synthesis and/or release of AEA (McFarland et al., 2004; Placzek et al., 2008).

For evaluating the process behind the recovery of AEA metabolites, intact cells in suspension were incubated with [3H]AEA and measured for the retention of tritium in the cell membranes. The method was established to be efficient both for evaluating FAAH inhibition and/or recovery of AEA metabolites, and were therefore utilized in our studies in both perspectives. Consistent with the experiment of AEA uptake in adherent cells, the accumulation of metabolites was time-dependent and was significantly inhibited by URB597.

Utilizing the method for evaluating the inhibition of FAAH in intact cells in suspension, a URB-sensitive accumulation of tritium was expressed. The retention of radioactivity attained by cells treated with URB597 (0.1 µM) was substracted from the radioactivity recovered on the filter paper in all other treatments. The effects of 4 flavonoids were examined in this method and were following the same order in potency as seen in the experiments performed with other preparation of FAAH as a substrate. The IC$_{50}$ value for 7OH-flavone and 3,7DiOH-flavone were 0.8 and 0.4 µM, respectively. The URB-sensitive accumulation of tritium was also significantly reduced by the isoflavones biochanin A, genistein and daidzein with IC$_{50}$ values low to submicromolar range (see figure 11).

In paper VI, the recovery of AEA metabolites in the intact cells were examined. URB597 decreased the accumulation of tritium significantly in
RBL2H3 cells without an effect in 3T3-L1 cells, suggesting that the retention occurs following hydrolysis of AEA by FAAH.

NO is an important signaling molecule in many processes and was tested for the possibility of modifying the recovery of AEA metabolites (Bryan et al., 2009). Several classes of NO donors were examined and the following compounds were able of increasing the amount of tritium recovered; SNAP, SNP, NOC-22, SIN-1. The effects of SNAP were significant ≥1 mM for RBL2H3 and ≥2.5 mM for the 3T3-L1 cells. URB597 diminished the effects of NO donors in all three cell lines used (RBL2H3, 3T3-L1 and b.End5 mouse endothelial cells) (see figure 11). NO donors had no effect upon the FAAH activity, indicating that the outcome was separated from FAAH.

![Graph showing the effect of pretreatment prior to tritium retention in RBL2H3 cell membranes following incubation with [3H]AEA, labelled in the arachidonate part of molecule. n=4. * p<0.001 versus the corresponding vehicle, Dunnett’s Multiple Comparison Test following significant one-way ANOVA for repeated measures. # p<0.001 for the comparison indicated in figure, Dunnett’s Multiple Comparison Test following significant one-way ANOVA for repeated measures.](image)

Figure 11. Effect of pretreatment prior to tritium retention in RBL2H3 cell membranes following incubation with [3H]AEA, labelled in the arachidonate part of molecule. n=4. * p<0.001 versus the corresponding vehicle, Dunnett’s Multiple Comparison Test following significant one-way ANOVA for repeated measures. # p<0.001 for the comparison indicated in figure, Dunnett’s Multiple Comparison Test following significant one-way ANOVA for repeated measures.
The increase of $[^3\text{H}]$AEA, labelled in the arachidionate part of the molecule, after pretreatment of NO donors, was also seen with $[^{14}\text{C}]$AA, $[^3\text{H}]$2-AG and, to some extent, $[^3\text{H}]$PEA but not in $[^3\text{H}]$AEA, labelled in the ethanolamine part of the molecule. These results indicated that the effects observed was mainly represented by possibly a strict regulation of arachidonic acid recovery (Marshall & Johnston, 1983).

In further experiments, the exact target for the effects of NO donors in the present study remains to be determined. Extraction of lipids from the cell membrane may reveal the incorporation of tritium into phospholipids, as separation of phospholipids from neutral lipids using specific chromatographic conditions is possible (Di Marzo et al., 1998). In addition, the involvement of MGL in the incorporation into membranes following cell incubation with radiolabelled 2-AG utilizing novel relatively selective and potent MGL inhibitors will also be investigated (Bisogno et al., 2009; Long et al., 2009).

**pERK staining (Paper V)**

Investigations of novel compounds for their anti-inflammatory properties in rodents generally involve intraplantar injections of irritants, such as formalin and carrageenan. We have applied the formalin model to anesthetized C57Bl/6 mice for investigations of the expression of pERK following treatment of the FAAH inhibitors biochanin A and URB597. URB597 has in previous studies, administrerated i.pl., producing benficial effects in several models of pain (Jhaveri et al., 2006; Sagar et al., 2008). In the formalin model used have we evaluated biochanin A and URB597 as analgesic agents and the involvent of CB$_1$ in this process. The highest dose of the two FAAH inhibitors tested, reduced the pERK expression in the dorsal horn of the spinal cord. The CB$_1$ antagonist AM251 had no effect on its own but, on the other hand, blocked the positive effects produced by biochanin A and URB597 (see figure 12).
Biochanin A has limited capability of penetrating the blood brain barrier to attain the brain; the effects seen in the formalin model may so consequently be a result of a restricted peripheral inhibition of FAAH. On the other hand, biochanin A is able of interacting with other targets, such as PPARα and COX-2, which also may contribute to the effects seen (Lam et al., 2004; Shen et al., 2006). Nevertheless, these results are rather promising for the development of novel pharmaceuticals in this field.
Conclusions

This thesis has been undertaken with the main goal of elucidating the mechanisms involved in the cellular processing of AEA. From the present experiments the following conclusions can be drawn:

- For investigating accumulation and transmembrane movement of AEA, liposomes are a simple and useful model and mimic the early properties of AEA accumulation, such as temperature-dependency and saturability.

- Many flavonones and isoflavones are potent FAAH inhibitors. However, these compounds, used in micromolar concentrations, have many biological effects. These may be useful as templates for designing novel drugs.

- The isoflavone Biochanin A produces mainly its effects in the periphery as brain levels of AEA is unaffected subsequent to treatment. Biochanin A may therefore be useful as template for designing novel peripherally acting inhibitors of FAAH.

- The recovery of AEA metabolites following hydrolysis by FAAH is a process that can be modified by NO and may be of significance for recycling of arachidonic acid.
The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation

Populärvetenskaplig sammanfattning


Kroppsegna cannabisliknande substanser (endocannabinoider) bildas och frisätts av celler vid olika sjukdomstillstånd, och har visats vara både antiinflammatoriska och smärtlindrande. De positiva effekterna är dock kortvariga eftersom kroppens celler snabbt tar upp och bryter ner endocannabinoiderna. Anandamid är en fettsyra som snabbt kan förflyttas över cellens membran, det är dock oklart om denna transport sker via ett transportprotein, likt andra signalsubstansers upptag i celler. Däremot är det väl känt att inne i cellen sker nedbrytningen av anandamid av enzymet fettsyraamidhydrolas (FAAH). FAAH kan spela en viktig roll för transporten av anandamid över cellmembranet, då den snabbt bryter ner anandamid och den låga halten på insidan av cellen gör att anandamid dras in i cellen. Detta är dock en av flera hypoteser om hur anandamid tas upp av celler. Syftet med denna studie var att undersöka de cellulära processes som ligger bakom den snabba inaktiveringen av endocannabinoider.

Inledningsvis studerade vi upptaget av anandamid i liposomer, vilket är artificiella membraner som har likheter med biologiska celler men saknar de proteiner som kan påverka upptaget. Vi fann att upptaget i liposomer var känslig för låga temperaturer, precis som i normala celler, men substanser som blockeras upptaget och/eller FAAH hade ingen effekt i liposomerna.

I nästa studie använde vi oss av odlade celler för att studera en process som kallas endocytos. Detta är en vanlig process i celler där membranet veckas och vänds inåt varvid matrial på utsidan av cellen fångas in i
membranstrukturen och transportereras in i cellen. Tidigare forskning har visat att anandamid kan tas upp på detta sätt. Vi fann att genistein, en substans som ska förhindra endocytos, påverkade upptaget. Men vi kunde också visa att genistein är en potent hämmare av FAAH, vilket gör det troligt att effekten av genistein beror av FAAH-hämningsnärings och hämning av endocytos.

Fyndet att genistein är en FAAH-hämmare är av särskilt intresse. Även om FAAH-hämmare för närvarande är under tidig klinisk prövning mot smärta, så är utfallet av dessa studier osäkra varför det finns ett behov av nya substanser som kan hämma FAAH. Genistein tillhör gruppen flavonoider, ett samlingsnamn på ett hundratal närbesläktade ämnen som finns i många olika växter, och har associerats med andra positiva hälsoeffekter, bl a vid klimakteriesvär. I studie tre, fyra och fem undersökte vi totalt ett 30-tal andra flavonoiders förmåga att hämma FAAH. Resultaten visade att sex stycken av dessa var potenta hämmare.

En av dessa flavonoider, biochanin A, valdes för att studera smärtlindring i möss. Den smärtlindrande effekten av biochanin A jämfördes med effekten av den kända FAAH-hämmaren URB597. Båda behandlingarna visade sig ha en förmåga att blockera de nervbanor som är involverade i smärsignaleringen. Den smärtlindrande effekten av biochanin A beror troligtvis på en ökad anandamidhalt, och därmed en ökad aktivitet vid cannabinoidreceptorerna eftersom effekten av biochanin A till viss del kunde blockeras av en antagonist till CB1-receptorn.

Förutom studierna kring återupptaget av anandamid, har vi även undersökt vad som sker med de nedbrytningsprodukter (metaboliter) som bildas av FAAH-metabolismen i cellen. Vi fann att metaboliterna ansamlades i cellens membran, via en process som kunde regleras med kväveoxid, en signalsubstans som också reglerar många andra funktioner i kroppen. Vår hypotes är att dessa metaboliter återanvänds vid bildandet av ny anandamid.
Acknowledgements

This work has mainly been performed at the Department of Pharmacology and Clinical Neuroscience, Umeå University. I am indebted to many people who have made this thesis possible by providing support in many ways.

First of all, my main supervisor Chris Fowler, thank you for always being supporting and inspiring. You have given me lots of opportunities that I never have dreamed of when I started my career at the Department; I will forever be in debt to you!

My co-supervisor Stig Jacobsson, thank you for always contributing with high-quality criticism in no matter what I have done and for constantly reminding me that I’m nothing special. 😊 Many thanks also for reading my thesis and proposing the relevant changes!

My co-supervisor Staffan Tavelin, thank you for teaching me everything I know about liposomes.

Gunnar Tiger, you are a true “pajsare”, but nevertheless an important figure at the Department. Thank you for contributing with your knowledge in both science and teaching together with your magnificent personality.

Very special thanks to Sofia Gustafsson for being my PhD-friend, a wonderful coach in figure skating, for the perfect Saturday night performances and for taking your time to listen whenever I needed. Although we occasionally have been in a fight about how long it takes for the culture medium to attain the correct temperature, you really are something special!

Thanks also to Olov Nilsson for teaching me all about the ceremonies at the University, for giving me a novel first name and for always being open for a discussion. I really miss you at the Department!
Sandra Holt and Kent-Olov “hiphop-Kenta” Jonsson, two previous PhD-students at the Department, thanks for all the help and pleasant time in the beginning of my PhD-period.

Many thanks also to Ingrid Persson, Eva Hallin, Britt Jacobsson, Emma Söderström and Alf Olsson for helping me with all the experimental work, making the everyday labwork easier and finding all the things I needed.

I would also like to take the opportunity to thank Rob, Ben, Judy, Yari, Mena and Mike for taking excellent care of me during my stay in St Louis. I really loved being around!

Thanks to all my darlings in “kollektivet”; Linda, Marre, Marran, Ewe, Martina, Therese, Annelie, Caisa, Kickan, Maria, Tracy, Åsa, Erik, Kicken, Macke, Sjölla, Nyllet, Stefan, Håkan, Ante, Johan, Elias, Hägget, Fluff, Matte, Magnus, Stenlund, Nicke for all outstanding events such as Midsummer’s Eve, Christmas Day and New Years Eve as well as the many Eurotours and evenings with movies, games and, of course, expeditions involving fascinating country roads!

Figure skating has been a big part of my life as long as I can remember. Here in Umeå; Jörgen From, Kristina Johansson, Emma Lindberg and, of course, all the skaters, have contributed to this thesis by allowing me to forget about science and having a great deal of fun for a couple of hours every day, thank you all!

So finally I would like to thank my family for always being there for me (not just during the day, but also when I have forgot about time and called in the middle of the night!). My mother Helene and father Ulf, who have contributed with love, time and joy, not just these years but all my life. My sister Åsa for your patience, its not easy having a little sister like me!
References


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


Costa B, Comelli F, Bettoni I, Colleoni M, Giagnoni G (2008). The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic effects in a murine...
model of neuropathic pain: involvement of CB(1), TRPV1 and PPARgamma receptors and neurotrophic factors. *Pain*.


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


Fowler CJ, Jonsson KO, Tiger G (2001). Fatty acid amide hydrolase: biochemistry, pharmacology, and therapeutic possibilities for an enzyme hydrolyzing anandamide,


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


The cellular processing of the endocannabinoid anandamide and its pharmacological manipulation


Do cannabis-based medicinal extracts have general or specific effects on symptoms in multiple sclerosis? A double-blind, randomized, placebo-controlled study on 160 patients. *Mult Scler* **10**: 434-41.

Cannabinoid mechanisms of pain suppression. *Handb Exp Pharmacol* **509-54**.

Dynamin, a GTPase involved in the initial stages of endocytosis. *Ciba Found Symp* **176**: 185-93; discussion 193-7.


Assessment of anandamide’s pharmacological effects in mice deficient of both fatty acid amide hydrolase and cannabinoid CB1 receptors. *Eur J Pharmacol* **557**: 44-8.


