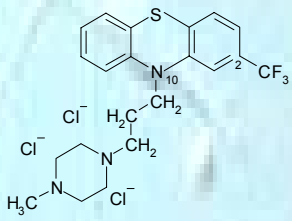

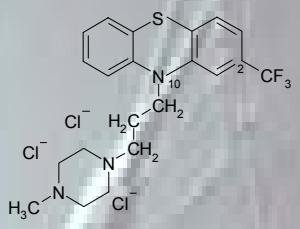

TRIFLUOPERAZINE:

EFFECTS ON POLYPHOSPHOINOSITIDE METABOLISM IN NEUROBLASTOMA CELLS AND ON GLYCEROPHOSPHOLIPID MONOLAYERS

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Bergen, May 2003

Anne Berit Ølmheim

CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CMP	cytidine monophosphate
CPZ	chlorpromazine
CTP	cytidine triphosphate
DAG	diacylglycerol
DAGK	diacylglycerol kinase
DPPC	dipalmitoylphosphatidylcholine
DPPE	dipalmitoylphosphatidylethanolamine
DPPS	dipalmitoylphosphatidylserine
DSPS	distereoylphospatidyserine
EFA	essential fatty acid
ER	endoplasmic reticulum
GDP	guanidine diphosphat
GTP	guanidine triphosphate
IP_3	Inositol triphosphate
NGF	nerve growth factor
PA	phosphatidic acid
PACT	phospatidic acid cytidine transferase
PAP	phospatidic acid phosphatase
PC	phosphatidylcholine
PE	phosphatidylethanolamine

PKC	Protein kinase C
Pi	inorganic phosphat
PI	phosphatidylinositol
PI4P	phosphatidylinositol 4-phosphate
PI4,5P2	phosphatidylinositol 4,5-phosphate
PI4K	phosphatidylinositol 4-kinase
PIP5K	phosphatidylinositol 5-kinase
PI4,5,PH	phosphatidylinositol 4-phosphatase
PI4PPH	phosphatidylinositol 5-phosphatase
PIS	phosphatidylinositol synthase
PLA2	phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PPI	polyphosphoinositide
PS	phosphatidylserine
SAPS	1-stearoyl,2-arachidonoyl- phosphatidylserine
SDPS	1-stearoyl,2-docosahexaenoyl-phosphatidylserine
SLPS	1-stearoyl,2-linolenoyl- phosphatidylserine
SOPS	1-stearoyl,2-oleoyl- phosphatidylserine
TFP	trifluoperazine

SUMMARY

The effects of trifluoperazine (TFP) on the phosphoinositide metabolism in the neuroblastoma cell line SH-SY5Y have been investigated. TFP is a cationic amphiphilic phenothiazine, which has been used in psychiatry for decades. Its exact mechanism of action is not understood, but a non-specific antagonism of a number of receptors in the CNS is reported. [^{32}P]Pi-prelabelled SH-SY5Y cells were incubated with TFP with varying concentrations and exposure times. The level of [^{32}P]Pi after 20 min exposure to 10 μM TFP in phosphatidylinositol (PI) increased 5 fold compared to controls. The level of [^{32}P]Pi phosphatidic acid (PA), increased 2 fold under the same conditions. The effects of TFP on the [^{32}P]Pi content of phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol-4,5-bisphosphate (PI4,5P$_2$) was seen as a small decrease in radioactivity in PI4,5P$_2$, and a low increase in PI4P (0,5 fold at 10μM TFP). To our knowledge this is the first demonstration that TFP interferes with the polyphosphoinositide metabolism in resting cells. Different concentrations of carbachol increased the rate of incorporation of [^{32}P]Pi into PA and PI and reduced the incorporation rate into PI4P and PI4,5P_2 in accordance with work performed by others(Quist and Satumtira 1987; Wei and Wang 1987; Morris, Cook et al. 1990; Frolich, Aarbakke et al. 1992; Limatola, Pacini et al. 1996; Sorensen, Linseman et al. 1998; Willars, Nahorski et al. 1998). Stimulation with carbachol concentrations between 0-5 μM lowered the [^{32}P]Pi contents in PI and PA produced by 5μM TFP alone. Atropine, a muscarinic receptor antagonist, also reduced the level of radioactive PA and PI produced by TFP. The amphiphilic character of TFP would allow it to interact with the membrane surrounding the enzymes in the polyphosphoinositide metabolism, possibly explaining why a change in enzyme activity is observed. To further investigate the possible membrane interaction of TFP, the Langmuir isotherm technique is used. The mean molecular area (mma) for a monolayer of the acidic phospholipids, phosphatidylserine (PS), was markedly increased by introducing the positively charged TFP (1 μM) into the subphase, while neutral phospholipids showed no interaction with TFP. The fatty acid composition in PS also affected its interaction with TFP. The mma

decreased with increasing unsaturation in the fatty acid in the *sn*-2 position, except in the case of 22:6 (n-3) where the mma was slightly higher than with 20:4 (n-6).

1 INTRODUCTION

1.1 Neuroblastoma cells (SH-SY5Y)

Human neuroblastoma is a cancer occurring in early childhood. It originates from areas around the adrenal medulla and the paraspinal site, where sympathetic nervous system tissue is present. It is an aggressive tumour that metastasis early and widely.
The SK-N-SH continuous cell line was established after a bone marrow aspiration in 1970 from a patient, a 4 year old girl, who had been treated for neuroblastoma cancer for 6 months with radiation and chemotherapy (Biedler, Helson et al. 1973).
In 1973 June L. Biedler and his colleges found that the SK-N-SH cell line contained two distinctively different cell types. One was a large epithelioid like cell the other was a denser and smaller cell, forming focal aggregates. They also found that the SK-N-SH cell line contain 47 chromosomes, with one was a structurally abnormal larger M1 chromosome from an unknown origin, and another, an abnormal M2 chromosome, which was proposed to originate from chromosome nr. 21-22, since one in this pair was missing (Biedler, Helson et al. 1973). Later three different distinct cell morphologies were characterised, the neuroblastic cells (N), the flat and substrate adherent cells (S) and morphologically intermediate cells (I) (Ciccarone, Spengler et al. 1989). (See table 1.1.1)

Table 1.1.1 *SK-N-SH clones: neuroblastic cell types (N), S-type morphology (S) and intermediat morphology (I) (Ciccarone, Spengler et al. 1989)*

Parental cell line	Subline designation	Cell type	Description
SK-N-SH	SH-SY5Y	N	Thrice cloned
	SH-EP	S	Clonal subline
	SH-EP1	S	Twice cloned
	SH-EP1E	S	Thrice cloned
	SH-IN	I	Clonal subline

SK-N-SH cells have been reported to show many indications of neuronal origin, for example they express high dopamine-β-hydroxylase activity, an enzyme found only in sympathetic nervous tissue (Biedler, Helson et al. 1973).

Some of the important cell signalling pathways in this cell line indicative of neuronal origin are listed in table 1.1.2 and below.

Table 1.1.2

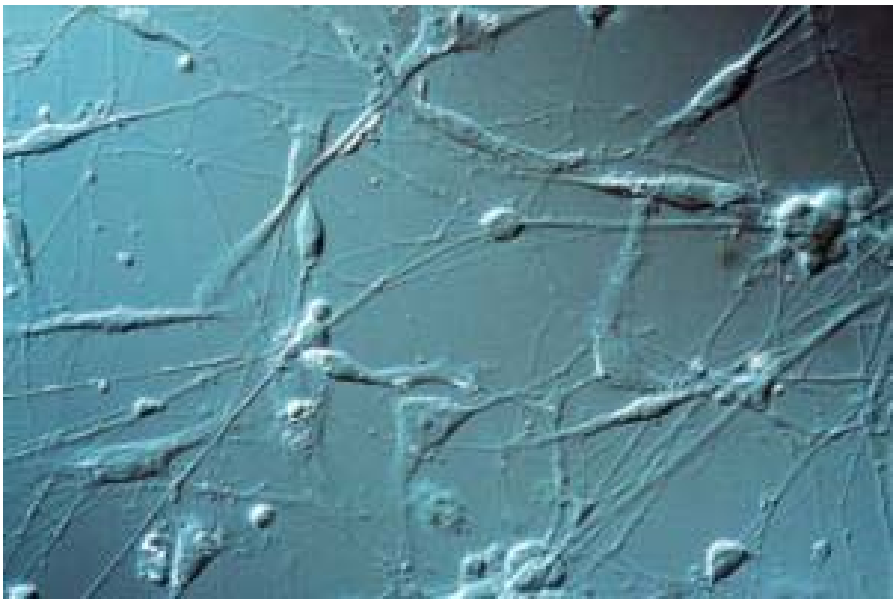
SIGNALLING PATHWAYS INDICATING NEURONAL ORIGIN.	
1) Dopamine-β-hydroxylase activity	6) No Fibronectin synthesis
2) Noradrenergic biosynthetic enzymes	7) Opiod (μ and δ) receptor expression
3) Norepinephrine uptake mechanism	8) Muscarinic M3 receptors
4) Tyrosine hydroxylase activity	9) Nicotinic receptors
5) Different response pattern for different growth factors; Neurite outgrow: NGF Mitogenic response: bFGF, EGF	

Table 1.1.2 shows that the SK-N-SH cell line possesses noradrenergic biosynthetic enzymes and norepinephrine uptake mechanisms. Tyrosine hydroxylase activity has been reported, but no tyrosinase activity is detected. They have no fibronectin synthesis and expression of

intermediate filament proteins of 68,000M_r, and 150,000 M_r (Ciccarone, Spengler et al. 1989). They express opioid (μ and δ) receptors (Sadee, Yu et al. 1987) and homogeneous M3 subtype muscarinic receptors associated with hydrolysis of polyphosphoinositides (Baird, Lambert et al. 1989) (Serra, Mei et al. 1988). Nerve growth factor receptors (Sonnenfeld and Ishii 1985; Rettig, Spengler et al. 1987; Sadee, Yu et al. 1987) are present but they do not respond equally to the different types of nerve growth factors, for example, basic FGF (bFGF) and endogenous epidermal grow factor (EGF) give mitogenic responses (Janet, Ludecke et al. 1995).

The SH-SY5Y cell line is of SK-N-SH origin (table 1.1.1) and exhibits a small rounded, loosely adherent cell body and has a large nuclear/ cytoplasm volume ratio (Ciccarone, Spengler et al. 1989). In addition, they show neurite outgrowth in the presence of nerve growth factor (NGF), tumour promoters and retinoic acid (Spinelli, Sonnenfeld et al. 1982). (See figure 1.1.1)

Figure 1.1.1



Neuroblastoma cells grown in the present of NGF *Taken from "Topical Treatment with Nerve Growth Factor for Corneal Neurotrophic Ulcers, published on the 24th April issue of "New England Journal of Medicine", 2003*

1.1.1 Molecular species of glycerophospholipids in SH- SY5Y cells

The phospholipid composition of the plasma membrane is known to affect receptor activities (Murphy 1990) and transmembrane signalling (Nishizuka Y 1984). The cell membrane lipid composition in SH-SY5Y cells is slightly different from its origin, the SK-N-SH cells, but both accumulate ω-9 fatty acids into phosphatidyl inositol (PI) indicating essential fatty acid (EFA) deficiency. EFA deficiency is associated with a reduction in ω-6 fatty acids and an accumulation of ω-9 fatty

acids such as (18:1(n-9) and 20:3(n-9) in lipids (Holman 1968). SK-N-SH cells accumulate 20:3(n-9) acids even when exposed to 20:4 (n-6) acid as supplement in the growth media (Lee).

The major phospholipid classes in SH-SY5Y cells are 60% PC, 15% PE, 10% PI and small amount of PS (Liepkalns, Myher et al. 1993). PC hydrolysis is activated by hormones and neurotransmitters such as thrombin and platelet-derived growth factor (PDGF), and contributes to cell signalling by producing PA via an active phospholipase D (PLD), and DAG via phospholipase C (PLC) (See Figure 1.1.2). PE contributes less to cell signalling than PC as Figure 1.1.3 shows, giving only free fatty acids upon stimulation of phospholipase A_2. PI is a major phospholipid in the PPI cycle and will be given great attention in section 1.1.2.

Figure 1.1.2

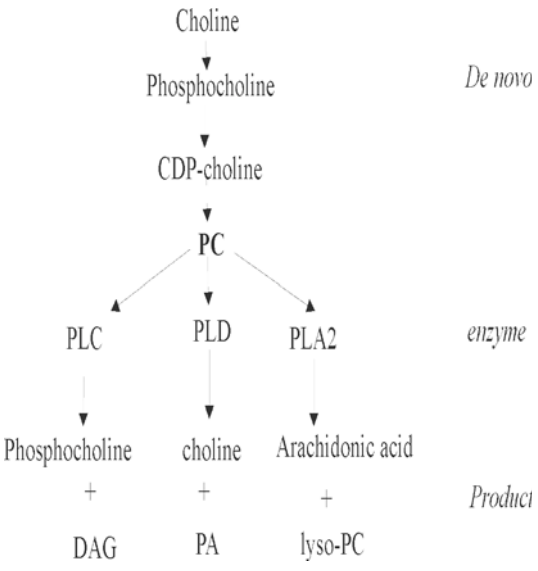


Figure 1.1.3

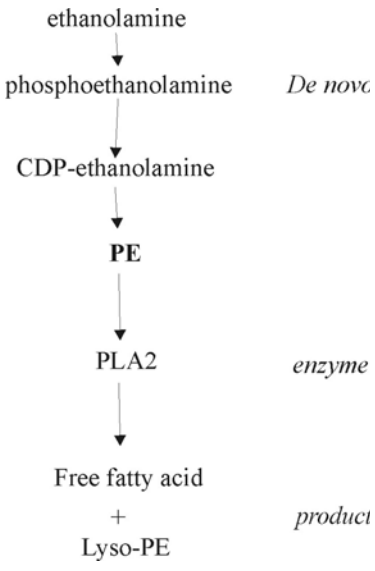


Table 1.2.1 *Molecular specie compositions for PC, PI and DAG in SH-SY5Y cells (Liepkalns, Myher et al. 1993)*

Molecular species	Retention time	Glycerolipid classes, mol%		
		CGPL[a]	IGPL[b]	DAG[c]
14:0–16:0	4.41	2.11 ± 0.73	1.60 ± 1.23	2.15 ± 0.15
	4.55	0.68 ± 0.15		
	4.83	0.30 ± 0.00		
15:0–16:0	4.92	0.76 ± 0.22		
34′	5.07	1.55 ± 0.12		
		1.08 ± 0.49		
16:0–16:0	5.48	5.57 ± 1.50	2.71 ± 2.04	3.48 ± 0.40
	5.67		1.12 ± 0.33	
16:0–16:1	6.63	8.07 ± 0.98	0.29 ± 0.10	4.88 ± 2.44
		1.67 ± 0.73	1.22	0.34 ± 0.34
	6.14	0.22 ± 0.03		
16:0–18:0	6.23	0.55 ± 0.14		2.71 ± 0.35
16:0–18:1	6.69	2.71 ± 0.89	1.48 ± 0.68	10.35 ± 2.46
16:0–18:1	6.95	38.45 ± 1.32	1.95 ± 1.36	4.49 ± 0.63
16:1–18:1	7.11	4.43 ± 0.53	0.93 ± 1.11	
16:1–18:1		0.86 ± 0.56	0.39	
16:0–20:3(*n* − 9)	7.62	0.56 ± 0.14		2.97 ± 0.08
18:0–18:1(*n* − 9)	8.58	8.16 ± 1.83	5.11 ± 0.38	2.38 ± 0.47
18:0–18:1(*n* − 7)	8.69	1.92 ± 0.14	1.02 ± 0.69	
18:1(*n* − 9)–18:1(*n* − 9)	8.88	7.72 ± 1.62	1.20 ± 0.36	4.43 ± 1.46
18:1(*n* − 9)–18:1(*n* − 7)	9.00	3.32 ± 0.15	0.75 ± 0.37	1.76 ± 0.07
	9.24	0.95 ± 0.11	0.45	
16:0–20:4	9.31	2.36 ± 1.27	1.39 ± 0.48	0.38 ± 0.38
	9.51	0.28 ± 0.07		
17:0–20:3(*n* − 9)	9.97	0.16 ± 0.02	0.27 ± 0.00	
17:0–20:4(*n* − 6)	10.49	0.19 ± 0.11	0.36 ± 0.30	
	11.00	0.67 ± 0.32		
	11.18	0.48 ± 0.15		
18:0–20:3(*n* − 9)	11.38	0.59 ± 0.25	11.70 ± 12.64	16.86 ± 9.07
18:1–20:3(*n* − 9)	11.53	0.71 ± 0.24	3.40 ± 3.23	5.03 ± 2.68
18:0–20:4	11.69	3.37 ± 1.59	46.23 ± 11.01	32.81 ± 2.44
16:0–22:4(*n* − 6)	12.10	0.64 ± 0.27		
	12.31	1.86 ± 1.09		
18:1(*n* − 9)–20:4(*n* − 6)	12.49	0.59 ± 0.32	7.79 ± 1.69	
18:1(*n* − 7)–20:4(*n* − 6)	12.67	0.20 ± 0.14	2.83 ± 0.53	1.33 ± 1.33
16:0–22:5/22:6	13.10	0.66 ± 0.33	2.28	
	14.55	0.28 ± 0.09		
18:0–22:3(*n* − 9)	15.29	0.35 ± 0.08	1.40 ± 1.43	2.50 ± 1.30
18:1(*n* − 9)–22:3(*n* − 9)	15.56	0.43 ± 0.22	1.65 ± 0.90	0.75 ± 0.75
18:1(*n* − 7)–22:3(*n* − 9)	15.91	0.18 ± 0.13	1.64 ± 0.57	1.83
	16.45	0.29 ± 0.10	0.89	
18:0–22:5/22:6	16.81	0.63 ± 0.30	1.52 ± 0.37	
	17.94	0.38 ± 0.16	0.51	

NOTE: Molecular species are identified by the paired fatty acids, which are specified by carbon:double bond number. The fatty acid indicated on the left usually occupies the *sn*-1-position of the glycerol molecule.
[a]Mean ± SD (n = 4).
[b]Mean ± SD (n = 3).
[c]Average ± range/2.

In PC, 16:0/18:1 dominate in the *sn*-1 and *sn*-2 position respectively, and in PI, 18:0/20:4 (n-6), 18:1 (n-9)/20:4 (n-6), and 18:0/20:3(n-9) species are most abundant. The large amount of

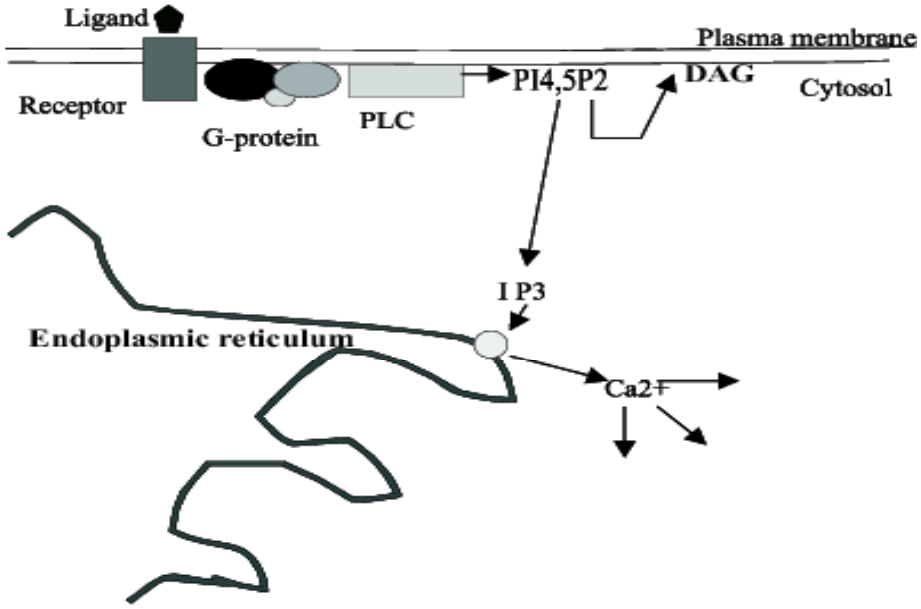
18:0-20:3(n-9) indicates again an EFA deficiency in these cells. E. B Stubbs and colleagues (1992) stimulated the muscarinic receptor with carbachol causing breakdown of $PI4,5P_2$, and showed that the 18:0/20:3(n-9) species is favoured in the PPI cycle.(Stubbs, Carlson et al. 1992).

1.1.2 Phosphoinositide signalling in SH-SY5Y

Many receptors for neurotransmitters, neuropeptides and neurohormones are coupled to the phosphoinositide signalling system. Agonist binding leads to an activation of phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-bisphosphate ($PI4,5P_2$) into the second messengers, diacylglycerol (DAG) and inositol-1,4,5-*tris*phospate (IP_3). DAG activates protein kinase C (PKC), and IP_3 stimulates Ca^{2+} release from endoplasmic reticulum (ER). (See Figure 1.1.4)

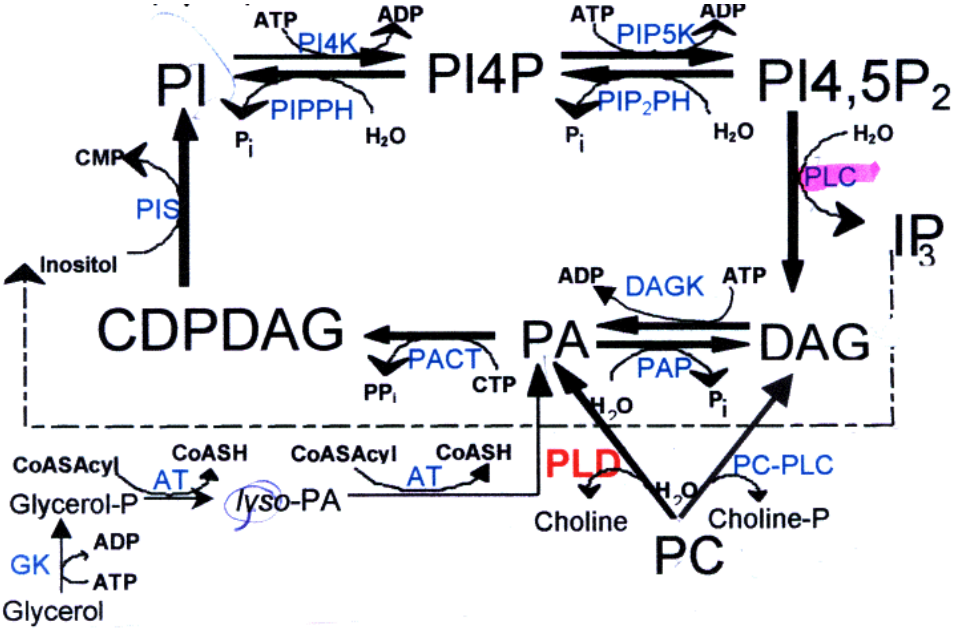
The receptors are coupled to G-proteins, and upon agonist binding a conformational change in the receptor takes place. This change activates the heterotrimeric G- protein which consists of α, β and γ subunits. The α subunit exchanges its GDP with cytosolic GTP, dissociates from the $\beta\gamma$ complex and activates PLC. In SH-SY5Y cells the different G-protein α subunit pattern is similar to that of the human brain cortex, with a high level of Go α and Gi α (Klinz, Yu et al. 1987). Three types of PLC have been identified, PLCα, PLCβ, and PLCγ, where PLCβ is the subtype associated with the G-protein coupled receptor system (Pacheco and Jope 1996).

Figure 1.1.4



G-protein receptor coupled activation of PLC.

Figure 1.1.5



The phosphoinositol signalling pathway (The PPI cycles). *Full names on all components involved are listed in; List of abbreviation and below.*

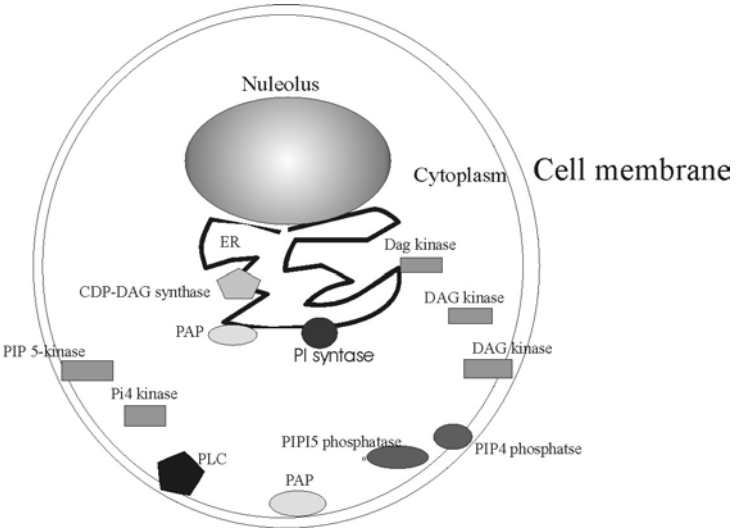
The major component among inositol phospholipids are PI followed by PI4P and $PI4,5P_2$. $PI4,5P_2$ makes up about 5% of the total cellular inositol phospholipid content under basal conditions(Willars, Nahorski et al. 1998). Agonist induced cleavage of $PI4,5P_2$ has been demonstrated to occur at the plasma membrane (Seyfred and Wells 1984; Uhing, Prpic et al. 1986) but questions has been proposed as to what extent the whole PPI cycle is localised in the plasma membrane. To answer this question S, J. Morris and colleges demonstrated in 1989 that only two of total five enzymes required for $PI4,5P_2$ synthesis (see figure 1.1.5) are situated in the cell membrane of murine neuroblastoma cells (N1E-115)(Morris, Cook et al. 1990). Most of the activity of specific $PI4,5P_2$ PLC is seen in the cell cytosol, but a small amount is located at the plasma membrane, being responsible for the agonist-sensitive hydrolysis of $PI4,5P_2$ (Morris, Cook et al. 1990). DAG is phosphorylated to phosphatidic acid (PA) by DAG kinase, which is present in

the cell cytosol, plasma membrane, and endoplasmic reticulum in the brain as well as in cultured cells of neural origin (glioma cells)(Morris, Cook et al. 1990). The membrane-bound DAG kinase in 3T3 cells shows specificity towards arachidonoyl-containing DAG produced from $PI4,5P_2$ hydrolysis (Seyfred and Wells 1984). The cytosolic DAG kinase exhibits no such acyl specificity, and is thought to play a role in the platelet derived growth factor (PDGF) stimulated formation of PA in these cells(Morris, Cook et al. 1990). PA is also generated de novo from lyso-PA, and from hydrolysis of PC by phospholipase D (PLD), another phospholipase that cleaves phospholipids at the diesterphosphate (see figure 1.3.2). PA can also bee dephosphorylated by phosphatidate phosphatase (PAP) located at the cytosolic part of the plasma membrane. PA accumulates in the plasma membrane (Bennett, Cockcroft et al. 1982) and has to be transported to the ER for further processing, but the mechanisms here are still not known (Whatmore, Wiedemann et al. 1999). The next step in the PPI cycle is formation of cytidine diphosphate diacylglycerol (CDPDAG), a liponucleotide intermediate, made from cytidine triphosphate (CTP) and PA by CDP-DAG synthase, followed by formation of PI by PI-synthase. It is believed that CDP-DAG and PI are synthesized in the endoplasmic reticulum (Hokin 1985; Bishop and Bell 1988), since there is no CDP-DAG synthase or PI synthase activity reported in neuroblastoma plasma membrane (Morris, Cook et al. 1990). Small fractions of these enzymes are reported to be present in the plasma membrane in some tissues(Takenawa, Saito et al. 1977; Imai and Gershengorn 1987),

suggesting the possibility that some activity in the plasma membrane can occur. This observation however is not supported by S.J Morris et al.,1989 who report no such findings in neuroblastoma cells. PI needs to be transported back to the plasma membrane by the soluble phosphatidylinostol-transfer protein (PITP) for further phosphorylation. The origin of resynthesised PI has been studied by J. Whatmore (1999) and she found that resynthesis of PI requires an active PLC, not PLD, since PA generated by PLD was not utilized in PI resynthesis (Whatmore, Wiedemann et al. 1999). Recent studies also show that DAG produced from PI4,5P_2 hydrolysis is resynthesised back into PI, whereas DAG from alternative sources is not (Sillence and Low 1994).The steady-state levels of the polyphosphoinositides are maintained by Mg^{2+} dependent kinases and phosphatases. PI 4-Kinase and PI4P 5-kinase are widely distributed among the different cellular compartments, and have also been reported to be present in the plasma membrane. These results are however somewhat inconsistent. S.J Morris et al. (1989) found no PI 4-kinase in the plasma membrane

but rather in the cytosol, associated with the Golgi apparatus. Phosphorylation of PI to PI4P can then still take place at the surface of the inner membrane leaflet. PI4P 5-kinase is exclusively localised in the plasma membrane of the glioma cells (Morris, Cook et al. 1990). PI4,5P_2 phosphatase is an entirely soluble enzyme not directly attached to the plasma membrane, but PI4P phosphatase, on the other hand, is dominantly active in the plasma membrane in glioma cells (Morris, Cook et al. 1990) These findings from the neuroblastoma and glioma cells indicate an incomplete ability to synthesis all components in the phosphoinositide signalling pathway in the plasma membrane alone. S.J Morris et al.,1989 does however not exclude the possibility for a small agonist induced recycling pool of the polyphosphoinositides at the plasma membrane (Morris, Cook et al. 1990).

Figure 1.1.6



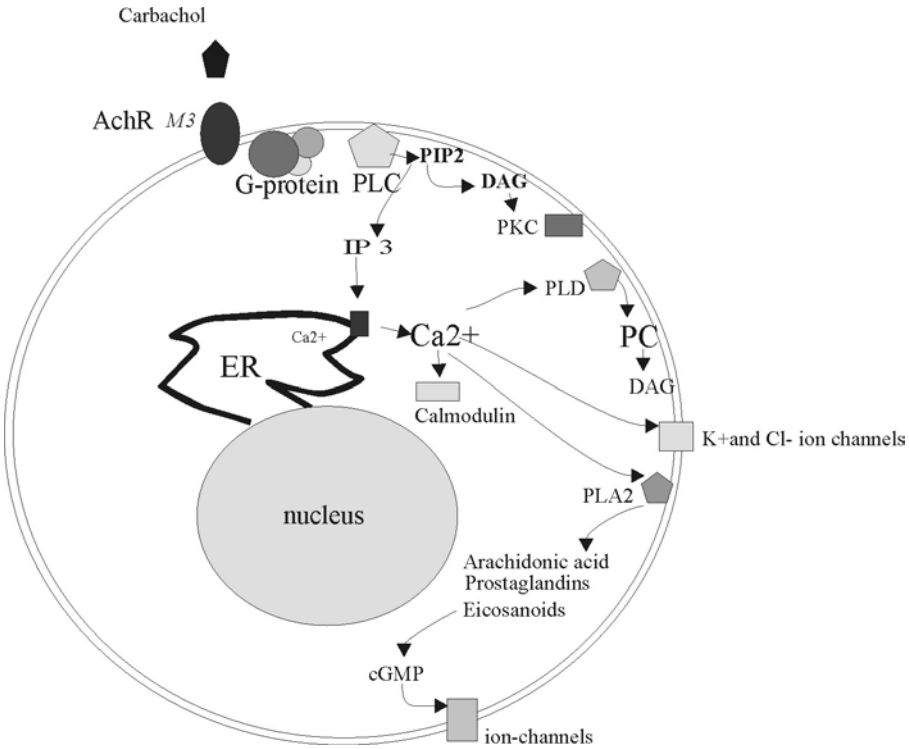
The cellular location of the different enzymes in the PPI cycle

Regulation of the PPI cycle depends upon many different regulation mechanisms at the receptor level as well as downstream from PLC. For example, PLC activity depends on Ca^{2+} mobilisation as a feedback regulation and an amplifier for $PI4,5P_2$ hydrolysis (Wojcikiewicz and Nahorski 1993). PLC-linked receptors are rapidly desensitised by phosphorylation and internalisation (Wojcikiewicz and Nahorski 1993; Sorensen, McEwen et al. 1997).

1.1.3 The Acetylcholine receptor in SH-SH5Y

The muscarinic receptors are involved in various neuronal functions in the CNS and the autonomic nervous system. Five different subclasses of the muscarinic receptors are identified; the M1, M2, M3, M4 and M5 receptor, and they are all G- protein coupled receptors activating different signalling pathways in different cell types.
In SH-SY5Y cells the M3 receptor is predominant among the muscarinic receptors (Lambert, Ghataorre et al. 1989), and it activates hydrolysis of $PI4,5P_2$, via G-protein activated PLC, generating the second messengers IP_3 and DAG. (See figure 1.1.7)

Figure 1.1.7.



Carbachol activation of the M3 receptor

In SH-SY5Y cells, the PI4,5P_2 mass has been determined to be 360±27 pmol/mg protein, and upon maximal agonist activation of the M3 muscarinic receptors this mass can be reduced to

about 70% of basal level(Willars, Nahorski et al. 1998), indicating increased phosphorylation of PI4P to meet the need for PI4,5P2 (Willars, Nahorski et al. 1998). With use of wortmannin, claimed to block the PI4P kinase and thereby PI4,5P2 supply, GB Willars and colleges (1997) showed that about 15% of basal level of PI4,5P2 was in an agonist insensitive pool (Willars, Nahorski et al. 1998).They also estimated the agonist-sensitive pool to turn over 12 times per minute under maximal agonist stimulation. Wortmannin is however known to block PI3-kinase specifically, these casting some debuts on the findings of Willars et al. 1998. Frølich et al.1992 also reported similar findings with human platelets where 45% of PI, PI4P and PI4,5P2 are estimated to be found in an agonist insensitive pools (Frolich, Aarbakke et al. 1992). The depletion of PI4,5P2 by wortmannin is followed by a loss of IP_3 generation. GB

Willars argue that despite the ability for some PLC isoforms to hydrolyze PI, PI4P and $PI4,5P_2$ (Rhee and Choi 1992), PI does not provide a substrate for PLC during stimulation with carbachol in SH-SY5Y cells(Willars, Nahorski et al. 1998). Activation of mAChR with carbachol in SK-N-SH cells has been reported to increase the cellular mass of DAG by 60%. The DAG formed shortly after agonist exposure arises from breakdown of the phosphoinositides, but after several minutes of receptor stimulation, PC becomes the main source of DAG (Stubbs, Carlson et al. 1992). (See figure 1.4.1)

Desensitization of the muscarinic receptor has regulation beyond receptor phosphorylation and PI4,5P2 depletion. This is reflected in the fact that upon addition of maximal effective concentration of agonist, $PI4,5P_2$ is consumed 3 times faster than during sustained receptor activation, where a partly desensitized receptor is used(Willars, Nahorski et al. 1998). Protecting mechanisms for the $PI4,5P_2$ pool are reflected in the different rate of recovery for the PI4P and $PI4,5P_2$ pools. Removal of agonist lead to a faster recovery to basal level for $PI4,5P_2$, than for PIP because when PI4P recovery starts, 60% of the $PI4,5P_2$ pool had already recovered (Willars, Nahorski et al. 1998).

The Ca^{2+} concentration in resting SH-SY5Y cells is estimated to be 199 +/- 14 nM (Murphy, Vaughan et al. 1991). Stimulation by an effective concentration of carbachol gives rise to a biphasic change in intracellular Ca^{2+}. An initial peak arises after only 4 seconds followed by a plateau phase with a significantly higher Ca^{2+} concentration than basal level (Murphy, Vaughan et al. 1991). This pattern is also seen with changes in the IP_3 concentration upon agonist stimulation (Wojcikiewicz, Tobin et al. 1994).

Carbachol is here more potent for the plateau phase (Lambert and Nahorski 1990). Increased levels of internal Ca^{2+} will activate a range of cellular responses. A direct activation of K^+ and Cl^- ion channels is one reported effect and activation of PLD, resulting in more DAG, is another. Ca^{2+}-dependent PLA_2 activation gives arachidonic acid, prostaglandins and eicosanoids, which again result in increased cGMP levels that regulate the influx of ions through ion channels. Finally Ca^{2+} together with calmodulin causes phosphorylation of many target enzymes. (See Figure 1.1.7)

These responses can be blocked by using a *non- specific* antagonists of the muscarinic receptors, atropine. Atropine inhibits the receptor-activated IP_3 formation leading to failure in release of Ca^{2+} from ER.

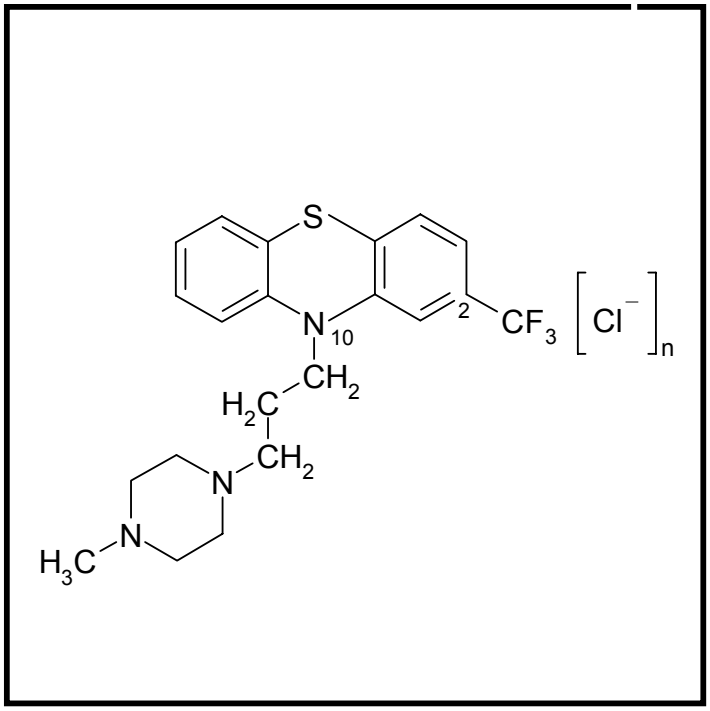
1.1.4 Trifluoperazine (TFP)

TFP is an antipsychotic agent in the phenothiazine group. Chlorpromazine (CPZ) was the first of the phenothiazine derivatives that was introduced into the practice of psychiatry in the mid 1950s, showing therapeutic effect in treating psychoses such as schizophrenia, a condition of hallucinations in all sense organs, delusion, split personality and seclusion. The phenothiazines have a general slowing effect on the activity in the central nervous system (CNS), putting the patients into a sedative state. Motor restlessness and non-specific ECG changes is also observed in patients receiving phenothiazines. The response is more rapid and effective if TFP and CPZ are given together, than if either drug is used alone (Martindale 1977).

There are many papers discussing the effects of TFP and CPZ on the neurotransmitter receptors involved in psychosis, including different types of dopaminergic, serotonergic, histaminergic and cholinergic receptors, most of them reporting inhibitory responses. Cholinergic hyperaction is believed to cause the negative symptoms of schizophrenia such as personal withdrawal and lack of motivation, energy and initiative. Muscarinic antagonists are then therapeutically useful in treating these symptoms (Tandon and Greden 1989). TFP is a muscarinic receptor antagonist, and there has been reported a TFP-induced reduction in the muscarinic receptor-

induced IP_3 level in SH-SY5Y, TFP removed the initial peak of IP_3 but did not alter the steady state level at the plateau phase significantly (Larsson and Alling 1995).

Figure 1.1.8



Molecular structure of Trifluoperazine

TFP, also known as Stelazine, is a cationic amphipilic, high-potency drug (Maziere, Maziere et al. 1988) with a hydrophobic three-ring structure where two planar benzene rings are linked together by a sulphur and a nitrogen atom which lie asymmetrically outside the planes (McDowell 1980).The average length between C-C, C-S, C-N, C-S-C and C-N-C is in agreement with those found in related compounds (McDowell 1976). In position 2 there is a trifluoromethyl group (–CF_3) which is a strong electron attracting group. The C-F bond length is here shorter than in normal C-F single bonds, with shorter bond distance associated with multiple F atoms on a single C atom (McDowell 1980). The trifluoromethyl group is strongly electron attracting, more than the -Cl atom in the same position on CPZ, giving it the strong therapeutic effect. The positively charged group on the nitrogen atom in

position 10 swings away form the CF_3 group (McDowell 1980), and is also of therapeutic significance. The length of the carbon chain linking the phenothiazine ring to the piperazine-ring affects the hydrophobicity of the phenothiazine ring and plays a role in the ability of the drug to interact fully with a lipid monolayer as determined by Langmuir isoterm technique,

the ideal length here being three carbons (Agasosler, Tungodden et al. 2001). The number of carbon atoms also plays a role in calmodulin binding efficiency, 4-5 carbon atoms give greater interaction than a three carbon chain (Gresh 1987). TFP has a membrane perturbing effect (Seeman 1972; Ferrell, Mitchell et al. 1988), and its amphiphilic property could explain that it partitions readily into the cell membrane (Landry, Amellal et al. 1981).

In human platelets phenothazines have been shown to display different effects according to drug concentration. At low drug concentrations below 25µM, secretory responses are stimulated. Concentrations between 25-50 µM inhibit these responses and concentrations between 50-100 and above 100µM cause permeabilization of the platelet plasma membrane (Tharmapathy, Fukami et al. 2000). In 1982 K.W.Frølich and colleges also argue that most of the work done with phenothiazines in the literature were done at concentrations ranging from 100-3000 µM, and the reported effect may reflect permeabilization artefacts rather than specific drug effects (Frolich, Aarbakke et al. 1992). In the present work we have been careful to operate in a low concentration range, 0- 30 µM TFP.

Phospholipids are important components of cellular membranes. Their composition and turnover may play a role in the regulation of membrane-bound enzymes by modifying the physico-chemical characteristics of membranes such as membrane potential (Snelling and Nicholls 1984). Protein conformation and exact function depend therefore on the milieu in which the proteins are embedded, and the membrane hypothesis give an alternative view explaining the abnormalities among the many neurotransmitter systems in schizophrenic patients. D.F Horrobin and colleagues argue that a marked changes in the lipid composition of cell membranes, especially the essential fatty acid (EFA) metabolism should be given greater attention (study of patient with low level of n-3 and n-6 fatty acid) (Horrobin 1994).

Phenothiazines are known to change lipid composition in model systems. It has been shown that TFP and CPZ inhibit PC synthesis in HeLa cells (Pelech, Jetha et al. 1983; Pelech and Vance 1984; Kolesnick and Hemer 1989; Rabkin 1989; Frolich, Aarbakke et al. 1992).TFP also alters the fatty acid composition in phospholipids in human fibroblasts. Preincubation with TFP for more than 6 hours, gives increased incorporation of oleic acid into PC, PE and

PI. The incorporation of different fatty acids was stimulated in this study but the total phospholipid composition remained unchanged. A direct effect of the drug on the activity of the enzymes involved is proposed rather than the importance of the total availability of phospholipid precursors (Maziere, Maziere et al. 1988).The ability of TFP and other

phenothiazines to alter the activity of membrane-associated proteins is seen in a numbers of papers reporting CPZ induced changes in enzyme activity in many different enzymes. Examples are that CPZ activates phosphatidate cytidyltransferase (Sturton and Brindley 1977) and CDP diacylglycerol inositol phosphatidyltransferase (Zborowski and Brindley 1983). CPZ also affects the activity of calmodulin-independent ATPases (Luthra 1982) and calcium pumps (Hirata, Suematsu et al. 1982), and inhibition and displacement of PAP leading to accumulation of PA (Brindley, Allan et al. 1975; Bowley, Cooling et al. 1977) (Hopewell, Martin-Sanz et al. 1985; Holmsen and Dangelmaier 1990).

1.2 The Langmuir isotherm technique

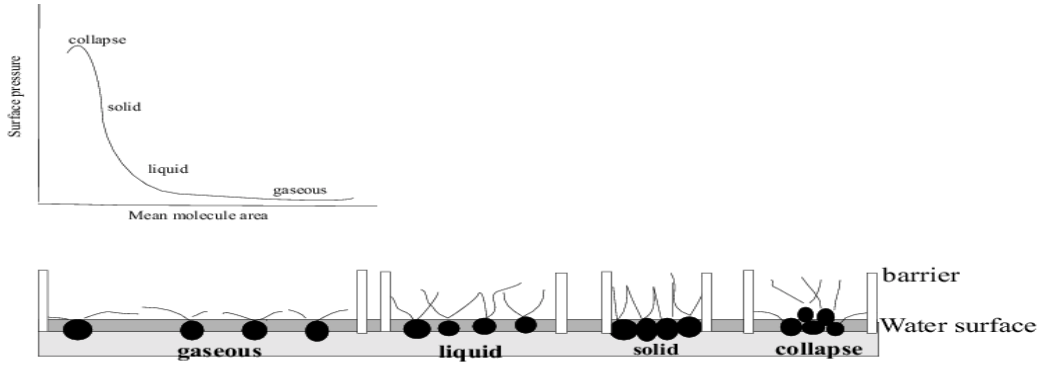
Trifluoperazine (TFP) has, as mentioned above, has been claimed to have a range of effects on many membrane bound receptors in the CNS. One mechanism of action could be the possibility for the drug to enter the phospholipid membrane and alter the milieu around the receptors, indirectly changing their reactivity. The bilayer cell membrane consists of two phospholipid monolayers, and the Langmuir monolayer technique works as a model system for one such layer giving the possibilities to vary and control the different molecule species present in the monolayer.
Much attention has been paid to CZP, a compound related to TFP, and its effect on the packing of phospholipids in monolayers. Much of Armelle Varnier Agasøster`s PhD work is concentrated around the interaction between different glycerophospholipid molecular species and CPZ. She has established that both the head group and acyl chain composition of the phospholipid affects the interaction between the drug and monolayers of different glycerophospholipid molecular species (Agasosler, Tungodden et al. 2001). Here we want to explore if some of the same principles are applicable for TFP.

1.2.1 The Monolayer

Monolayer-forming molecules are mostly of amphiphilic character, and phospholipids have hydrophilic head-groups and hydrophobic acyl-chains. The hydrophilic part of the molecule dissolves in the water subphase, while the hydrophobic acyl-chains lie on top of the water

surface. The surface tension for water at 20°C is 73 mN/m, a high value caused by the unique large number of hydrogen bonds between the water molecules in the air/ liquid interface. When phospholipid molecules are spread onto a water surface with a surface area large enough to let the phospholipid molecules stay far apart in a gaseous state, the surface tension for water will not be much influenced. As the two barriers (see Figure 3.2.1) on the trough decrease the surface area available to the phospholipid molecules, they enter a denser, liquid state. This makes the water molecules to rearrange their hydrogen bond connections leading to a decrease in the surface tension for water and an increase in surface pressure. As the available surface area continues to decrease the lipid molecules enter a condensed, almost solid state, seen by a marked increase in surface pressure, the surface tension for water is now minimal since the air/liquid interface is covered with phospholipids. (See Figure 1.2.1) As the surface area decrease further the uniform phospholipid monolayer will collapse, seen by a small decrease in the surface pressure.

Figure 1.2.1

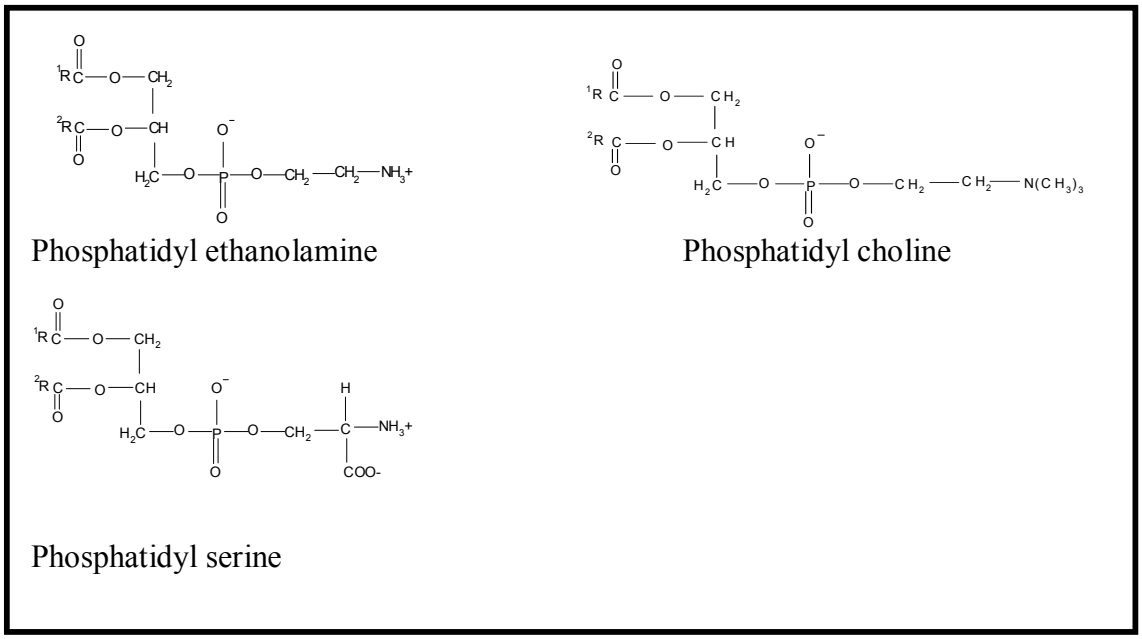


$\Pi - A$ ***Isoterm diagram*** *for a typical phospholipid monolayer at the liquid/air interface and a illustration of the different states of the monolayer during compression.*

1.2.2 1,2-diacylglycerophospholipid (Phospholipids)

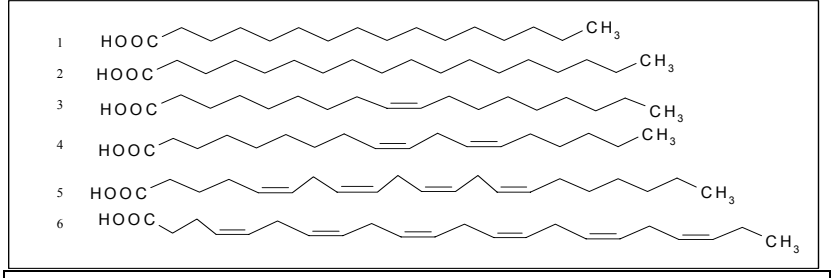
1,2-diacylglycerophospholipids consist of two fatty acids in *sn*-1 and *sn*-2 position and an esterified phosphate group in position *sn*-3 on a glycerol backbone. (Figure 1.2.4) We find different alcohols on the phosphate, defining the glycerophospholipid class. At neutral pH phosphatidylcholine (PC) and phosphatidylethanolamine (PE) have a net zero charge while phosphatidylserine (PS) and phosphatidylinositol (PI) carry a net negative charge (-1). The acyl-chains in position *sn*-1 and *sn*-2 vary in length of the carbon chains and the degree of saturation. The fatty acids in position *sn*-1 are normally saturated, and in position *sn*-2 we find different unsaturated fatty acids.

Figure 1.2.2



Chemical formulas of the 1,2-diacylphospholipids PE, PC and PS

Figure 1.2.3



		Acyl group	Melting point for free fatty acid
1	16:0	Palmitoyl	T_m 62,9 °C
2	18:0	Stearoyl	T_m 69,2 °C
3	18:1 (n-9)	Oleoyl	T_m 13,0 °C
4	18:2 (n-6)	Linolenoyl	T_m –10,0 °C
5	20:4 (n-6)	Arachidonoyl	T_m – 49,5 °C
6	22:6 (n-3)	Docosahexaenoyl	T_m -? °C

*The **glycerophospholipid acyl chains** used in this study*

Fatty acids have different phase transition temperatures depending on length and the degree of unsaturation. At temperatures above T_m the fatty acids are in a liquid state and at T below T_m we find them in a gel phase. This gel-to-liquid transition is important to have in mind when looking at the isotherm diagrams of the different glycerophospholipid monolayers since a phase transition occurring at T_m is said to involve a trans-to-gauche rotational isomerization of methylene groups about the C-C single bonds in the acyl chain (Wang, Li et al. 1999), influencing the conformation and regularity in packing of the phospholipid.

1.3 Aim of the project

Human platelets have for decades been used as a model system for serotonergic neurons when the effect of different antipshycotic medicaments action on the PPI metabolism is investigated. Even though they are from human origin and have several advantages

concerning measurable parameters and access, the neuroblastoma cancer cells SH-SY5Y are most likely more close to neurons in morphology and composition, and may give a more representative responses to drug exposure. The knowledge about the effect of phenothiazines on the PPI cycle in human platelets has been well established over the years, but the same is not the case for the SH-SY5Y cells. The PPI metabolism is expected to be the same and results obtained from the to cell types may then easily be compared.

In the present study SH-SY5Y neuroblastoma cells are grown to confluence and labelled by incubation with [^{32}P]Pi (0.1 mCi / dish) for one hour at 37^{o}C, and treated with TFP, charbachol and atropine, in a concentration- and time-dependent manner.

The glycerophospholipids are extracted and separated by thin layer chromatography. The radioactivity of the [^{32}P]Pi labelled glycerophospholipids are detected and quantified. The effects of stimulation on the level of radioactive PA, PI, PI4P and PI4,5P_2 are compared to published findings from human platelets treated in the same manner.

The hypothesis is that TFP not only acts directly on the receptor but also may change its activity indirectly by interaction with the membrane phospholipids surrounding the receptor. To further support the idea that TFP interacts with the cell membrane, the langmuir isotherm technique is used. It is a well-established technique in our laboratory, and has showed that TFP interact with monolayer of acidic phospholipids like PS, PA and PI. The saturation of the fatty acid in position *sn*-2 on these phospholipids are also of importance concerning interaction with TFP, and in this work the unsaturation is changed systematically from cero to six *cis*-double bonds in phosphatidylserine (PS).

2 MATERIALS

2.1 Neuroblastoma cells (SH-SY5Y)

The SH-SY5Y cell line was purchased at European Collection of animal cell cultures, Centre for Applied Microbiology & Research, Porton Down, Salisbuty, Wiltshire, UK (ECACC Ref. No: 94030304) and stored in liquid nitrogen.

SH-SY5Y human neuroblastoma cell-line was grown in Ham`s F 12:DMEM w:w/o Gln (1: 1) serum from KEBO LAB A/S. Added 10 % Fetal Bovine serum, 1 % non essential amino acids and 2 mM glutamine. No penicillin.

The fetal bovine serum was from Bio Whittaker Cat N 14- 701F B-4800 Verviers Belgium; the non essential amino acids were from Flow Laboratories, Scotland Cat.No 16-810 49, and L-glutamine was from E, MERCK Germany Cat. No. 1,00289

2.1.1 Radioavtive phosphate

[^{32}P]Pi Orthophosphate (carrier-free) in dilute hydrochloric acid, pH 2-3, 10 mCi/ml, was purchased from Amersham Pharmacia Biotech Buckinghamshire, England. Decay time; ~14 days.

2.1.2 TFP

Trifluoperazine-dihydochloride (TFP) was from Smith & Kline, Cat.no. B 82130024. TFP was dissolved in Hepes buffer and stored in the dark at +4 C^{o} for not longer than two months.

2.1.3 Carbachol

Carbamylcholine chloride from Sigma Chemical CO, St. Louis, MO, USA. Cat. No. C4382, was dissolved in Hepes-buffer and stored at +4 C^{o} for two months.

2.1.4 HEPES- buffer

Concentration		**Mw**
10 mM	Hepes	238.3
4.9 mM	KCl (Potassium Chloride)	74.56

139 mM	NaCl (Sodium Chloride)	58.44
5.5 mM	Glucose	198.17
0.9 mM	$CaCl_2$ (Calcium Chloride)	111

Hepes, KCl, NaCl and Glucose were dissolved in distilled water and adjusted pH to 7.2. $CaCl_2$ and distilled water were added up to 1 Liter. Sterile filtered and stored at +4 C^o

2.1.5 PBS (Phosphate –buffered Saline) pH 7.3.

Concentration		**Mw**
2.67 mM	Potassium Chloride (KCl)	75
1.47 mM	Potassium Phosphate Monobasic (KH_2PO_4)	136
138.0 mM	Sodium Chloride (NaCl)	58
8.10 mM	Sodium Phosphate Dibasic (Na_2HPO_4)	268

2.1.6 DPBS (Phosphate –buffered Saline) containing Ca^{2+} and Mg^{2+} pH 7.3

Concentration		**Mw**
2.67 mM	Potassium Chloride (KCl)	75
1.47 mM	Potassium Phosphate Monobasic (KH_2PO_4)	136
138.0 mM	Sodium Chloride (NaCl)	58
8.10 mM	Sodium Phosphate Dibasic (Na_2HPO_4)	268
0.9 mM	Calcium Chloride($CaCl_{2)}$	111
	Magnesium Chloride ($MgCl_2$)	

Materials

2.1.7 Separation of Phospholipid classes by Thin layer Chromatography

Silica gel TCL aluminium sheets 20 x 20 come from MERCK.

2.1.8 The methylamine system

Chloroform ($CHCl_3$)	60 ml
Methanol (CH_3OH)	36 ml
Distil water (dH_2O)	5 ml
Methylamine (40%)	5 ml

2.2 The Langmuir isotherm technique

2.2.1 Glycerophospholipids

DPPE, DPPC, DPPS, DSPS, SOPS, SLPS, SAPS and SDPS were all from Avanti Polar Lipids Inc. Birmingham, AL, USA.

3 METHODS

3.1 Neuroblastoma cells (SH-SY5Y)

3.1.1 Culture Method

The SH-SY5Y human neuroblastoma cells were cultured in small plastic flasks with 5 ml Ham's F 12:DMEM w:w/o Gln (1: 1) medium, containing 10 % Fetal Bovine serum, 1 % non essential amino acids and 2 mM glutamine. No penicillin was added. The incubator was a CO_2 Water Jacketed Incubator from Forma Scientific, Inc. model: 3110. with the temperature set at 37^oC , 5% CO_2 level, and high humidity. To ensure high air quality inside the incubator, Forma HEPA filter airflow system had been installed.
At confluence, the medium was removed from the SH-SY5Y cells and the cells were incubated in 5 ml divalent cation-free PBS for 10 minutes. The flask was then carefully shaken to loosen the cells. The cell suspension was centrifuged at 300xg for 5 minutes, and the pellet containing the cells was dissolved in Ham's medium and seeded in new flasks at low cell concentration. The passage number is recorded for every splitting.
The SK-N-SH cell line has an average population doubling time of 44 hours and reaches high cell density (more than 10^6 cells/sq cm) upon confluence. In contrast, different human fibroblastic cell lines reach a maximum cell density 13 fold lower (Biedler, Helson et al. 1973).Culture conditions alter viability and growth. Both seeding density and medium change are of importance to ensure optimal growth conditions. High seeding density, compared to low seeding density, increases the percent confluence and cell number over time and reduces the percentage of cells with fragmented DNA (Carlson and Ehrich 2000). Frequent medium changes increase the number of cells in S and G_2/M phase and decreased the number of cells in G_0/G_1 cell cycle phase over time compared to cells with no media change(Carlson and Ehrich 2000). Under growth conditions where the cells are cultured on a 2D plastic surface, the cells form monolayers, give no cell clusters and do not form contacting neurites. On 3D support such as a 1 mm thick gel, on the other hand, the cells assume a more regular shape and form ganglia –like clusters with targeting neuritis (Hahn, Glass et al. 2000).

3.1.2 Experimental set-up

The cells were loosened from the flask surface by PBS as described above, and seeded on ϕ 60 mm plastic growth dishes containing 5 ml Ham`s medium. They were grown to near confluence and the medium was not changed during this growth period.
The medium was removed and 3 ml of HEPES buffer containing [^{32}P]Pi (0.033 mCi/ml) was added. The cells were incubated for 60 minutes.
The purpose of adding [^{32}P]Pi is to label the ATP turning over in these cells, and to promote incorporation of radioactive phosphate into the phospholipids in the phosphoinositol signalling pathway (Figure 1.3.2).
The HEPES containing the radioactivity was removed and replaced by 3 ml HEPES buffer without phosphate. TFP or/and carbachol were then added to desired concentration and left for the times of interest. The HEPES containing TFP or/and carbachol is then removed and the dishes were washed with ice-cold DPBS containing divalent cations, stopping cellular reactions. The DPBS was removed and the dishes were left overnight in – 20 C^{o}.

3.1.3 **Glycerophospholipid extraction**

We used the E.G. Bligh & W.J. Dyer (1959) lipid extraction and purification method to extract all the glycerophospholipids from the cells(Bligh 1959). The cells were loosened from the dishes with 2x1 ml of a mixture of ice-cold methanol and 4.75 M HCl (2:0,8) and transferred to lipid extraction glass tubes. Pure chloroform (0.7 ml) was added to the cell: methanol: HCl mixture and vortexed for 30 seconds. 0.71 ml dH_2O and a new portion of 0.70 ml chloroform were added with vortex between each adding. The mixture was centrifuged at 300xg for 3 minutes for phase separation, and the chloroform layer was transferred to a Kimberly tube and the chloroform evaporated under argon gas.

3.1.4 **Separation of the different glycerophospholipid classes**

The dry lipids were dissolved in 60µL chloroform and 30µL was applied manually on a TLC sheet and developed with the methylamine system at room temperature for two hours. The

methylamine system is especially designed to separate inositol polyphospholipids, and consisted of chloroform: methanol: methylamine: dH_2O in a 60:35:5:5 proportion respectively.

3.1.5 Detection and quantification

The radioactive spots on the TLC plate were detected and quantified directly with an Instant Imager (Packard, Meriden, CT, USA).

3.1.6 Statistics

Working with biological material gives rise to many statistical errors, and working with the neuroblastoma cell line has been no exception. The variation in the values of CPM and percent of control among the parallels was large in some cases.
The goal was to at least have three parallels for each experimental value, but in some of the experiments only one or two were performed. The experiments were time consuming and here the *tendency* in the results was all we wanted to look for.
In the case where the time of TFP and carbachol incubation was keept constant (20 minutes) and TFP and carbachol concentration were varied, up to five parallels were obtained from the same experimental set up performed on different days.
Looking at the CPM values alone, great variation can be seen among these parallels. The reason for this stems most likely from the fact that there were variations in the total number of cells per dish. During the process of selecting dishes for an experiment we found that the number of cells varied between dishes. We did not succeed in finding a satisfying method to determine the quantity of cells in each dish. At first, the total amount of phosphate in the lipid extracts was used as a measure of the total quantity of glycerophospholipids in each dish. This test has great sensitivity but did not, for unknown reasons, give the correction we hoped fore; the variation among the parallels remained the same. Then we looked at how we seeded the cells onto the dishes, good homogeneity must be reached in the cell suspension to facilitate an accurate number of cells per dish.

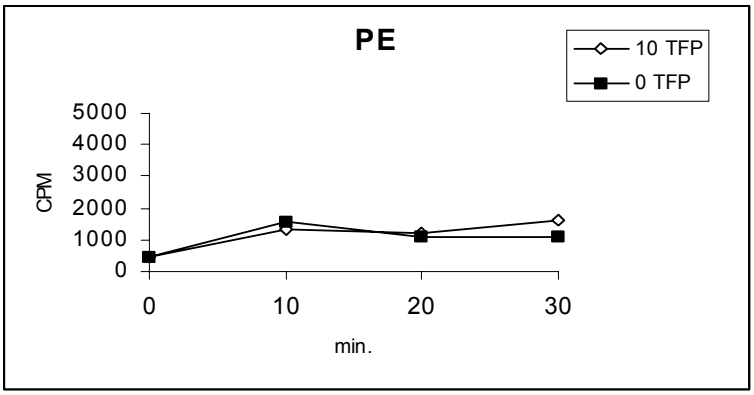
Methods

One of the first experiments performed was to determine exposure time to TFP. The TFP concentration was keep constant (10μM) and the exposure time was varied from 0 to 30 minutes. The seeding density in this experiment was satisfactory and looking at the variation of PE radioactivity over time, compared to control, little or no influence of TFP is seen.

(Figure 3.1.1) We decided to use this fact as a internal standard of cell quantity per dish. PE is not known to be a precursor to lipid signal molecules in cells, and the incorporation of radioactive [^{32}P]Pi occurs through the de novo syntheses of PE.

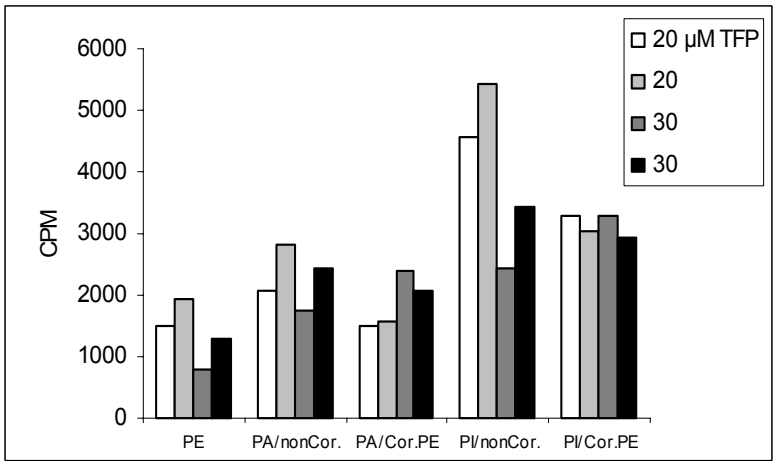
Figure 3.1.2 show an example of how the variation in PE-radioactivity, correlate with *variation* in CPM for PA and PI. When corrected for this phenomenon we see a tendency toward more equal parallels.

Figure 3.1.1



TFP influence on radioactive PE over time Results obtained in the same experimental set up from the same day

Figure 3.1.2.



Variation in CPM before and after PE correction Results obtained in the same experimental set up from the same day.

All the results in the following were corrected for variation in cell quantity in the same manner as described above, and in the case where the are three or more parallels, a simple Q-test was performed to exclude the values that differed from the average with a 90 % degree of confidence. The mean was calculated for the remaining values (three or more) and a simple standard derivation was performed.

3.1.7 Cell adhesion test

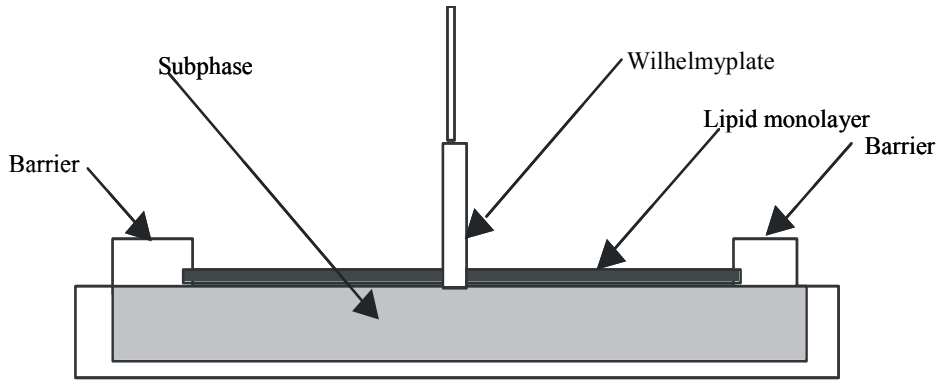
A cell adhesion test was performed to see if any concentration of TFP loosens cells from the dishes they were grown on. Cells were seeded in wells on an Elisa plate and cultured to confluence. The medium was removed and replaced with different concentrations of TFP dissolved in Ham's medium with a final volume of 200µL per well. The exposure time was one hour. The TFP-containing medium was removed and the wells washed with 150 µl DPBS per well. 100µl 1% glutaraldehyde was added to each well and left for 15 minutes, again the wells were washed again with 150 µl DPBS. 100µl of filtered crystal violet (0.1%) was added to each well and left for 30 minutes to stain the remaining cells. The plate was washed with excess double distilled H_2O, and 100µl of 0.25% Triton was added and left for more than 12 hours. The plate was read at 595 nm.

3.2 The Langmuir isotherm technique

3.2.1 The Langmuir apparatus

$\Pi - A$ Isotherms are the most frequently used Langmuir monolayer parameter. At constant temperature the glycerophospholipid monolayer is spread onto a liquid surface, the surface pressure (Π) is continuously detected and plotted against mean molecular area (mma(Å^2)), giving a $\Pi - A$ Isoterm diagram (See figure 1.2.1)

Figure 3.2.1



The Langmuir apparatus

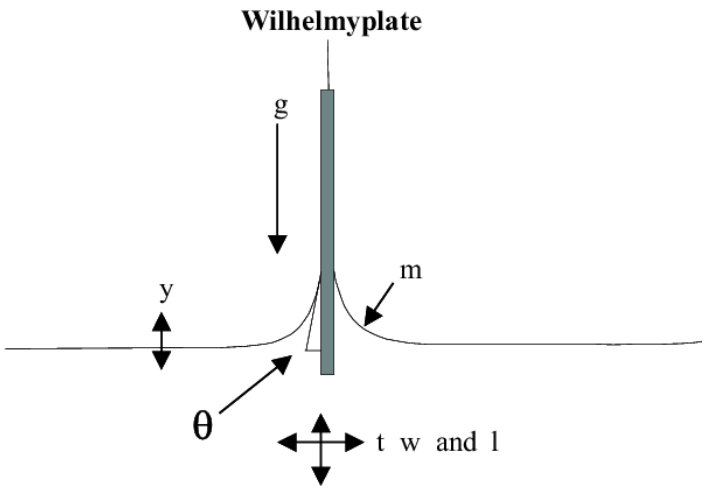
The langmuir apparatus gives the possibility to control the area available to the monolayer-forming molecules between two barriers on each side of the trough. The surface pressure is measured with the Wilhelmy plate.The Langmuir apparatus used is a KSV Minitrough (Helsinki, Finland) of dimensions 75 (w) x 364 (l) x 5 (h) (mm). It is coated with polytetrafluorethylene (PTFE), a highly hydrophobic and inert material that prevents the subphase from running over the edges. The film area is compressed with two barriers on each side of the trough. The barriers are coated with a polyacetal hydrophobic material Delrin, which prevents the monolayer from sliding under the barriers. The barriers are driven symmetrically along the long side of the trough, by a motor controlled by the KSV Minitrough software. Both the trough and barriers are carefully cleaned after each run with 96% ethanol and destilled water.

A Wilhelmy plate, which consists of a 10x20 mm piece of platinum, is connected to an electrobalance above the trough, and immersed about 5 mm into the subphase. The plate is also cleaned twice with 96% ethanol and destilled water and stored in 96% ethanol.

3.2.2 Principle of the Wilhelmy plate

The measurement of the monolayer surface pressure is done trough the Wilhelmy plate. Changes in the composition of the liquid surface will cause changes in the forces working on the plate.

Figure 3.2.2



y is the surface tension, g is the gravitational force, m is the mass of the lifted liquid, t is the thickness of the plate, w is the width of the plate and l is the length of the plate, θ is the liquid contact angel.

When the Wilhelmy plate is lowered down into the subphase, three types of forces are working on the plate in total, the capillary force and gravitation working downwards, and the buoyancy of the plate upwards. When disregarding the buoyancy both in water and air, we get the equation 1.2.1

Equation 1.2.1 $F = F_a + \gamma p$

where F is the force pulling the plate down in water and F_a in air. γ and p are surface tension and perimeter respectively. The perimeter is the contact area between the liquid and the plate.

The equation for the surface tension can then be written:

Equation 1.2.2 $$\gamma = \frac{mg}{2(l+t)}$$

where γ is the surface tension, m is the weight of the lifted liquid, g is the gravitational force, l is the length of the plate, t is the thickness of the plate.

There is a liner relationship between the liquid surface tension and the strength of the capillary forces in action. This relation can be used to measure changes in the liquid surface by recording the weight of the lifted water with a very accurate and sensitive balance. Looking more closely at the forces working on the plate we get the equation:

Equation 1.2.3 $$F = mg - \{2\gamma(t+w)\cos\theta + \rho_1 + V_1 g\}$$

Here m is the mass of the plate, g is the gravitational force, t is the thickness of the plate and w is the plates width. γ is surface tension, ρ_1 is liquid density , and V_1 is the volume of the plate lowered into the liquid. θ is the liquid contact angle if we disregard the buoyancy in air. If the θ is zero ($\cos\theta$=0), the surface pressure Π can be calculated by weight measures from the equation:

Equation 1.2.4 $$\Pi = -\Delta\gamma = [\Delta F / 2(t+w)]$$

3.2.3 The subphase

The subphase was MilliQ-water purified with Pak Millipore, S.A.S, (France) at 18 megohm/cm at 25^{o} C. The MilliQ-water was prepared and used at once to avoid acidification by atmospheric CO_2 and contamination occurring with time. We did not adjust pH in this series of experiments.

The subphase and the trough were preheated to 37 ^{o}C, and all the recordings were carried out at this temperature. No ions of any kind were added to the subphase. All control experiments were carried out on pure MilliQ-water. Different concentrations of Trifluoperazine (TFP) were added to the subphase , 0.1 μM, 1.0μM and 10μM. In experiments varying the head group of the glycerophospholipids, keeping the acyl-chains constant, only 1,0 μM TFP was used. When looking at the role of the different acyl-chains-saturation and carbon chain length, keeping the head group constant, all the above concentrations of TFP was used.

3.2.4 The lipid monolayer

The 1,2-diacylglycerophospholipids were diluted to a concentration of around 1 mg/ml in chloroform ($CHCl_3$) and stored at –20 ^{o}C. A volume of 10 μl was added to the subphase surface, with the barriers at start (outmost) position, and left for 30 seconds to allow the chloroform to evaporate, leaving the pure glycerophospholipids as a monolayer in the gaseous state at the liquid/ air interface. The compression of the barriers is started and the recording of surface pressure (π mN/m), against mean molecule area (mma), are performed by the computer. To determine the role of the phospholipid head group, dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylserine (DPPS) were spread onto the subphase with and without 1,0μM TFP added. The role of the acyl-chain composition was studied with distearoylphosphatidyserine (DSPS), 1-palmitoyl, 2-oleoyl-phosphatidylserine (POPS), 1-steraoyl, 2-oleoyl-phosphatidylserine (SOPS), 1-stearoyl, 2-lioleoyl-phosphatidylserine (SLPS), 1-stearoyl, 2-arachidonoyl-phosphatidylserine (SAPS) and 1-palmitoyl, 2-docosahexaenoyl-phosphatidylserine (SDPS) molecular forms.

3.2.5 Statistics

The mean from three parallels, are preformed for each glycerophospholipid at each concentration of TFP.

4 RESULTS

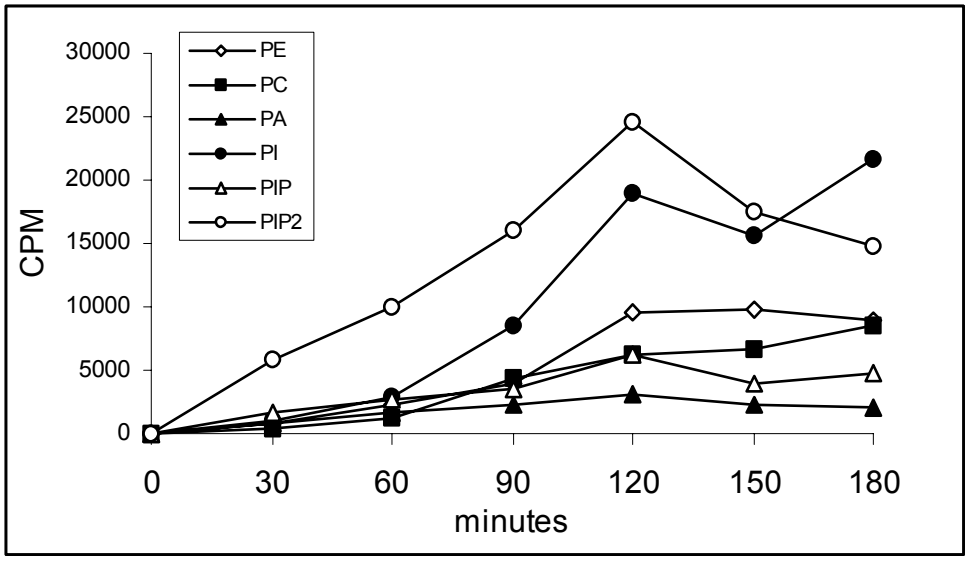
4.1 Neuroblastoma cells (SH-SY5Y)

Most of the work written about the effects of phenothiazines on the PPI cycle, at our laboratory, has been performed on thrombin-stimulated human platelets. Micromolar concentrations of the phenothiazines enhance the thrombin-induced [^{32}P]Pi incorporation into PA and PI4P in a-time-and-concentration dependent manner, leaving the thrombin induced level of PI and PI4,5P_2 unchanged (Holmsen and Dangelmaier 1990; Frolich, Aarbakke et al. 1992; Daasvatn and Holmsen 1999; Tharmapathy, Fukami et al. 2000). In this study, the effect of TFP on the incorporation of [^{32}P]Pi into the PPI cycle intermediates in SH-SY5Y cells is of interest. When shifting the attention from platelets to SH-SY5Y neuroblastoma cells, a suitable PPI cycle agonist had to be found, since our assumption was that TFP does not have an influence on cell activity alone. Nerve growth factor (NGF), insulin and carbachol, have been tested, all activating hydrolysis of PI4,5P_2 and stimulate PPI turnover.
As mentioned under Methods, section 3.1.6, some of the experiments in this study show great variation in response among the parallels performed on different days. The tendency in the results remained the same and many of the results are therefore represented as one single experiment. (See section 3.1.6 and 5.1.1 for further details.)

4.1.1 [^{32}P]Pi incorporation over time in resting SH-SY5Y cells

The [^{32}P]Pi incorporation pattern in resting SH-SY5Y cells is the first that needs to be looked at in order to establish how to set useful incorporation times for later experiments (Figure 4.1.1).

Figure 4.1.1



[^{32}P]Pi incorporation over time in resting SH-SY5Y cells. *0.1 mCi ^{32}P[P_i]per dish. One ϕ10 cm dish, for each half hour. Passage number used: P15*

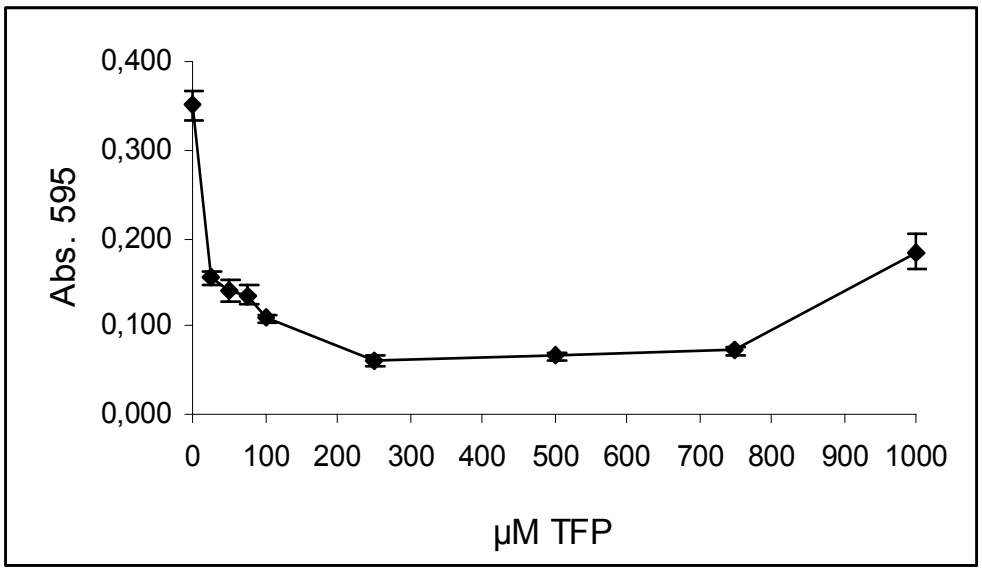
Figure 4.1.1 shows the incorporation rate of radioactive phosphate into different glycerophospholipids in the SH-SY5Y cells exposed to 0.1 mCi/dish [^{32}P]Pi over a time period of three hours. It is clear that the different glycerophospholipids have different [^{32}P]Pi incorporation rate. PI4,5P_2 had a high degree of incorporation already at 30 minutes of exposure to 0.1 mCi/dish [^{32}P]Pi compared to the other phospholipid classes. The high, almost liner incorporation rate continue for about two hours before it start to decrease. PI also showed a high degree of [^{32}P]Pi incorporation. Here a delay time of about one hour was seen before a marked increase in incorporation started. It increased steadily throughout the length of the experiment. PE, PC and PI4P showed an almost similar incorporation pattern; they increased in radioactivity slowly and evenly for two hours. After two hours a more diverse patterns was seen. PE seem to reach a plateau phase at about two hours while PI4P decreased the incorporation rate after two hours. PC continues to incorporate [^{32}P]Pi evenly throughout the experiment. PA was the metabolite with the lowest [^{32}P]Pi incorporation rate of all the phospholipids. Only a small amount of radioactive PA was present in the resting cells independent of three hour exposure to 0.1mCi [^{32}P]Pi.

One the basis of this experiment the incorporation time of [^{32}P]Pi for all other following experiments was set to 60 minutes.

4.1.2 The effect of TFP on the SH-SY5Y cell adhesion

Based on the knowledge that high doses of TFP leads to cell lysis (Tharmapathy, Fukami et al. 2000), an adhesion test indicating cell lysis or even cell apoptose was performed (Figure 4.1.2). Different concentrations of TFP from 0 µM to 1 mM dissolved in Ham's medium was used on cell seeded into the wells on an ELISA plate. The drug exposure time was set to one hour.

Figure 4.1.2



Adhesion tests for SH-SY5Y cells exposed to different concentrations of TFP. *The mean of six duplicates with a corresponding standard error derivation.*

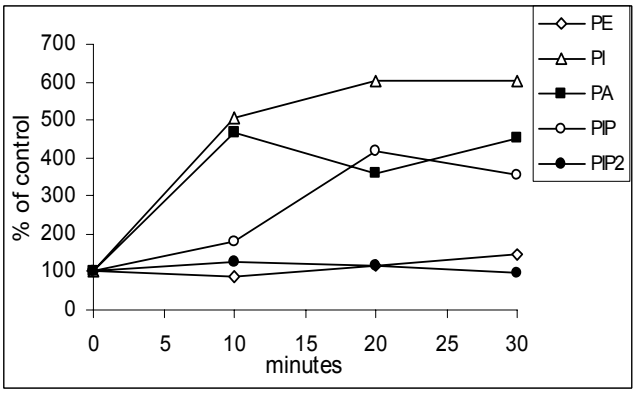
Figure 4.1.2 shows that the cells started to loosen from the wells already at TFP concentration around 25 µM. The test was also performed on TFP concentration from 0µM to 10µM (results not shown) and showed no significant loosening of cells at this low concentration range. We used 0 to 30 µM TFP in all subsequent experiments.

Results

4.1.3 The effect of 10µM TFP over time

This study was performed to establish an incubation time that would revel effects of different concentrations of TFP.

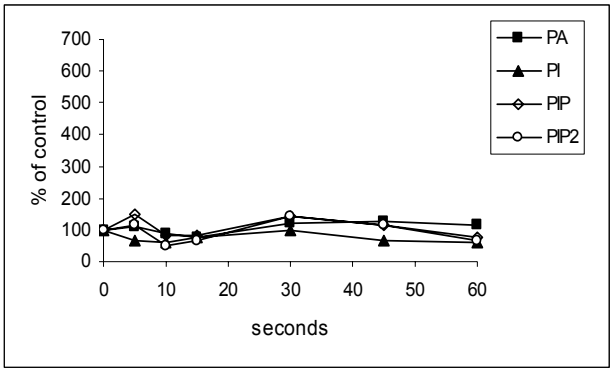
Figure 4.1.3



SH-SY5Y cells exposed to 10 µM TFP over a time period of 30 minutes.

SH-SY5Y cells were grown to confluence in ϕ 6 cm dishes. Celss were prelabelled for 60 minutes incubation with 0.1 mCi [32P]Pi per dish. Two dishes were treated for each timepoint (+/- 10µM TFP). Passage number used was P18. Results were obtained from one single experiment.

Figure 4.1.4



SH-SY5Y cells exposed to 10 µM TFP over a time period of 60 seconds.

Experiment was carried out as in Fig.4.2.3

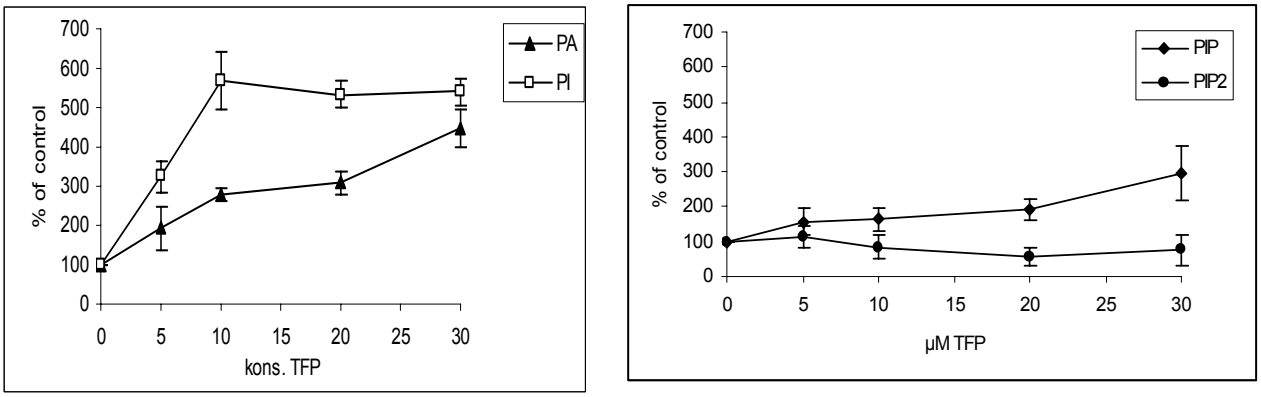
Figure 4.1.3 shows the effect of 10 µM TFP on the PPI metabolites over 30 minutes in non-stimulated SH-SY5Y cells. PA and PI increased their incorporation rate with 500% after only 10 minutes, and reached a plateau phase around ~ 400 (PA) and ~ 600 (PI) percent of control after 20 minutes. This experiment has been done twice, and the plateau phase seen here did not appear in the second experiment, but the values after 30 minutes were still 500% and 600% above control for PA and PI respectively (result not shown). PI4P increased its [32P]Pi incorporation rate with 300 % after 30 minutes in this experiment. This high value has not been reproduced and experiment number two, showed a max PI4P value of 200% above control after 30 minutes (results not shown). PI4,5P_2 and PE seemed not to be affected by the addition of 10µM TFP at any time in this experiment, and this finding is in accordance with the second experiment. The time of drug exposure was set to 20 minutes for later experiments. To rule out the possibility of altered [32P]Pi incorporation within one minute of drug

exposure, an experiment exploring the effect of 10 µM TFP over a time period of 60 seconds was done (Figure 4.1.4). Here no visible increase in radioactivity in any of the phospholipids of the PPI cycles was found.

4.1.4 The effects of different concentrations of TFP

Phenothiazines dose not seem to exhibit an effect on the PPI cycles metabolites in human platelets at any concentration alone (Holmsen and Dangelmaier 1990; Frolich, Aarbakke et al. 1992; Underhaug 2001). Together with a PPI activating stimuli such as thrombin, CPZ and TFP amplify the incorporation of [32P]Pi into both PI4P and PA but have less effect on PI and PI4,5P_2 (Holmsen and Dangelmaier 1990; Frolich, Aarbakke et al. 1992; Underhaug 2001). Figure 4.1.4 shows the changes in [32P]Pi incorporation into PA, PI, PI4P and PI4,5P_2 when SH-SY5Y cells were exposed to different concentrations of TFP for 20 minutes. As mentioned in the introduction, and shown in figure 4.1.2, concentrations of TFP above 30 µM lead to a loss of cell material from the dishes under the experiment, and the highest concentration of TFP was limited to 30 µM to avoid this problem.

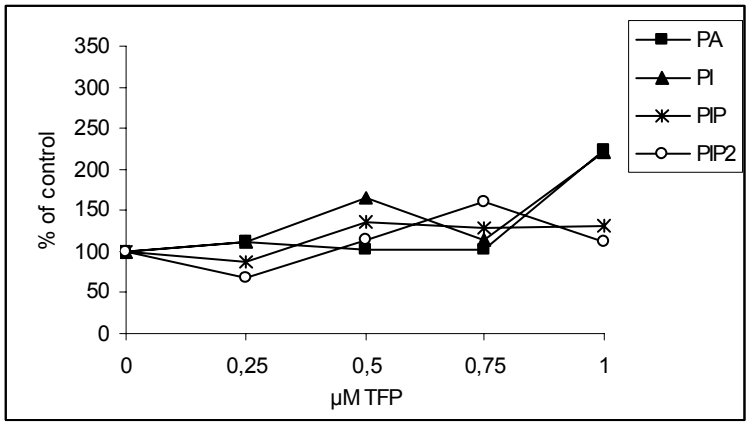
Figure 4.1.5



The effect of different concentrations of TFP at constant exposure time. (20 minutes)
Cells were labelled as describe in Fig. 4.1.3. Three or more dishes weres used for each concentration of TFP. Passage number used was P 18.Results obtained from three experiments, ±s.d., n=3.

To our surprise TFP gave increased activity in the PPI cycle by itself. As Figure 4.1.5 shows, PA had a gradual increase in radioactivity as the concentration of TFP was increased, reaching 350 % ± 46 above control at 30µM TFP. PI reached a plateau phase and a maximum radioactivity near 600 % ± 73 of control already at 10µM TFP. The level of [^{32}P]Pi incorporation into PI is higher at all concentration of TFP than the [^{32}P]Pi incorporation rate into PA. PI4P and PI4,5P_2 seemed less affected; PI4P increased with ~150 % ± 37 above control at 5µM TFP and did not increase further. PI4,5P_2 decreased to about 50 % ± 33 of control values at 10 µM TFP and this lasted throughout the experiment.
In conclusion, TFP alone caused an increase in radioactivity of PI>PA> PI4P and decreased the radioactivity of PI4,5P_2

Figure 4.1.6



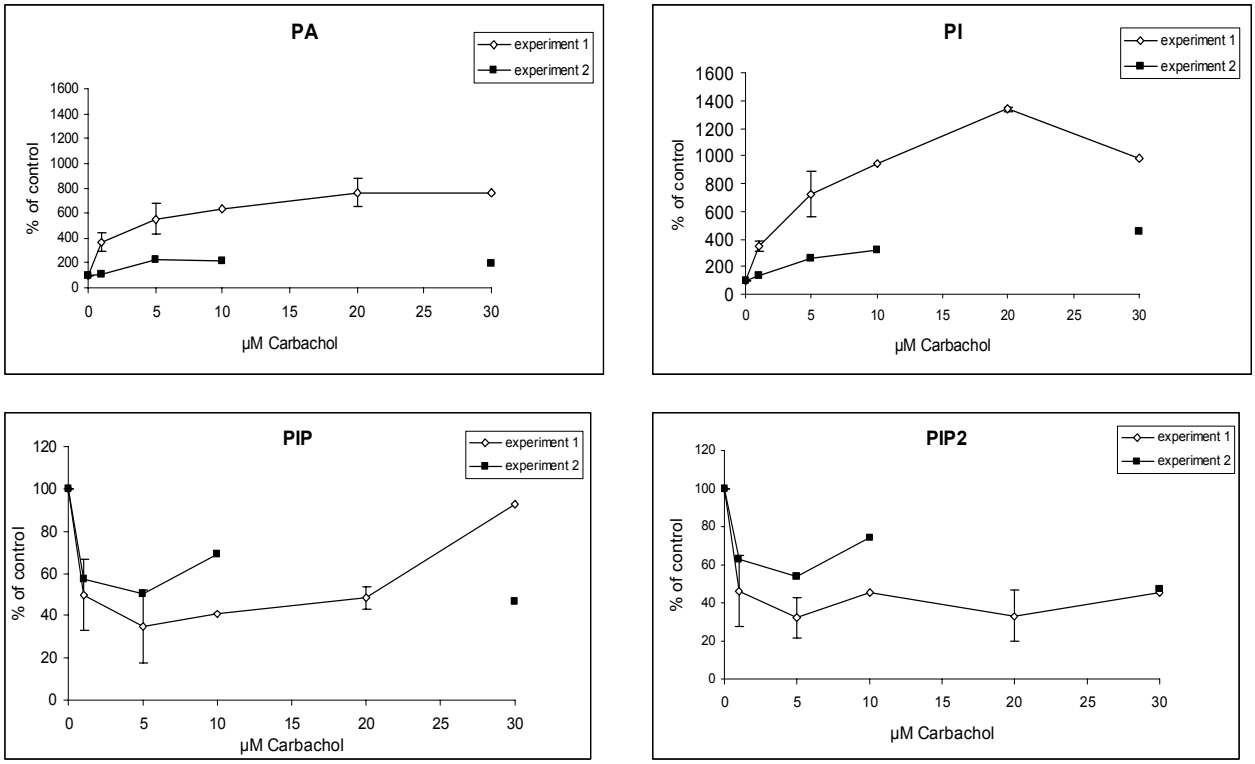
TFP concentrations from 0 µM to 1,0 µM *Cell labelling conditions were as described previously. One dish wass used for each concentration of TFP. Passage number used: P18. Results obtained from one experiment.*

An experiment was performed to look at the effects of low concentrations of TFP (0 µM to 1µM) on [^{32}P]Pi incorporation into the PPI metabolites. The result is shown in Figure 4.1.6. Here we see no large impact on [^{32}P]Pi incorporation at any of the applied TFP concentrations, only a marked increase in PA and PI at 1µM TFP to 250% above control. We therefore continued to use concentrations of TFP above 1µM.

4.1.5 The effects of different concentrations of carbachol

With the work done on platelets in mind (see above) we looked for any enhancing effect of TFP on an already stimulated PPI cycle. NGF and insulin are known to stimulate the PPI turnover by activating PLCγ, but in this study we failed to detect any influence of insulin and NGF on [32P]Pi incorporation (result not shown). Our attention was then shifted towards activation of PLCβ by carbachol via the acetylcholine receptor.

Figure 4.1.7



The effect of different concentrations of carbachol at constant exposure time. (20 minutes)*.Cell labelling conditions were as described previously. Three or less dishes were used for each concentration of carbachol. Passage number used: P19. Results obtained from two experiment performed on different days.*

Much attention has been paid to the effect of carbachol on the PPI turnover in SH-SY5Y cells. The results obtained in figure 4.1.7, show the same effect as reported by others, an

increase in PA and PI and a reduction of PI4P and PI4,5P2 in a concentration-dependent manner (Quist and Satumtira 1987; Wei and Wang 1987; Morris, Cook et al. 1990; Frolich, Aarbakke et al. 1992; Limatola, Pacini et al. 1996; Sorensen, Linseman et al. 1998; Willars, Nahorski et al. 1998). The cells were preincubated with [^{32}P]Pi (0.1 mCi/dish) for 60 minutes. After removal of the [^{32}P]Pi, the cells were stimulated with increasing concentrations of carbachol. Three experiments have been performed on different days with varying numbers of parallels. Figure 4.1.7 shows that the difference in potency with respect to control produced great differences in degree of activation from experiment to experiment. Two of the three experiments performed are represented in figure 4.1.7. In experiment 1, the three parallels allow us to perform a standard derivation calculation, but two dishes containing 10 µM and 30 µM carbachol were lost. Experiment 2 was done to compromise for the lost parallels in experiment 1 (again the dish containing 20 µM carbachol was lost), but a great discrepancy in effect appeared.

An increase in radioactivity for both PA and PI was seen, but the results gave no clear indication of how great the increase in [^{32}P]Pi incorporation into PA and PI was. For PA the variation at 10 µM carbachol is almost 300%, and there is six times as much radioactive PI in experiment 1 than in experiment 2 at 10 µM carbachol. [^{32}P]Pi incorporation into PI4P and PI4,5P_2 were clearly reduced already at low concentrations of carbachol but again we were faced with the same problem of the degree of activation.

A third experiment was performed to see if it gave the same results as one of the two others, but again the degree of stimulation varied (result not shown).

In conclusion, carbachol caused an increase in radioactivity for PI>PA and decreased the radioactivity for PI4,5P_2 > PI4P.

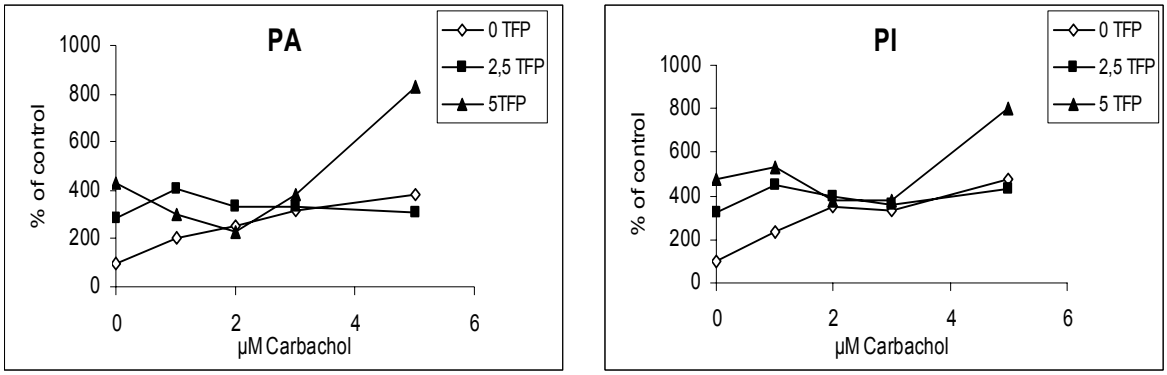
4.1.6 The effect of different concentrations of carbachol and TFP together

As shown above in section 4.1.4, different concentrations of TFP alone gave, surprisingly, changes in the PPI turnover. Carbachol, as expected, influenced the levels of radioactive PPI metabolites in accordance with that, which has been reported by others (section 4.1.3).

Results

Next we wanted to explore if the two compounds influenced each other, in a manner similar to the synergism experienced with platelets stimulated with thrombin in the presence of TFP, as cited above (section 4.1.) (Holmsen and Dangelmaier 1990; Frolich, Aarbakke et al. 1992; Daasvatn and Holmsen 1999; Tharmapathy, Fukami et al. 2000). Experiments performed earlier with a concentration gradient of carbachol from 0 to 30 µM, adding 5 µM TFP, gave no indications of an enhancing effect at any of the chosen concentrations of carbachol used. Three experiments performed on different days gave the same results, a flat curve for the [32P]Pi levels in PA and PI made by 5µM TFP. All the experiments lacked one or more controls and the results are not shown. A new experiment was designed with lowered concentration of carbachol, 0 µM to 5 µM, making sure that maximal stimulation was avoided (Figure 4.1.8).

Figure 4.1.8



The effect of TFP and different concentrations of Carbachol on the PPI cycle *Cell labelling conditions were as described previously. Passage number used: P19. Results obtained from one single experiment.*

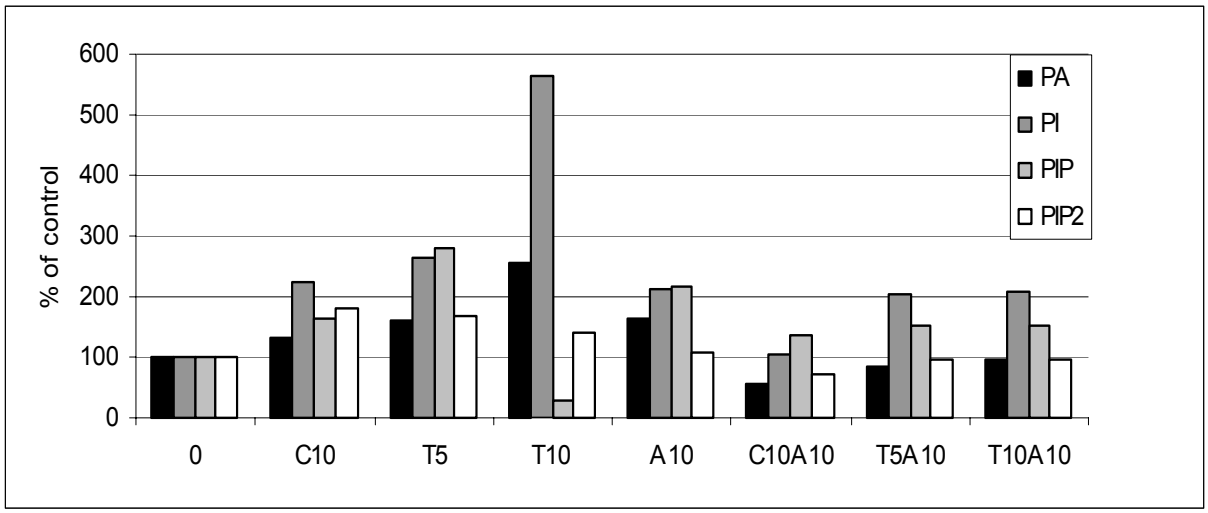
As evident from Figure 4.1.8 both 2,5 µM and 5µM TFP, without carbachol present, increased the incorporation rate of [32P]Pi into PA by 300% and 400% respectively. Adding different concentrations of carbachol to dishes containing 2.5 and 5.0 µM TFP gave a decrease in radioactive PA stimulated by TFP, similar to the [32P]Pi incorporation pattern found for different concentrations of carbachol alone (~250% at 2 µM Carbachol). This phenomenon was also seen for radioactive PI. Again 5µM TFP gave more radioactive PI with respect to control than 2.5µM TFP, without carbachol present, 450% and 350% respectively. Addition of carbachol results in a lower level of radioactive PI, similar to the effect of

carbachol alone (300% of control at 2 µM carbachol) For PI4P and PI4,5P_2 we saw no effect of carbachol on any of the concentrations of TFP. All values obtained remained close to control (results not shown).

4.1.7 The effect of Atropine on the [^{32}P]Pi incorporation induced by TFP

Atropine is a known nonspecific acetylcholine receptor antagonist. In this experiment we looked for a possible influence of atropine on the action of TFP.

Figure 4.1.10



Cell labelling conditions were as described previously. 10 µM Atropine was added 30 seconds before 5 and 10 µM TFP and 10 µM Carbachol. Passage number used: P18. Results obtained from one experiment.

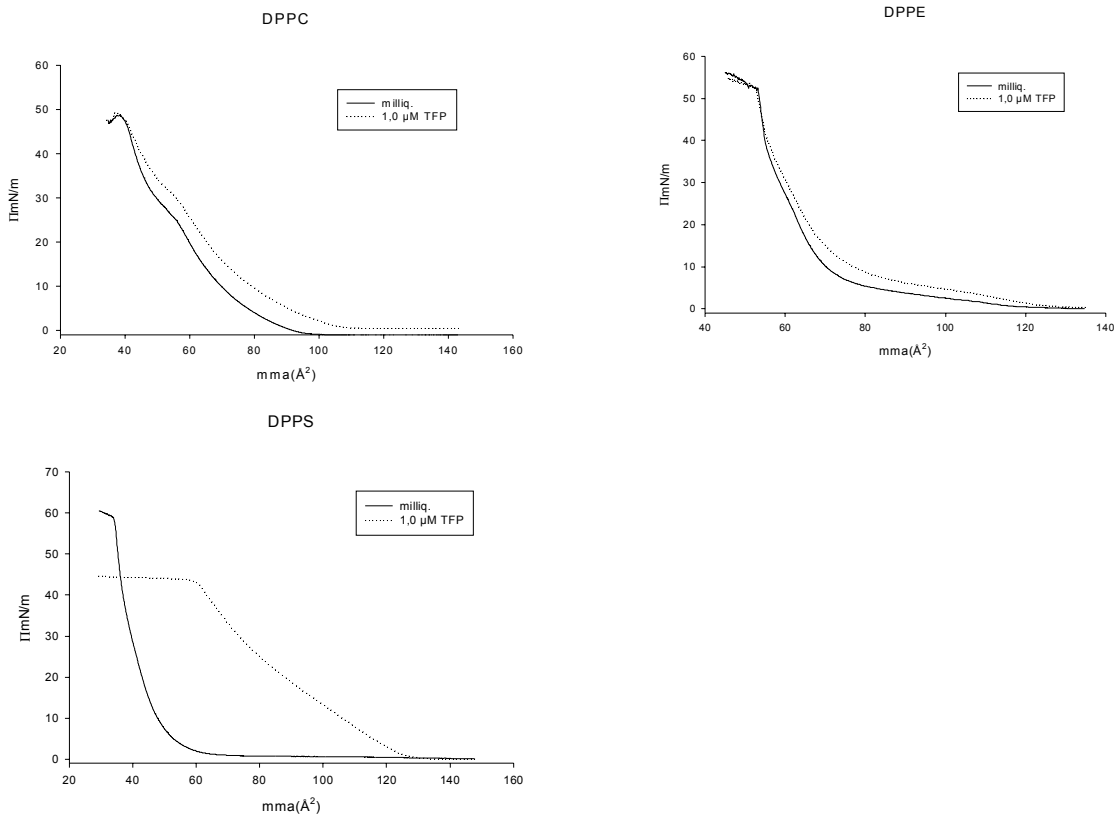
Figure 4.1.10 shows that atropine gave a reduction in carbachol stimulated [^{32}P]Pi incorporation into PI, PA, PI4P and PI4,5P_2 by 100% compared to the effect of 10 µM carbachol alone. 10 µM carbachol and 10µM atropine by themselves contained equal amounts of radioactive PI and PA, 200% and 150% respectively, but the values were expected to be higher for 10µM carbachol. 10 µM TFP alone caused an increase of 250% in PA and 500% in PI; 10 µM TFP together with 10µM atropine resulted in a reduction of almost 150% for PA and 300% for PI, values similar to the level caused by 10µM atropine alone.

4.2 The Langmuir isotherm technique

4.2.1 The role of the phospholipid head group

The effect of CPZ on monolayers of phospholipids depends, as mentioned before, on both the ionisation of the phospholipid head group, and the length and saturation of the two acyl-chains making up the phospholipids (Agasosler, Tungodden et al. 2001).
Figure 4.2.1 shows the π-A isotherm diagrams from experiments where the role of the phospholipid head group on the interaction of 1 µM TFP with phospholipid monolayers was determined. The acyl-chains (palmitoyl) are kept constant.

Figure 4.2.1



Surface pressure isotherms at 37°C, of DPPE, DPPC and DPPS on MilliQ-water and 1µM TFP. (Mean of three parallels for each experimental variation.)

Table 4.2.1

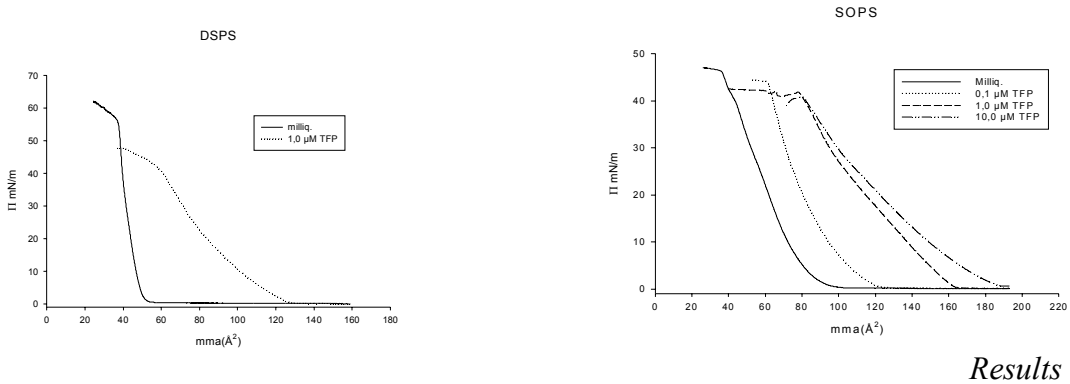
% increase molecular surface area, mma (Å^2) at 20 mN/m for DPPE, DPPC and DPPS.

	% increase in mma(Å^2) at 20 mN/m		
	DPPE	DPPC	DPPS
1,0 µM TFP	4.0	9.0	108.0

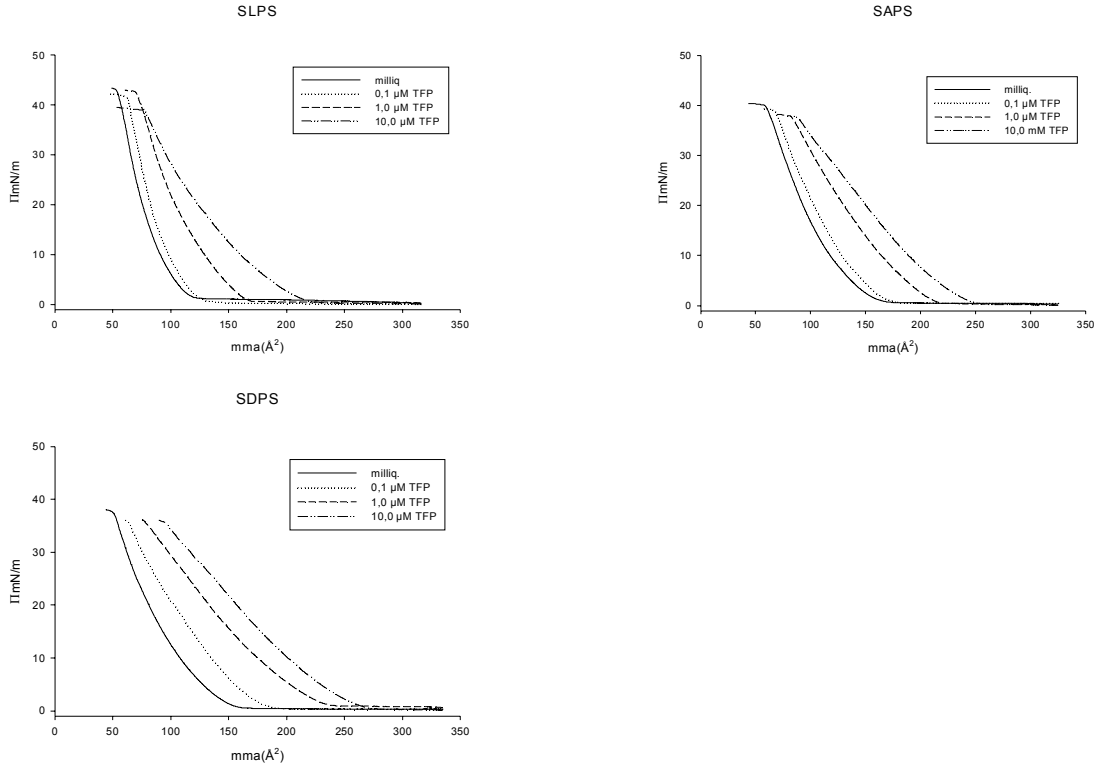
From figure 4.2.1 we can see that 1 mM of TFP in the subphase resulted indifferent increases in mean molecule surface area mma(Å^2) throughout the entire compression of the monolayer depending on the head group of the glycerophospholipid used. With neutral phospholipids DPPE and DPPC, TFP induced little effect on the mean molecule surface area (mma), only an increase of 4 and 9 % respectivly at 20mN/m (Table 4.2.1). With the negatively charged phospholipid DPPS, TFP caused a marked increase in molecular surface area compared to the neutral ones, 108 % at 20mN/m. The collapse point occurs at surface pressure 50 mN/m for DPPC and 54 mN/m for DPPE, independently of the presence of TFP. DPPS, on the other hand, showed a collapse point at 59 mN/m without TFP present and lowered the collapse point in the presence of TFP to 45 mN/m.

4.2.2 The role of phospholipid acyl chain composition

Figure 4.2.2



(Figure 4.2.2 continue)



The π-A Isotherm diagrams from monolayers of PS with different acylchain composition, in contact with various concentrations of TFP. Surface pressure isotherms at 37°C, of DSPS, SOPS, SLPS, SAPS and SDPS on MilliQ-water and various concentrations of TFP. (Mean of three parallels for each variations.)

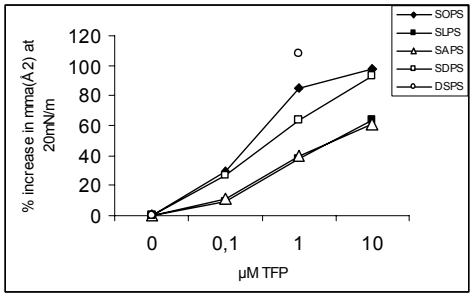
Table 4.2.2

% increase molecular surface area, mma (Å^2) at 20 mN/m for DSPS, SOPS, SLPS, SAPS and SDPS.

% increase in mma(Å^2) at 20 mN/m					
	DSPS	SOPS	SLPS	SAPS	SDPS
0.1 µM TFP		30.0	9.0	11.0	27.0
1.0 µM TFP	91.0	85.0	38.0	40.0	64.0
10.0 µM TFP		98.0	64.0	61.0	93.0

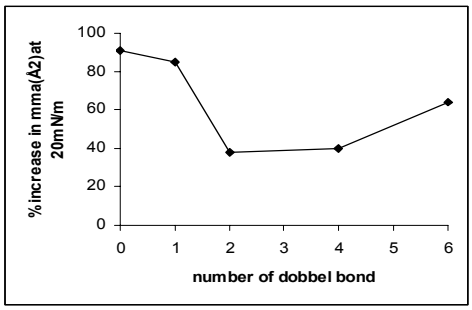
From figure 4.2.2 and table 4.2.2 we see that increasing the concentration of TFP affects the packing of the different PS species. Already at 0,1 µM TFP we see an effect, with greatest impact on the monolayer of SOPS, with an increase in mean molecular area (mma($Å^2$)) of 30% at 20 mN/m. As the number of double bonds increases, the mma($Å^2$) at 20mN/m decreased, with SLPS and SAPS giving the lowest values of mma($Å^2$) on all concentrations of TFP. SDPS with six double bonds had a slight increase in all mma($Å^2$) values at 20 mN/m compared to SLPS and SAPS. The results from Figure 4.2.2 are replotted in Figure 4.2.3, and it can be clearly seen that there is a general lowering in mma($Å^2$) values for all concentrations of TFP for SLPS and SAPS compared to SOPS and SDPS. Another replotting of the data is shown in Figure 4.2.4. Here only the value from one concentration of TFP (1µM) is plotted against number of double bonds in the *sn-2* acyl-chain in PS, keeping the *sn*-1 acyl group; stearoyl, constant. Again we note a slight increase in values for DSPS.

Figure 4.2.3



% increase in mma($Å^2$) at 20 mN/m for monolayer on different µM TFP.

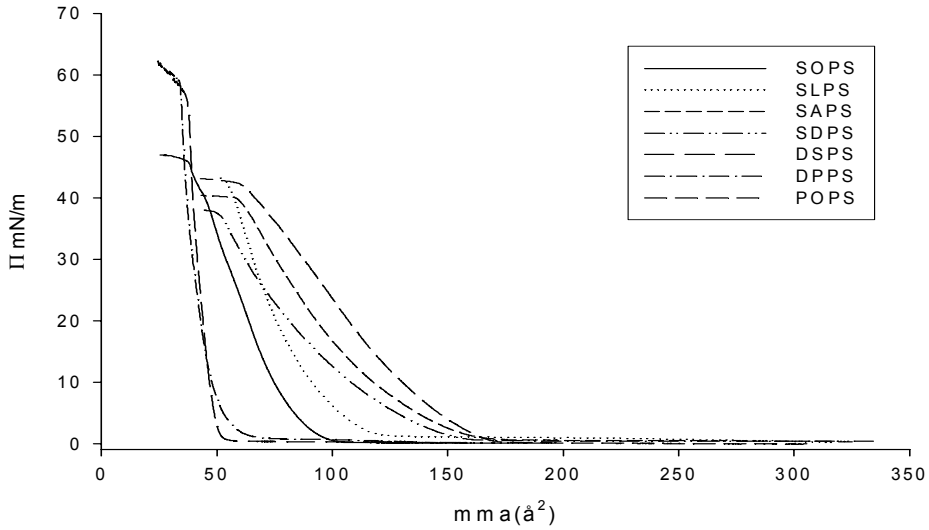
Figure 4.2.4



% increase in mma(Å2) at 20mN/m for monolayer on 1 M TFP
Number of double bond being
0 = SDPS, 1 = SOPS,
2=SLPS, , 4 = SAPS, 6 = SDPS

In all the π-A isotherm diagrams in figure 4.2.2 we see a change in both the collapse point and the mma range where the monolayers are in a condensed phase. The collapse point with *pure* MilliQ-water decreased when the length of the acyl chains was increased and the saturation of the acyl chains was decreased at the same time. This phenomenon is easily seen when compared in the same π-A Isotherm diagram (Figure 4.2.5).

Figure 4.2.5



Isotherm plots *for different glycerophospholipid monolayers on pure MilliQ-water. (no TFP) Reconstructed from figure 4.2.2*

Figure 4.2.5 shows that the different monolayers, without TFP, enter the condensed phase at different mean molecular areas, mma(Å^2), and have different values for the collapsing point, noting that all the monolayers were applied in the same manner and contains the same phospholipid concentration. The phospholipids with saturated fatty acid in the *sn*-2 position (DPPS and DSPS) had the mma (50 Å^2) lowest value of for both, and with a narrow range value around 10 mma(Å^2) where the condensed phase exist. The collapse point occurred at surface pressure around 59 mN/m. SOPS entered the condensed phase at 100 Å^2, which existed over 50 Å^2. The collapse point was at 40 mN/m. SLPS has the same tendency as SOPS, with the collapse at 45 mN/m; the condensed phase starts at 120 mma(Å^2) but only lasted over 30 mN/m. For SAPS and DSPS the collapse points are seen at 43 mN/m and 38 mN/m, respectively. The curves were less steep, and it is difficult to talk about a condensed phase lasting over more than 50 mma(Å^2). Between 150 mma(Å^2) and 100 mma(Å^2) the monolayers entered a liquid state, and the condensed phase was seen under 100 mma(Å2).

5 DISCUSSION

5.1 Neuroblastoma cells (SH-SY5Y)

All the methods applied in this thesis have been used for decades on human platelets at our laboratory. For this reason most of the results obtained by using these methods on the neuroblastoma cell line will be compared to corresponding results found in platelets. SH-SY5Y differs greatly from human platelets in both morphology and cellular activity, but the polyphosphoinositol metabolism in general was expected to be the same (Figure1.3.2). The variation in PA, PI, PI4P and PI4,5P_2 will be discussed separately from PE and PC, which are not directly involved in the PPI cycle, but first the main focus will be on the methods applied.

5.1.1 Methods

Alexander Staeffler, a former Ph.D. student was the first who started working with neuroblastoma cells in our laboratory. He transferred most of the methods to study the PPI turnover in humane platelets to the cells. After some time, some obvious methodological differences occurred. One great difference between the two cell types is the cell quantity in each experimental sample. For blood platelets each sample is taken from the same stock with known concentration, and used directly in the experiment. With the SH-SY5Y cells, the period near confluence, after about a week, is determined to be a good time to start the experiment. Complete confluence may turn the cells into a resting phase, which again may influence the cellular effects we are looking for. Large variations in cellcounts per dish can occur if careful attention is not paid to cell numbers at seeding. As mentioned in section 3.1.6 the cell count problem in this study was solved by using the amount of radioactive PE in each sample as an internal standard of total number of cells.

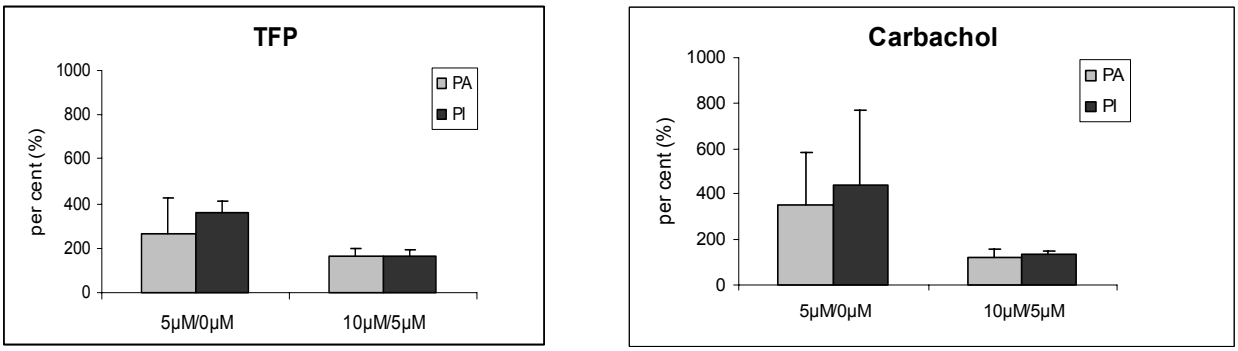
Another important issue appeared when the method to terminate drug stimulation in platelets could not be applied on the neuroblastoma cells. Stimulated platelets are stopped with stop-solution containing chloroform, and taken directly into lipid extraction. The lipid extraction for SH-SY5Y cells is more complicated since the cells are grown on plastic dishes and the first stopsolution in lipid extraction, which

contains chloroform, cannot be utilized as a stop solution since the chloroform will dissolve the plastic. The cells are instead washed with ice-cold DPBS containing Ca^{2+} and Mg^{2+} and stored at –20 ^{o}C until lipid extraction is performed. These cells are then scraped off with methanol and HCl, and chloroform is added after the cell suspension is transferred to a glass tube. This storage procedure is debated at our laboratory, since it is uncertain whether it stops the cell signalling rapidly and efficiently. However it saves time, and makes the whole procedure easier. Every step in the activation procedure is dependent on time and only three dishes can be handled at a time. It takes more than two hours from the first dish in the experiment is ready until the last one is finished. It seems impossible to start lipid extraction at the same time as the stimulation procedure is carried out. Here is a great challenge for those who follow later, making the experiment more practical, solving the time, storage and cell number problems.

Another important phenomenon concerning the methods applied, is the cells access to phosphate during the 60 minutes of cell labelling. [^{32}P]Pi has a half-life of 14 days. Using different batches of [^{32}P]Pi stock solution may provide the same phosphate radioactivity but will most likely differ in the concentration of total phosphate depending on the day of use. Both the radioactive (hot) and the cold phosphate are taken up by the cell and used in ATP turnover. The degree of [^{32}P]Pi labelling will not be equal at each experiment if the concentration of cold phosphate varies.

In the current study, the control dishes differ greatly in [^{32}P]Pi labelling and it influences the outcome of the results. The different plots in Figure 4.1.7 reveal a similar shape but different degrees of incorporation rate due to varying control values. This is illustrated further in figure 5.1.1. Here the concentration gradient is given with respect to variation among the different concentrations.

Figure 5.1.1



Variation among the different concentrations for TFP and carbachol *Cell labelling conditions were as described previously.. Passage number used: P18. Results obtained from three experiments.*

Figure 5.1.1 shows that there was great variation in the [32P]Pi incorporation rate from 0µM to 5 µM carbachol for both PA and PI. However from 5 µM to 10 µM less variation was seen, and there was a similar rise in the incorporation rate independent of the day the experiments were performed. The same tendency is seen with different TFP concentrations. Any correction for this phenomenon is not obtained in this study.

5.1.2 The effect of TFP and carbachol on PE and PC

In this study little or no attention is paid to the changes in [32P]Pi radioactivity in PE and PC. The radioactivity in PE was assumed not to be influenced by stimulation of either TFP or carbachol. [32P]PE is formed only through de novo synthesis (see figure 1.2.2), and in platelets little or no radioactive PE is seen after 60 minutes incubation with 0.1mCi/ml [32P]Pi. In SH-SY5Y cells, PE becomes [32P]Pi labelled and Figure 4.1.1 show a small but steady increase in radioactive PE over time. As mention under section 3.1.6, PE is used as an internal standard to determining the total quantity of cell material in each sample (see section 3.1.6). PE is not known to act as a precursor for any signal molecule in the PPI cycles, and all observations working with [32P]Pi labelled SH-SY5Y cells indicate a stable and equal incorporation rate for PE independent of activation of the PPI cycle, seen clearly in Figure 3.1.1.

PC shows a similar [^{32}P]Pi incorporation pattern as PE, and is [^{32}P]Pi -labelled through the de novo syntheses (figure 4.1.1 and 1.2.1). PC is known to influence the level of DAG and PA in SH-SY5Y cells after long-term stimulation with carbachol, but here, changes in radioactive PC is for simplicity not considered any further in this discussion.

5.1.3 The effect of TFP on the PPI cycle

As mentioned before under section1.1.4, TFP has a amphiphilic character and has been reported to have a membrane perturbing effect (Seeman 1972; Ferrell, Mitchell et al. 1988). The hypothesis is that the diverse effects of TFP go beyond the direct receptor-ligand interaction reported by many, and interact with the membrane surrounding the receptors and membrane associated enzymes affecting their reactivity indirectly. The duration of drug exposure is claimed to play an important role on membrane lipids (Daasvatn and Holmsen 1999). Short-term incubation (less than one hour), does not alter the lipid composition of PE, PC, and PI, but may affect the labelling incorporation rates for the individual phospholipids. Long-term stimulation (two hours or more) may alter the phospholipid composition in cell membranes. For example, exposure to TFP for more than 6 hours caused degradation of PC (Kolesnick and Hemer 1989). Twenty minutes incubation with TFP, as used in this study, will then not be expected to alter the lipid composition in SH-SY5Y cells. In resting human platelets no effect is seen by TFP alone on any of the PPI cycle metabolites, except a slight increase in radioactive PI (Tharmapathy, Fukami et al. 2000). To our surprise we found that in SH-SY5Y cells, TFP alone increases the incorporation rate of [^{32}P]Pi into both PA and PI and less so into PI4P and PI4,5P_2, in a time- and concentration-dependent manner (see Figures 4.1.4 and 4.1.5). Phenothiazines affect the activity of many enzymes in the PPI cycle. Inhibition of PAP (Brindley, Allan et al. 1975; Bowley, Cooling et al. 1977) (Hopewell, Martin-Sanz et al. 1985; Holmsen and Dangelmaier 1990), PI4P-4 phosphatase and PI4P 5-kinase(Frolich, Aarbakke et al. 1992) and activation of phosphatidate cytidyltransferase (CDP-DAG syntase) (Sturton and Brindley 1977) and CDP diacylglycerol inositol phosphatidyltransferase (PI-syntase)(Zborowski and Brindley 1983) have been reported. All three enzymes inhibited by the phenothiazines are located in or close to the plasma membrane. The proposed entry of the

drug into the cell membrane, changing enzyme activity, may then be one possible explanation of TFPs action. The same explanation cannot be logically applied when looking at the localization of the enzymes TFP is supposed to activate. They are all located at the endoplasmic reticulum in SH-SY5Y cells. An indirect activation of these enzymes by TFP must be explained by a possible entry of the drug into the cell cytosol. For decades, TFP was reported to bind to and inhibit calmodulin (Weiss B 1980). For this to be possible, TFP must enter the cell cytosol where calmodulin exists. On the otherhand, many of the papers reporting calmodulin inhibition have not carried out experiments on intact cell preparations, but rather in solutions containing free calmodulin.

In resting SH-SY5Y cells, [^{32}P]Pi incorporation into PA is very low, indicating restrict regulations (see figure1.3.2). An increase in radioactive PA can, as mentioned in the introduction, be produced from hydrolysis of PC via an activated PLD, and/or from phosphorylation of DAG. TFP is reported to activate PLD in a dose- and time- dependent manner (del Carmen Boyano-Adanez and Gustavsson 2002). The high level of [^{32}P]PA after exposure to different concentrations of TFP, seen in this study, can then be explained by PLD activation. Displacement and inhibition of PAP may be another explanation for the increase in [^{32}P]PA. PA produced from PC is a substrate for PAP while PA produced from hydrolysis of PI4,5P_2 by PLC most likely is not (Tysnes, Verhoeven et al. 1988). PA from PI4,5P_2 is enriched with 18:0/20:4 fatty acid combination and a conservation of the 18:0/20:4 fatty acids in the inositol phospholipids has been reported (Fukami 1995). PI, on the other hand, must be synthesized from PA at the ER complex, and transported back to the plasma membrane for further phosphorylation. 300% more PI than PA is [^{32}P]Pi labelled during incubation with 5µM TFP. PA produced through PLC activation is utilised in PI synthesis, but PA made from PLD is not (Whatmore, Wiedemann et al. 1999). A change in the PPI turnover due to PLC activation could lead to an increased turnover for PI as well, but PLC inhibition by TFP has been reported (Larsson and Alling 1995). TFP alters the initial production of IP_3 but leaves the plateau phase of IP_3 less affected. Calmodulin antagonism is said to be the cause of this PLC inhibition. Again, TFP is assumed to enter the cytosol, and if we stay with this explanation, an activation by TFP of the enzymes producing PI at the ER (mentioned above) is possible, or if not the increased level of PI must be explained by an ability of TFP to activate PLC at the receptor level. The time of incubation with a labelling precursor, such as

[32P]Pi, is of importance when considering the effects of drug stimulation. Daasvatn and Holmsen reported in 1999 that a complete [32P]Pi labelling of PI corresponding to its relative mass, in resting platelets, is reached after 90 minutes. Stimulation of platelets in this pre steady-state condition leads to an increased levels of radioactive PI, without affecting the total PI mass (Daasvatn and Holmsen 1999). In this study PI is not pre-labelled with [32P]Pi corresponding to its relative mass, and the increase in radioactive PI is due to increased [32P]Pi ATP turnover.

Both PI4P and PI4,5P_2 in platelets are after 60 minutes incubation with [32P]Pi said to be labelled so that the total radioactivity corresponds to their relative mass (Frolich, Aarbakke et al. 1992). In SH-SY5Y cells both PI4P and PI4,5P_2, are not completely labelled, as we see in figure 4.1.1, since the [32P]Pi incorporation rate continues to increase after 60 minutes pre-incubation. In platelets, PI4P is the one component in the PPI cycles that is greatly affected by phenothiazine exposure. Thrombin concentration of 0.05U/ml give a 300% increase in [32P]PI4P and addition of 10 µM CPZ caused an additional 200% increase (Underhaug 2001). The high level of [32P]PI4P was explained by PI 4-kinase stimulation or PI4P 5 kinase inhibition (T 1988; Huseby ES 1989), the levels of radioactive PI4P and PI4,5P_2 were less affected by TFP in SH-SY5Y cells. PI4P increases by only 200% after 20 minutes exposure to 10 µM TFP, while PI4,5P2 decreased by 50% compared to the control. The activity of the four enzymes regulating the level of PI4P and PI4,5P_2 may be affected by TFP. Figure 4.1.3 shows the effect of 10µM TFP over a time period of 30 minutes, and here the PI4P level was more affected, 400% above control. No other results on later occasions have been able to reproduce this increase, which suggest that this was a one-time artefact.

5.1.4 The effect of carbachol on the PPI cycles

Insulin and NGF did not cause activation of the PPI turnover in this experimental set up, and our attention was therefore shifted toward an activation of the PPI turnover by carbachol. A carbachol concentration range from 0-3µM was used. Carbachol increased the [32P]Pi incorporation into PA and PI and decreased the incorporation into PI4P and PI4,5P_2 in accordance with work done by others

(Quist and Satumtira 1987; Wei and Wang 1987; Morris, Cook et al. 1990; Frolich, Aarbakke et al. 1992; Limatola, Pacini et al. 1996; Sorensen, Linseman et al. 1998; Willars, Nahorski et al. 1998). A reduction in radioactive $PI4,5P_2$ to 70% of basal level upon maximal agonist stimulation is for example reported by G. B Willars and colleagues (Willars, Nahorski et al. 1998) The reduction in PI4P and $PI4,5P_2$ was not seen in platelets stimulated with thrombin. On the contrary, an increase in [^{32}P]PI4P and [^{32}P]$PI4,5P_2$ was seen instead. An increase in radioactive PA was reported in both SH-SY5Y cells and human platelets upon PLC activation. The level of radioactive PI was less affected by thrombin stimulation (less than 200% above control) in platelets, than with carbachol stimulation in SH-SY5Y cells (more than 200% above control).

A biphasic change in the level of radioactive PPI metabolites is seen in platelets, where low doses of agonist stimulate [^{32}P]Pi incorporation into PI4P and $PI4,5P_2$, indicating activation of the platelets, while high doses inhibit platelet responses, seen as a decrease in the same incorporation (Underhaug 2001). This biphasic response to different doses of agonist was not seen in SH-SY5Y cells.

A rapid and sustained desensitisation of the M3 receptor upon maximal agonist stimulation has been reported in many papers, for example references; (Xu and Chuang 1987; Wojcikiewicz, Tobin et al. 1994; Willars and Nahorski 1995; Sorensen, Linseman et al. 1998; Szekeres, Koenig et al. 1998; Edwardson and Szekeres 1999). Maximal desensitisation was reached within minutes, and although 1 mM carbachol was used in most articles, one research group has claimed that even a small amount of an agonist can cause sustained receptor internalisation after short-time stimulation (Sorensen, Linseman et al. 1998).

This phenomenon has received little or no attention in experiments performed on human platelets. It is not certain whether receptor desensitisation affects the incorporation rate of [^{32}P]Pi into the PPI cycle or not. It reduces the Ca^{2+} and IP_3 levels to a steady state or plateau phase slightly above the concentration level seen in resting cells, but does not seem to have a opposite effect on $PI4,5P_2$. In SH-SY5Y cells the $PI4,5P_2$ level was lowered as a result of PLC activation (figure 4.1.7), and upon massive receptor stimulation the turnover for PI and PI4P was altered to meet the need for $PI4,5P_2$. In Figure 4.1.7, a plateau phase was seen in the [^{32}P]Pi incorporation rate into PI4P and $PI4,5P_2$. Radioactive PA and PI also seemed to

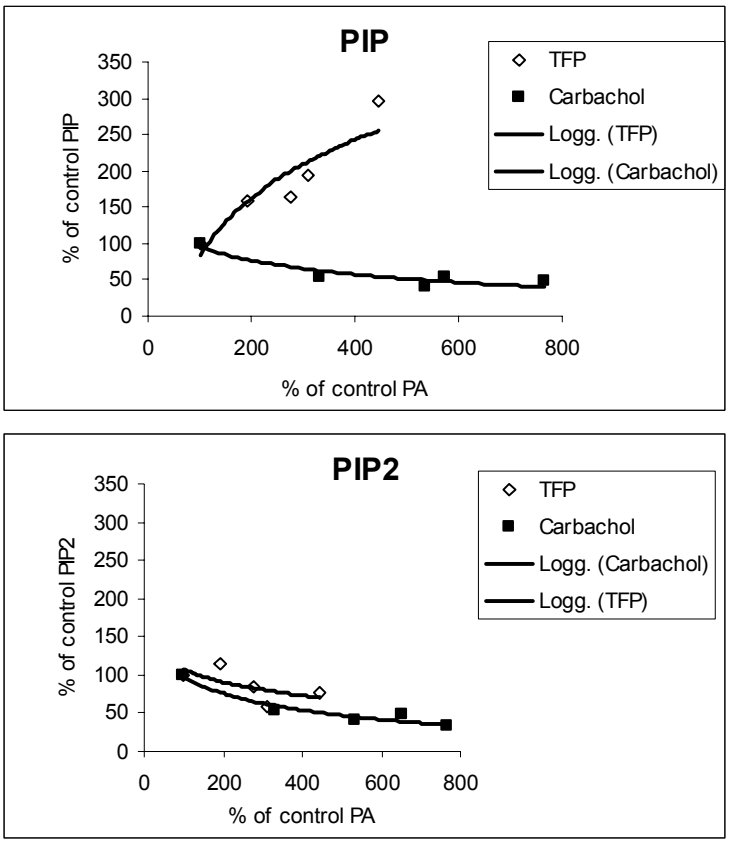
reach a plateau phase with steady-state concentrations greatly elevated above controls. Continuous stimulation with carbachol seems therefore to result in a steady-state condition in the PPI cycle and is only altered by removal of the agonist or addition of an antagonist.

5.1.5 The effect of different concentrations of carbachol and TFP together

TFP is, as mentioned earlier thought to influence the [32P]Pi incorporation rate into PPI metabolites produced by stimulation with an agonist such as carbachol. An enhancing effect was expected in accordance with that, which has been found on platelets stimulated with thrombin and TFP. This expected tendency was not seen in Figure 4.1.8.
It became important to use concentrations of both stimuli that did not give maximal effect on their own, to make sure that any enhancing effect of TFP was detected. 2.5 µM and 5µM TFP were used and the carbachol gradient was limited from 0 to 5 µM. Low concentrations of carbachol (0-3µM) lowers the effect TFP has on its own, as seen in both PA and PI. At carbachol concentrations above 3µM the effect of TFP is gone. No effect was seen on [32P]PI4P and [32P]PI4,5P_2, at any of the applied concentrations of carbachol and TFP. TFP have been reported to create a decrease in carbachol responses in SH-SY5Y cells, indicating antagonistic properties ((Wei and Wang 1987; Wakamori, Hidaka et al. 1993; Larsson and Alling 1995).The incorporation rate of [32P]Pi into the PPI cycle are controlled by a set regulations of the different enzymes involved, for example, by a change in the cytosolic Ca^{2+} concentration, and/or by feedback mechanisms reflecting the amount of PPI metabolites. In 1989 V. Steen, O.B Tysnes and H. Holmsen claimed that there is a internal regulation in the PPI cycle, producing a tight relationship between the level of PA, PI4P and PI4,5P_2. Any amount of PA will occur with a corresponding amount of PI4P and PI4,5P_2, independent of stimulation. A defined pattern in this relationship will emerge as the PLC activated hydrolysis of PI4,5P_2 is started. Any stimuli activating other enzymes in the cycle beside PLC will give alterations in this pattern, and can be used as a measure to determine the point of action for any given stimuli (Steen, Tysnes et al. 1989). This idea was carried out on the results obtained in this study to look for any possible explanation for the action of TFP. Looking at figure 5.1.2 we

see that an increase in PA lead to a reduction in the PI4P level in carbachol stimulated cells, but an increase in PI4P in TFP incubated cells. The corresponding $PI4,5P_2$ values decreased in both carbachol and TFP stimulated cells. Carbachol is known to give PLC activation and a decrease in PI4P, while TFP seem to influence the level of PI4P in a different manner comparable to PA, indicating an influence on the enzymes controlling the level of PI4P. These are the PI 4- and PI4P5- kinases and phosphatases, all situated near to or in the plasma membrane. PI-4 kinase stimulation and PI4P5 kinase inhibition are reported for CPZ (Huseby ES 1988; T 1988; Huseby ES 1989). The $PI4,5P_2$ level was less influenced by TFP compared PI4P, suggesting that TFP may have an effect on the enzymes controlling the step from PI to PI4P rather than from PI4P to $PI4,5P_2$.

Figure 5.1.2



Experimental values are taken from figure 4.1.5 and 4.1.7 and visualised with a logarithmic trend line.

5.1.6 The effect of Atropine on the [32P]Pi incorporation induced by TFP

Adding the acetylcholine receptor antagonist atropine to cells stimulated with TFP and carbachol was expected to decrease the level of PA and PI produced by carbachol, leaving the effect of TFP unchanged. Figure 4.1.8 shows the antagonistic effect of atropine on TFP induced increase in the level of radioactive PA and PI. Atropine has a membrane intercalating property on multilamellar vesicles of DPPC (Hao, Xu et al. 1998) and acts as a non-specific antagonist on the muscarinic receptor, inhibiting the carbachol stimulated accumulation of Ca^{2+} in SH-SY5Y cells (Heikkila, Jansson et al. 1991). Atropine decreases the Ca^{2+} concentration at the start of stimulation but does not influence the Ca^{2+} plateau phase (Oras, Jarv et al. 1999). Similar findings are reported for TFP (Wakamori, Hidaka et al. 1993).
The radioactive level of PI and PA induced by carbachol is in this experiment lower than expected, probably due to the fact that the actual carbachol concentration applied was lower than 10 μM.
The results obtained suggests that TFP not only influences the activity of the enzymes down stream from PLC in the PPI cycles, but may also act as an activator at the receptor and/or PLC level.

5.2 The Langmuir isotherm technique

TFP is an amphiphilic drug carrying a positive charge at the "tail" of the molecule and a hydrophobic area made by the three-ring structure, exactly like the related phenothiazine CPZ. TFP is also known to be more therapeutically effective than CPZ, and was expected to have the same, or even stronger, effect on monolayers of acidic glycerophospholipids.
Earlier work performed in our laboratory has shown that CPZ changes the packing of acidic glycerophospholipids in monolayers, increasing the mean molecular area (ma ($Å^2$)), as the concentration of the drug applied in the subphase was increases.
On the contrary, CPZ gives no significant increase in surface pressure (mN/m) at any concentration, on monolayers of the neutral glycerophospholipids DPPE and DPPC (Agasosler, Tungodden et al. 2001).

Some important experimental parameters are at this point *not* taken into account when searching for the effects of TFP. *1)* The subphase used here is free from other components than TFP, leaving out other monovalent and divalent cations. Two former master degree students at our laboratory, Armelle Varnier Agasøster and Anja Underhaug have established the effect of adding cations to the subphase and found it to decrease the general effect of CPZ on acidic phospholipids monolayers (Agasosler, Tungodden et al. 2001; Underhaug 2001). When adding Mg^{2+} to the subphase under monolayers of DSPS and DOPS using CPZ, no change in the collapse point, and only a small decrease in molecular surface area was observed throughout the isotherm diagram (Underhaug 2001). *2)* pH adjustments were *not* performed. The MilliQ-water contains no ions and determining the pH is impossible without adding ions like KCl. At this point the maximal effect of TFP is of interest and adjusting pH to 7.3 will most likely decrease the observed effects of TFP. The variation in mean molecule surface area was explored at 20 mN/m where the effects of TFP are large. Some will argue for using the value 30 mN/m since this pressure simulates more closely the actual biological pressure in the cell membrane. On later occasions where the biological relevance is being explored, ions, pH and relevant surface pressure would be considered. The temperature was set to 37^{o}C being the only variable close to biological conditions.

5.2.1 The role of glycerophospholipid head group

From these results, it becomes clear that ionisation forms of the glycerophospholipid play a major role in the degree of interaction of TFP on the monolayers. At 1 µM TFP we observed an increase in mma from 4% to 108 % at 20 mN/m for DPPE and DPPS, respectively. For DPPS this can be explained by electrostatic interactions between the positively charged part of TFP and the negatively charged phosphatidylserine. The TFP induced increasment in mma of DPPE and DPPC was not so dramatic as for DPPS. There was a small effect from TFP at the lower parts of the isotherm diagram, but when the surface pressure was increased and the monolayer reached the condensed phase and collapse point, no effect was seen for TFP. An explanation for this phenomenon may be that the high surface pressure seems to be able to squeeze out the few TFP molecules present in the monolayer. We cannot talk about a great

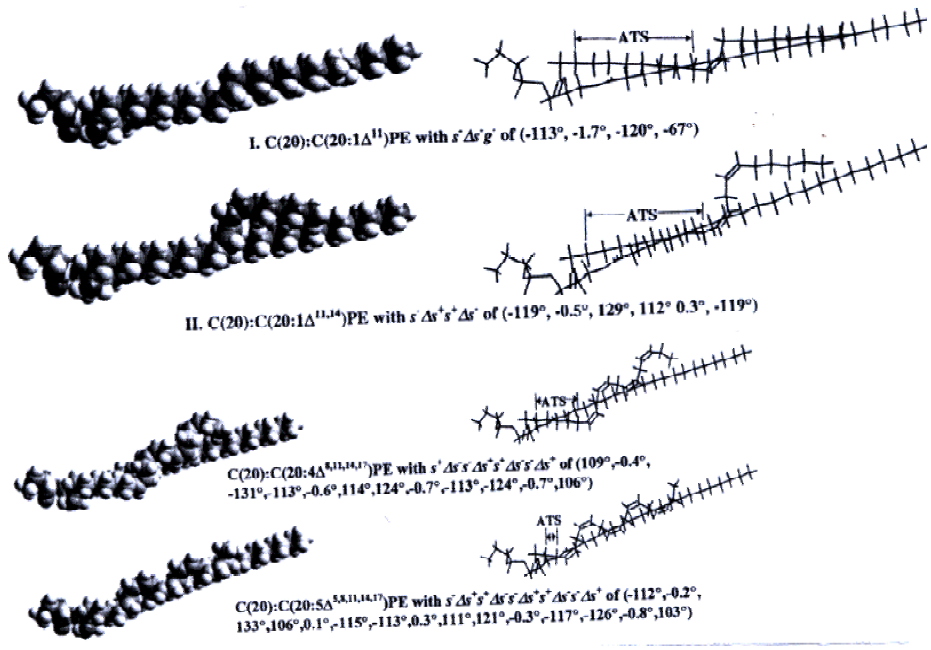
electrostatic interaction and the hydrophobic "force" alone seems not to play a major role in keeping the TFP molecules present in neutral glycerophospholipid monolayers. A totally different isotherm diagram for DPPS was seen when TFP was present in the suphase and indicated that the electrostatic forces present may play an important part in keeping the TFP molecules within the monolayer. The mma was elevated at all values of the surface pressure and the collapse point was lowered dramatically, all indicating that the TFP molecules remain in the monolayer throughout the compression.

5.2.2 The role of the glycerolphospholipid acyl chains

By keeping the head group constant and varying the acyl-chain in position *sn*-2 both in length and degree of saturation we investigated the role of unsaturated fatty acid molecular forms. In Figure 4.2.2 one notices the general lowering of the collapse point for monolayers on pure MilliQ-water. Figure 4.2.5 demonstrates this clearly. To be able to explain this observation we had to look at the difference in molecular conformation among the phosphatidylserine species, and their possible influence on monolayer packing. The first thing to keep in mind is that the isotherm temperature is set at 37^{o}C. At this temperature all phosphatidyl species used, except DPPS (T_m = 54^{o}C) and DSPS (T_m = 68^{o}C), are exposed to a temperature above the melting point; hence they are in a highly dynamic and disordered state, and the chain-chain interactions between the different fatty acids in the *sn*-1 and *sn*-2 position is minimal. There was a varying degree of interaction between TFP (independent of drug concentration) and monolayer species depending on the fatty acid unsaturation, seen like a saddle pattern. (See Figure 4.2.4) The phosphatidylserines with saturated fatty acids in *sn*-2, like DPPS and DSPS, show higher degrees of interaction than PS with only one double bond in the *sn*-2 position. SOPS has introduced one double bond in the acyl chain in position *sn*-2 and already here a slight decrease in mma at 20mN/m for 1µM TFP, from 98% for DSPS to 85% for SOPS was seen. The introduction of two, and four double bonds resulted in even less interaction with the drug, and was independent of TFP concentration. SLPS with two double bonds, and SAPS where there are 4 double bonds and the length of the acyl chain extended with two CH_2-groups, gave not more than a half of the mma value at 20mN/m for 1µM of TFP, i.e., from 85% for SOPS, to 38% for SAPS. SDPS has six double bonds and the longest acyl-chain, four extra methylene groups compared to

DSPS, but here we saw a slight increase in mma at 20mN/m for 1µM TFP, from 40% with SAPS to 68% for SDPS. In early 1990, Keough and colleagues showed that the introduction of one and two cis double bond into the *sn*-2 position of 20:0/20:0-PC gave a decrease in T_m, and introduction of a third (or more) cis double bound (s) caused again an small increase in T_m, giving a "down and up" trend in the T_m profile (Coolbear, Berde et al. 1983; Keough, Giffin et al. 1987; Keough 1990). The same pattern was found by G. Wang and S. Li et.al.1989 who looked further at the effect of number and position of cis double bonds at the *sn*-2 position in phosphatidyletholamine (PE), and their effect on chain melting temperature T_m. They found that the changes in T_m depended critically on the position and number of cis double bond in *sn*-2 position and they attributed the energy-minimised structural differences among the phospholipid molecular species as one explanation for the changes in T_m. Figure 5.2.1 shows some of their modelled structures with T under T_m (Wang, Li et al. 1999).

Figure 5.2.1



Molecular structures *of different PE species (T under T_m) (Wang, Li et al. 1999) From the top: PE 20:0/20:1(n-9), PE 20:0/20:2(n-6), PE 20:0/20:4(n-3), PE20:0/20:5(n-3).*

Figure 5.2.1 gives indications of a slightly more disordered conformation for molecular species with one, two and three double bonds, than for those with four and more.
The double bond unit, C-CH=CH-C, is coplanar and the double bound is rotationally restricted, while the C-C single bonds maintain their rotational flexibility even at low temperatures – 10^{o}C (Li, Wang et al. 1993).
Introducing a higher degree of unsaturation will give the chain less rotational freedom and a more rigid molecular conformation. Appelgate and Glomset (1991) studied the effect of acyl chain unsaturation on structural conformations depending on the number of cis double bounds in the *sn*-2 chain of diacylglycerols. They found three energy-minimised conformations that gave distinctively different types of packing arrangements in glycerolphospholipid monolayers. Both 22:6(n-3) and 18:2(n-6), gave regular but different packing patterns, and 18:1(n-9) gave a regular but much looser packing pattern then the two above(Applegate and Glomset 1991).
Knowing the influence of molecular structure on the melting point and the monolayer packing it is tempting to speculate whether or not increasing the unsaturation in the series of phosphatidylserine species can explain the “down and up” phenomenon observed. Perhaps the degree of disorder among the monolayer forming molecules affects the interaction of TFP with molecules in the monolayer. The state of the molecules in SLPS and SAPS monolayers at 37^{o}C are more flexible and disordered, giving the TFP molecules a less stabile environment for insertion, than in SOPS and SDPS monolayers.

5.3 Future perspective

Comparing the effects of TFP on human platelets and SH-SY5Y cells revealed some important differences concerning incorporation of radioactive phosphate into the metabolites in the PPI cycle, and showed that further investigation of the phenothiazines action on the PPI cycle in SH-SY5Y cells is preferable. Some methodological adjustments must be done to correct for differences in cell number at seeding, but beside this, the SH-SY5Y cells provide a good model system for human neurons.
The effect of TFP on the PPI cycle in the SH-SY5Y cell *nucleolus* would here be another project of interest.
Jet another project would be to establish if the phenothiazines, due to their amphiphilic character, would be able to crosses the cell membrane, since this possibility is not ruled out by the methods applied to day.

Further, the nature of the interaction between TFP and different phospholipids would be of interest. Anja Underhaug, together with Holm Holmsen and Willy Nerdal has established by solid-state nuclear magnetic resonance on liposomes of different mixtures of DPPC and PS species, that certain ration of these phospholipids give better interaction with CPZ.
The same idea could be used in Langmuir studies, varying phospholipids composition in monolayers.

Far into the future atomic force microscopy may give the possibility to study the interaction of TFP with cell membranes by visualising the intact membrane surface area and reveal the possible insertion of TFP molecules.

6 CONCLUSIONS

The current study demonstrates that TFP clearly changes the PPI cycle turnover in SH-SY5Y cells, seen as changes in radioactivity in the metabolites involved. The question to answer is; where is the action of TFP situated? Does TFP act at the receptor level or on the enzymes downstream from the receptor? TFP is an amphiphilic molecule possessing both a hydrophobic and a hydrophilic region. Because of this property our hypothesis has been that TFP will enter and alter the membrane milieu in such a way that enzymes embedded in the membrane will have their activity changed. In SH-SY5Y cell many of the enzymes producing metabolites in the PPI cycles is situated at the cytosolic part of the cell, in or near the ER and Golgi apparatus. A direct influence by TFP on these enzymes requires cell entry. Assuming that TFP does not pas the cell membrane but interact with the extracellular side of the cell membrane, makes activation of embedded enzymes and receptors indirectly a possible point of action. The TFP induced increase in radioactive PA and PI is inhibited with low concentrations of both carbachol and atropine, indicating actions at the receptor level for TFP. The Langmuir study did support the idea that TFP has a membrane-interacting property, but showed at the same time the importance of an opposite charge present on the corresponding phospholipids for interaction to occur. The unsaturation and length of the acyl-chain in position *sn*-2 indicate a possible influence on the degree of interaction between the acidic phospholipids and TFP. Molecular species with high disorder and a low melting point give a less stable environment for interactions with TFP.

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