

Interplay between AnnexinA6 and Phospholipase D1 during the process of exocytosis in PC12 cells Le Duy Do

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INTERPLAY BETWEEN ANNEXIN A6 AND PHOSPHOLIPASE D1 DURING THE PROCESS OF EXOCYTOSIS IN PC12 CELLS

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Abstract:

Interplay between AnnexinA6 and Phospholipase D1 during the process of exocytosis in PC12 cells

The regulated exocytosis is a key process allowing cell-cell communication through the release of hormone and neurotransmitters. In neurons and neuroendocrine cells, it is strictly controlled by extracellular signal such as transmembrane potential and ligand bindings to receptors. Substantial progress has been made to understand the molecular mechanism of exocytosis. Major components of secretory machinery have been brought to light. Now the emergent question concerns the role of scaffolding proteins that are thought to coordinate the action of each other. In the case of annexin family well known to be involved in exocytosis, their modes of -sequential or concerted- interactions with other proteins, and their regulatory effects on exocytosis are not very well established. Previous findings indicated that Annexin A6 (AnxA6) affected calcium homeostasis and dopamine secretion from PC12 cells, used as cellular model of neurosecretion (Podszywalow-Bartnicka et al., 2010). To determine the inhibitory effect of AnxA6 on exocytosis of dopamine, we were looking for molecular partners of AnxA6 in PC12 cells. We hypothesized that AnxA6 interacts with phospholipase D1 (PLD1), an enzyme involved in the fusion step. By using confocal microscopy and total internal reflection fluorescence microscopy, we found that isoform 1 of AnxA6 and Phospholipase D1 are both recruited on the surface of vesicles upon stimulation of PC12 cells. AnxA6 inhibited phospholipase D activity as revealed by our enzymatic assay based on infrared spectroscopy. To conclude, we propose that AnxA6 is not only implicated in membrane organization by its capacity to bind to negative charged phospholipids and to cholesterol, but AnxA6 is also affecting PLD1 activity, changing membrane lipids composition.

Keywords: Exocytosis, PC12 cell, Annexin A6, Phospholipase D1, Total internal reflection fluorescence microscopy, Laser scanning confocal microscopy, Calcium imaging, Infrared spectroscopy

Résumé:

Relation entre l'annexine A6 et la phospholipase D1 pendant le processus d'exocytose dans les cellules PC12

L'exocytose régulée, est un processus qui permet la communication entre les cellules à travers la sécrétion des hormones et des neurotransmetteurs. Dans les neurones et les cellules neuroendocrines, l'exocytose est strictement contrôlée par des signaux extracellulaires tels que le potentiel trans-membranaire et la fixation des ligands sur des récepteurs. Des progrès substantiels ont été effectués afin de comprendre le mécanisme moléculaire de l'exocytose. Les composants majeurs de la machinerie de sécrétion ont été dévoilés. Maintenant, la question qui émerge concerne le rôle de la plateforme de protéines qui semble avoir une action coordonnée entre chaque protéine. Dans le cas de la famille des annexines, qui est bien connue pour son action dans l'exocytose, leurs modes d'interactions séquentielles ou concertées avec d'autres protéines ainsi que leurs effets régulateurs sur l'exocytose ne sont pas encore bien établis. Des résultats précédents indiquent que l'Annexine A6 (AnxA6) affecte l'homéostasie du calcium et la sécrétion de la dopamine à partir des cellules PC12, utilisées comme un modèle cellulaire de neurosécrétion (Podszywalow-Bartnicka et al., 2010). Afin de déterminer l'effet inhibiteur de l'AnxA6 sur l'exocytose de la dopamine, nous cherchons des partenaires moléculaires de l'AnxA6 dans les cellules PC12. Nous faisons l'hypothèse que l'AnxA6 interagit avec la PLD1, une enzyme active dans l'étape de la fusion des vésicules avec la membrane plasmique. En utilisant la microscopie confocale et la microscopie à onde évanescente, nous avons trouvé que l'isoforme 1 de l'AnxA6 et la PLD1 sont tous les deux recrutés sur la surface des vésicules au cours de la stimulation des cellules PC12. AnxA6 inhibait l'activité de la PLD comme indiqué par notre méthode d'analyse enzymatique au moyen de la spectroscopie infrarouge. En conclusion, nous proposons que l'AnxA6 n'est pas seulement impliquée dans la réorganisation des membranes par ses capacités à se lier avec des phospholipides négativement chargés et avec le cholestérol, mais elle influence également l'activité de la PLD1, changeant la composition lipidique des membranes.

Mots-clés : Exocytose, Cellule PC12, Annexin A6, Phospholipase D1, Microscopie à onde évanescence, Microscopie confocale, Imagerie calcique, Spectroscopie infrarouge.

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ABBREVIATIONS

Abbreviations

A431 cells	human epithelial carcinoma cell line
Anx	annexin
ATP	adenosine triphosphate
Ca ²⁺	calcium ion
CgA	chromogranin A
CHO cells	Chinese hamster ovary cells
DA	dopamine
DMPC	dimyristoyl phosphatidylcholine
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EP	epinephrine
ER	endoplasmic reticulum
FBS	fetal bovine serum
GAP	GTPase activating protein
GEP	GTP exchange protein
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS	horse serum
IP ₃	inositol-1,4,5-triphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IR	infrared
LB	lysogeny broth
LDCV	large dense-core vesicles
NCX	Na ⁺ /Ca ²⁺ exchanger
NE	norepinephrine
NGFR	nerve growth factor receptor
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PC12 cells	cell line derived from a pheochromocytoma of the rat adrenal medulla
PE	phosphatidylethanolamine
PG	phosphatidylglycerol

PH domain	pleckstrin homology domain
PI	phosphatidylinositol
PIP ₂	hosphatidylinositol 4,5-bisphosphate
РКСα	protein kinase Ca
PLA2	phospholipase A2
PLD	phospholipase D
PS	phosphatidylserine
PX domain	phox homology domain
SNAP	soluble NSF attachment protein
SNAP-25	synaptosomal-associated protein 25
SNARE	SNAP receptor
SOCE	store-operated calcium channels
TBS	tris buffered saline
TGA	trans-Golgi apparatus
VAMP	vesicle associated membrane protein
VDCC	voltage-dependent calcium channel

CHAPTER I: INTRODUCTION

I. Introduction

1. Exocytosis

In humans, the most abundant catecholamines are: dopamine (DA), norepinephrine (NE), and epinephrine (EP). They are synthesized from tyrosine in neurons or in neuroendocrine cells including chromaffin cells (from adrenal medulla) as well as in tumorous PC12 cells. Catecholamines act as neurotransmitters in the peripheral and central nervous systems (Laverty, 1978). Secretion of catecholamine is crucial for cell survival (Noh *et al.*, 1999). They are stored in vesicles whose secretion is strictly regulated by external signals.

Secretory vesicles are initially formed by budding from trans-Golgi apparatus (TGA). (Chromogranin A (CgA) has been identified as a key component in the initiation of this process thanks to its capacity to interact with and aggregate membranes (Huttner *et al.*, 1991). Silencing of CgA in PC12 reduced granule number (Kim *et al.*, 2001). Next, these vesicular buds need to be excised from TGA to form premature vesicles. The mechanism is unclear but cholesterol and dynamin II are implicated in the excision (Wang *et al.*, 2000; Yang *et al.*, 2001). Due to the cone-shape form, cholesterol may induce negative curvature of Golgi membrane and facilitate the budding (Bacia *et al.*, 2005), (Corda *et al.*, 2002), (Orci *et al.*, 1980). In addition, cholesterol, together with other lipids, forms "lipid rafts" where proteins necessary for vesicle formation, e.g. chromogranin (Kim *et al.*, 2006), are recruited. Thus, imbalance of intracellular cholesterol may negatively affect vesiculation of TGA (Cubells *et al.*, 2008).

Once formed, immature vesicles undergo maturation process as long as they are transported along the microtubule network toward plasma membrane by means of kinesin (Hamm-Alvarez *et al.*, 1997), (Senda *et al.*, 1999), (Varadi *et al.*, 2003). The maturation consists of interfusion to generate bigger and denser vesicles and in parallel, elimination of misassorted compounds by budding of clathrin-coated vesicles (Burgoyne and Morgan, 2003). The molecular machinery of vesicle-vesicle fusion (homologous fusion) is similar to the one that drives vesicle-plasma membrane fusion (heterologous fusion) and is dependent of the action of a SNAP (soluble NSF attachment protein) receptor (SNARE complex) (Tooze *et al.*, 1991; Urbé *et al.*, 1998). The maturation step might consist of elimination of SNARE proteins involved in vesicle-vesicle fusion from premature vesicles (Burgoyne and Morgan, 2003) because

they can undergo both homologous fusion as well as heterologous one (Tooze *et al.*, 1991). In contrast, mature vesicles cannot fuse with each other.

At the extremity of the microtubule secretory vesicles are transferred onto the cortical actin network at the vicinity of the plasma membrane (at a distance of 200 nm from the plasma membrane). Myosin V is implicated in the transfer by interacting with microtubule-based motors (Huang *et al.*, 1999; Brown *et al.*, 2001 and 2002). The same protein may also help the vesicle to move through the actin cortex (Rosé *et al.*, 2003; Rudolf *et al.*, 2003; Eichler *et al.*, 2006). However reorganization of actin has a non-negligible contribution. Under basal conditions, cortical actin acts as a barrier, retaining vesicles and preventing their contact with the plasma membrane. Upon cell stimulation, this network is reorganized to allow vesicles to penetrate (Nakata and Hirokawa, 1992; Vitale *et al.*, 1995; Giner *et al.*, 2005). Vesicles may diffuse to the plasma membrane but the actin network is guiding the vesicles toward specific exocytosis sites (Wang and Richards, 2011).

Once the actin network crossed, the vesicles associate slightly with the plasma membrane. This state is called vesicle tethering and is characterized by the capacity of vesicles to associate/dissociate spontaneously with the plasma membrane. Once docked, vesicles cannot move freely and they are ready to fuse with the plasma membrane upon cell stimulation. Rab 3a and Rab 27a, two members of Rab GTPase family, mediate tethering and docking (Park and Loh, 2008). The fusion step is essentially carried by three proteins: VAMP (vesicle associated membrane protein)/synaptobrevin, syntaxin1 and SNAP25 (synaptosomal-associated protein 25) (Söllner et al., 1993; Sutton et al., 1998). VAMP/synaptobrevin is found on the docked vesicle membrane (Trimble et al., 1988) while syntaxin1 (Bennett et al., 1992) and SNAP25 (Oyler et al., 1989) associate with the plasma membrane at the docking site. Upon cell stimulation, these proteins will interact together and form the SNARE complex which brings vesicle and plasma membrane close to each other and allows their fusion (Hanson et al., 1997). Typical SNARE complex is the assembly, by hydrophobic interaction, of one α -helix from VAMP, one from syntaxin1 and two from SNAP25 (Poirier et al., 1998; Sutton et al., 1998).

Calcium is the most well-known second messenger which couples external signal with the triggering of exocytosis. In chromaffin cells, elevation of cytosolic calcium concentration is a prerequisite for the transport of granules carrying catecholamines, their fusion with plasma membrane and release of catecholamines to the extracellular milieu (Burgoyne and Morgan, 2003). Upon cell stimulation the cytosolic calcium concentration rises up due to either the calcium released from endoplasmic reticulum (ER) and/or the uptake of calcium from the extracellular milieu (Burgoyne and Morgan, 2003). Inositol-1,4,5-triphosphate (IP₃) mediates ER calcium release which is followed by calcium entrance via store-operated calcium channels (SOCE) (Fomina and Nowycky, 1999; Inoue *et al.*, 2003). On the contrary, cell depolarization usually activates voltage-dependent calcium channels (VDCCs) which allow massive influx of extracellular calcium (Garcia *et al.*, 2006). Na⁺/Ca²⁺ exchanger (NCX) working in the reverse mode also contributes to calcium uptake (Annunziato *et al.*, 2004). Synaptotagmin and syntaxin are intracellular calcium sensors of the exocytotic machinery (Sudhof and Rizo, 2011). Synaptotagmin, once bound with calcium, triggers exocytosis by interacting with the SNARE complex

Exocytosis is a highly synchronized process every single step of which is intensively investigated. However, much less effort has been spent to identify which proteins connect and coordinate these events.

2. Role of Annexin A6 in exocytosis

a. Protein structure

Annexin A6 (AnxA6) belongs to the annexin family of proteins, characterized by the capacity to bind reversibly to negatively charged phospholipids in a calcium-dependent manner thanks to the presence of the so-called annexin core domain at the C-terminus. Generally these conserved domains are composed of a tandem of four annexin repeats (70-80 amino acids), each containing five α -helices (Raynal and Pollard, 1994). The particularity of AnxA6 is the presence of two annexin cores and of a connecting linker region which is absent in other annexin members (Fig.I.1) (Smith et al., 1994; Benz et al., 1996; Kawasakia et al., 1996). The fusion of AnxA5 and AnxA10 gene during evolution is thought to give birth to AnxA6 gene containing 26 exons (Kaetzel et al., 1994). Alternative splicing of exon 21 encoding the seventh annexin repeat results in two isoforms: AnxA6-1 and AnxA6-2 which differ by six amino acids: ⁵²⁵VAAEIL⁵³⁰ (Moss and Crumpton, 1990; Smith et al., 1994). The larger isoform, AnxA6-1, is more hydrophobic but has less affinity to calcium than the second isoform (Kaetzel et al., 1994). In normal tissues AnxA6-1 is the most abundant isoform (Kaetzel et al., 1994) while AnxA6-2 is highly expressed in neoplastic cells (Edwards and Moss, 1995). This suggests that two isoforms have distinctive function.

The crystal structure of AnxA6 showed six calcium binding sites (Fig.I.1) located at repeats II, III, IV, V, VI, VIII of the annexin cores (Raynal and Pollard, 1994). These calcium ions are the link which serves to connect AnxA6 to the hydrophilic heads of negatively charged phospholipids.



Figure I.1: 3D structure of bovine AnxA6 (1AVC). The protein is constituted mostly from α -helices. The color code is as follows: annexin repeat I and V in green, repeat II and VI in blue. repeat III and VII in cyan, repeat IV and VIII in magenta, the linker in grey, calcium atoms in yellow. AnxA6 is composed of two annexin cores which are connected by a linker region

containing the grey helix. Each core is formed from four annexin repeats and each domain is composed of 5 helices. The Two annexin domains of AnxA6 are roughly perpendicular to one another. There are six calcium-binding sites in the AnxA6 crystal structure. This figure was created after (Avila-Sakar *et al.*, 1998) by using YASARA software (YASARA-Bioscience).

b. Role of Annexin A6 during exocytosis

It has been observed that there was a correlation between high level of AnxA6 expression and secretory activity of certain cells including the islet cells of the pancreas, the Leydig cells of the testis and the cells of the adrenal cortex (Clark *et al.*, 1991). The authors showed furthermore that AnxA6 was absent in epithelial cells with the exception of epithelium of secreting organs suggesting that AnxA6 regulates secretion. *In vitro* experiments indicated that AnxA2, AnxA6, and AnxA7 could induce aggregation of chromaffin granules but they had different calcium requirement (Zaks and Creuzt, 1990). Later on, AnxA6 was found to aggregate phosphatidylserine liposomes *in vitro* in a calcium and ATP-dependent manner (Bandorowicz-Pikuła and Pikuła, 1998). Further investigation demonstrated that at low calcium concentration (60-150 μ M) AnxA6 binds to PS (phosphatidylserine) liposomes but is not able to aggregate them, while at high calcium concentration (2 mM) AnxA6 can bind simultaneously to two different membranes and induce aggregation (Buzhynskyy *et al.*, 2009).

In addition to its capacity to bind to acidic phospholipids in a calcium-dependent manner, overexpression of AnxA6 in cardiomyocytes decreased the resting cytosolic calcium concentration ($[Ca^{2+}]_c$) and inhibited calcium entry upon cell stimulation and therefore impaired cardiomyocyte contractility (Gunteski-Hamblin et al., 1996). In A430 cells which naturally lack AnxA6, overexpression of this protein inhibited epidermal growth factor (EGF)-dependent Ca²⁺ entry (Fleet et al., 1999). Furthermore, involvement of AnxA6 in the regulation of the sarcoplasmic reticulum ryanodinesensitive Ca²⁺ channel (Diaz-Munoz et al., 1990) and neuronal K⁺ and Ca²⁺ channels (Naciff et al., 1996) has been revealed. Recently, it was reported that overexpression of AnxA6 in PC12 cells reduced calcium uptake upon cell stimulation and lowered catecholamine secretion (Podszywalow-Bartnicka et al., 2010). So far, this is the first direct proof of direct involvement of AnxA6 in exocytosis. Since exocytosis is a calcium dependent process, it is hard to conclude without further investigation whether AnxA6 inhibits the exocytotic machinery or if the reduced secretion is the consequence of alteration in calcium homeostasis. To explain the AnxA6-dependent mechanism of exocytosis we wondered whether AnxA6 can interact with phospholipase D1 (PLD1) since is one of important components of secretory machinery. The following chapter will bring some information on PLD1 and its protein machinery implicated in exocytosis.

3. Role of PLD1 in exocytosis

a. PLD functional domains

PLD hydrolyzes the phosphodiester bonds of membrane phospholipids, producing phosphatidate (PA) and alcohol. In mammalian cells, the most frequent substrate of PLD is phosphatidylcholine (PC) (Liscovitch, 1994). In humans, two genes, pld1 and pld2, encoding the PLD enzyme were found. pld1 encodes the 124 kDa protein PLD1a (1074 amino acids), and an alternatively spliced form PLD1b (1036 amino acids), which lacks 38 amino acid residues and is the best characterized variant. Both isoforms are capable of hydrolyzing PC, PE, PS but are not capable of hydrolyzing PI, phosphatidylglycerol (PG) or cardiolipin (Selvy *et al.*, 2011). It is usually stated that PLD1 and PLD2 are expressed in nearly all mammalian tissues (McDermott *et al.*, 2004; Selvy *et al.*, 2011), and that PLD modulates cellular function.

PLD1 belongs to a large subset of enzymes sharing a conserved HxKx4Dx6GSxN motif (HKD) (Ponting *et al.*, 1996), which is responsible for catalytic activity (Selvy *et al.*, 2011). This motif is found in the conserved domains II and IV of PLD1.

PLD1 has two other conserved domains, I and III, together with one phox homology (PX) domain, one pleckstrin homology (PH) domain and two polybasic PIP₂-binding domains. PX and PH are phospholipid-binding domains. The PX domain binds polyphosphoinositides with high specificity (Stahelin *et al.*, 2004) but this domain has also been implicated in protein interactions with regulatory proteins (Selvy *et al.*, 2011). The PH domain binds anionic phospholipids with low specificity (Selvy *et al.*, 2011). It contains also two palmitoylation sites localized at cysteine residues and this post-translational modification promotes membranous localization of PLD1 (Sugars *et al.*, 1999).

b. Action of PLD1 during exocytosis

PA directly produced by PLD activity is considered as a fusiogenic phospholipid because it alters membrane curvature and triggers synthesis of other signaling molecules, which control membrane traffic and cytoskeleton dynamics (Cross *et al.*, 1996; Bi *et al.*, 1997; Honda *et al.*, 1999; Jones *et al.*, 1999; Liscovitch *et al.*, 2000). The action of PLD in regulated exocytosis has been evidenced in many cell types, for example in HL-60 cells (Stutchfield and Cockcroft, 1993), platelets (Haslam and Coorsen, 1993), neutrophils (Morgan *et al.*, 1997) and RBL-2H3 basophilic leukemia

cells (Brown *et al.*, 1998), chromaffin cells (Galas *et al.*, 1997; Caumont *et al.*, 1998). Only ARF-controlled membrane-bound PLD1, but not PLD2, has been activated in a PIP₂ and PKC α -dependent manner in chromaffin cells during stimulation for exocytosis (Vitale *et al.*, 2001). The authors suggested that PLD1 activation, subsequent to the cytoskeleton-mediated recruitment of secretory granules to the plasma membrane, produced an important quantity of PA at the site of exocytosis inducing a negative curvature of the plasma membrane which might allow hemi-fusion of docked vesicles with the plasma membrane. This hypothesis was later confirmed (Zeniou-Meyer *et al.*, 2007).

4. Annexin A6 and phospholipase D1

It's known that annexins can modulate phospholipase activity. AnxA1 and AnxA5 inhibited the enzymatic activity of secreted phospholipase A2 (sPLA2) by binding to the acidic phospholipids and then preventing the enzyme from binding to its substrate (Buckland and Wilton, 1998). Besides its secreted form, PLA2 has a cytosolic form (cPLA2) which is also inhibited by these annexins. However, they exert the inhibitory effect by direct interaction with cPLA2 and not by preventing substrate binding as observed for the secreted form (Kim *et al.*, 2001). Interestingly, AnxA6 also showed an inhibitory effect on cPLA2 in chinese hamster ovary (CHO) cells by lowering Golgicholesterol level as reviewed by Enrich *et al.*, 2011.

So far, neither direct nor indirect functional links between PLD1 and the annexin family has been described. A known regulator of PLD1 activity is protein kinase C alpha (PKCa). In vitro PKCa activated PLD1, surprisingly, not by phosphorylation but via direct interaction (Hammond et al., 1997). PKCa binding sites were found in both the Nand C-termini of PLD1, while in PKCa both catalytic and regulatory domains participate in the interaction (Brown et al., 1995; Min et al., 1998; Sung et al., 1999). Phosphorylation of PLD1 entails its inactivation (Hammond et al., 1997; Min et al., 1998). In A431 cells expressing AnxA6, AnxA6 served as a scaffold facilitating the recruitment of PKCa to the plasma membrane to regulate the epidermal growth factor receptor (EGFR) (Koese et al., 2012). Taken together these findings provoke the following question: is the PLD1 activity regulated by AnxA6-mediated recruitment of PKCα or does PLD1 interact with AnxA6? This question is has not yet been addressed, in contrast to other mechanisms of exocytosis which are well described. Let us review here some of these mechanisms. Small GTP-binding proteins such as Rho family proteins (Rho, Rac1, Cdc42) or Arf family proteins are considered as activators of exocytosis (Momboisse *et al.*, 2010). Historically, Rho members were identified as proteins that regulated actin cytoskeleton remodeling while Arf as proteins that control vesicle budding from Golgi apparatus (Takai et al., 2001). In addition to that, these small G proteins have been shown to regulate PLD1, but not PLD2 (Exton, 2002). Small G proteins exist under 2 forms: active one which binds GTP and inactive one that binds GDP. They are activated by a GTP exchange protein (GEP) and down regulated by a GTPase activating protein (GAP). GEP induces the release of GDP from the binding pocket of an inactive small G protein and allows the binding of GTP (HernándezAlcoceba *et al.*, 2000). In contrast to GEP, GAP accelerates GTP hydrolysis activity of the small G protein. AnxA6 can inhibit Ras, another G-protein involved in signal transduction from membrane receptors (EGFR, NGFR), by recruiting Ras-GAP to the plasma membrane. The interaction is localized in a flexible region of AnxA6 and the C2 domain of p120 Ras-GAP (Grewal and Enrich, 2006). Although GAPs are specific for each family of small G proteins, the cross-talk between them has been described (Yang *et al.*, 2009). Until now, only a few GEFs activating a PLD-related small G-protein have been identified, i.e. ARNO (Vitale *et al.*, 2002) for Arf6, PIX (Zeniou-Meyer *et al.*, 2005) for Rac/Cdc42. On the other hand the interaction of GAPs with other proteins are less known. There are three possible modes of interactions between AnxA6 and PLD1 (Fig.I.2). 1) AnxA6 may interact via PKCa affecting PLD1 activity. 2) AnxA6 may recruit p120 Ras-GAP (an Arf/Rac-GAP) to regulate the RaIA (small G protein) activation as well as the subsequent signaling cascade affecting PLD1 activity (Fig.I.2) The action of AnxA6 through small G proteins is supported by the fact that RaIA, a downstream effector of Ras, associates directly and actives PLD1 in humans (Luo *et al.*, 1997).



Figure I.2: Hypotheses about the link between AnxA6 and PLD1 in the process of exocytosis: 1) It is known that AnxA6 binds and targets PKC α to the plasma membrane in a Ca²⁺-dependent manner. Although ΡΚCα the role of is still controversial, involvement of AnxA6 in PKCa-regulated PLD1 activity is conceivable. 2) AnxA6 can associate with and assist the recruitment of p120GAP to silence Ras activity. Ras is the upstream activator of RalA which binds and activates PLD1.

Pathways in which AnxA6 inhibit other small GTPases regulating PLD1 activity might be considered. 3) AnxA6 may interact directly with PLD1 affecting its activity. We presume that one of these pathways could provide a possible link between AnxA6 and exocytosis through a direct or indirect action on PLD1. This hypothesis is substantiated by the fact that both proteins are implicated in exocytosis.

5. PLD activity assays

To date, as reviewed (Selvy *et al.*, 2011; Morris *et al.*, 1997), there is no enzymatic assay to directly determine PLD activity using natural substrates. The PLD activity assay is usually based on a headgroup release of water soluble [³H] methyl-choline from labelled phosphatidylcholine (Brown *et al.*, 1993 and 2007; Colley *et al.*, 1997;

Hammond et al., 1995 and 1997) or on a transphosphatidylation reaction with radiolabelled or fluorescent phosphatidylcholine (Bligh et al., 1959; Cook et al., 1989; Daniel et al., 1993; Rose et al., 1995). N-acylphosphatidylethanolamine hydrolysis by PLD has been determined by using zirconium precipitation of anionic substrate phospholipids (Petersen et al., 2000), radiolabelling (Fezza et al., 2005) or liquid chromatography-tandem mass spectrometry (Guo et al., 2010). In all cases extraction of PLD-catalysed reaction products and their quantification is required. Coupled assays using choline kinase or choline oxidase (Carman et al., 1981; Das et al., 1986; Imamura and Horiuti, 1978; Krzystanek et al., 2010; Muma et al., 1985; Murray et al., 1990; Nanjee et al., 1991; Nie et al., 1993; Takrama and Taylor, 1991) are effective, however, additional controls are required to take into account competitive activities and to maintain saturated concentrations of substrates for optimal activity measurements. Real time pH conductimetric (Mezna and Lawrence, 1994) and membrane surface changes (Rawyler and Siegenthaler, 1989) have been also used to determine PLD activity. It has been proposed that infrared (IR) spectroscopy could be used to follow PLD activity (Estrela-Lopis et al., 2001; Yamamoto et al., 1995) but there were no quantitative determinations. Therefore, there is a need to develop a cost effective, label free assay using natural substrates to determine directly PLD activity. Among the experimental techniques available, we selected infrared spectroscopy since this technique makes possible direct determination of PLD activity using natural substrates. So far, IR spectroscopy, as reviewed in (Jencks, 1963; Kumar and Barth, 2010) has been successfully employed to monitor activities of alkaline phosphatase (Wong and Armstrong, 1992), amidase (Pacheco et al., 2003 and 2005) α-amylase (Krieg et al., 1996; Schindler et al., 1998), amyloglucosidase (Schindler et al., 1999), Ca²⁺-ATPase (Barth et al., 1990 and 1991; Barth 2002), creatine kinase (Raimbault *et al.*, Thoenges and 1997), diisopropylfluorophosphatase (Gäb et al., 2009), β-fructofuranosidase (Cadet et al., 1995, Schindler et al., 1998), fructose 1,6 biphosphatase (López-Sánchez et al., 2009), glucose oxidase (Karmali et al., 2004a), glutamic pyruvic transaminase (Wright et al., 1997), βlactamase (Fisher et al., 1980), oxalate decarboxylase (Muthusamy et al., 2006) and urease (Karmali et al., 2004b) activities. Taken together these findings suggest that infrared spectroscopy could be used to monitor PLD activity using natural substrates such as PC.

CHAPTER II: AIMS

II. Aims

As a PhD student of the International program in Neurobiology funded by Polish Foundation for Science and European Union, I had opportunity to share my time between two laboratories: the ODMB team, ICBMS, Lyon, France and the laboratory of Biochemistry of Lipids, Nencki Institute, Poland. In Poland, thanks to Bioimagine project, supported by an EU FP7 Capacities Programme (Research potential), I have access to cell imaging facilities including confocal microscopy, electron microscopy and TIRFM microscopy. Therefore I spend half time at the Nencki Institute where cell imaging was processed and at Lyon-I University where I developed an enzymatic assay to determine phospholipase activity. The Ph D thesis is divided in three parts.

The first part of the Ph D thesis performed at the Nencki Institute was to elucidate the molecular mechanism of the inhibitory effect of AnxA6 isoforms on secretion of catecholamine in PC12. We formulated and tested the hypothesis that AnxA6 may modulate the activity of PLD1, a key enzyme controlling the fusion step of exocytosis. In the first step, we aimed to verify cell localization of AnxA6 and PLD1 in either fixed or living PC12. Confocal microscopy is the method of choice by offering high. However exocytosis is an extremely fast process which occurs at specific sites on the plasma membrane. Therefore, we used TIRF microscopy which allows rapid and continuous monitoring of movement of secretory vesicles when they come close to plasma membrane. It served to track vesicles and quantify secretion. Furthermore TIRFM which eliminates noise from deeper structure by offering a very thin sectioning power was also employed to know if AnxA6 colocalized with the secretory vesicle during cell stimulation for secretion. PLD1 known to act in vesicleplasma membrane fusion, was used as positive control. For this purpose, secretory vesicles needed to be labeled with Neuropeptide Y-mRFP while AnxA6 and PLD1 were separately labeled with EGFP. Pearson's coefficient was used to quantify colocalization. Moreover, by specifically labeling one of two isoforms of AnxA6, we verified if there is any difference in their localization at the plasma membrane.

The second and third parts of the Ph D thesis were performed in France, where the ODMB team is known for its expertise in phospholipase and FT-IR spectroscopy. To reveal the possible interactions between PLD1 and AnxA6, it is essential to verify if AnxA6 can affect on PLD1 activity. We needed to obtain a sufficient amount of human recombinant PLD1 so that its activity can be accurately measured. The second part of my Ph D thesis was centered on the production of recombinant PLD, which is a risky project since most of the papers

failed to report a method of production of sufficient amount of recombinant PLD. Only PLD activity was detected and they were a lack of Western blot confirming the presence of recombinant PLD. Therefore I tried to improve the production of the recombinant h-PLD1-b. However I failed to obtain protein expression. The third part of my Ph D thesis was to develop a direct assay to determine PLD activity. Indeed, most of the PLD assays consist of either indirect multi-enzyme colorimetric assay or no-continuous radioactivity assay necessitating to stop the enzymatic reaction and to extract the radiolabeled product. They are not perfectly suitable for screening PLD activity. I developed an infrared assay which determined PLD activity by using natural substrates. Since I was not able to obtain sufficient amount of human recombinant PLD, PLD from Streptomyces *chromofucus* was employed instead of the recombinant enzyme. The infrared assay served to monitor the effects of hAnxA6 on the activity of PLD from Streptomyces *chromofucus*

CHAPTER III: MATERIALS AND METHODS

III. Materials and Methods

1. Materials

a. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, horse serum were ordered from ATCC (USA). The following chemicals were ordered from Sigma-Aldrich (USA): penicillin, streptomycin, ampicillin, trypsin, NaHCO₃, KH₂PO₄, glucose, HEPES, MgCl₂, CaCl₂, NaCl, KCl, Fura-2/AM, ionomycin, EGTA, EDTA, Triton X-100, Tris-HCl, protein inhibitor cocktail, glycerol, SDS, β-mercaptoethanol, bromophenol blue, acrylamide, glycine, methanol, non-fat milk, Tween 20, lysozyme, BSA, sucrose, dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE) lysophosphatidylserine from egg (lysoPS), lysophosphatidylglycerol from egg (lysoPG) and dimyristoylphosphatidate (DMPA), RNase, DNaseI, traskolan, pepstatin A, hydroxyapatite, paraformaldehyde, glutaraldehyde, chloroform, PLD from S. chromofucus, asolectin, choline, Bradford assay reagent, NH₄Cl, Coomassie brilliant blue R-250. Rat-tail collagen, LB broth (powder) and isopropyl β-D-1-thiogalactopyranoside (IPTG) are products of Life Technologies (USA). Nucleofector® Solution V, special cuvette for electroporation were ordered from LONZA (Switzerland), while Ø-35mm glass-bottom dishes were bought from Mattek (USA). LR White resin/propylene oxide was ordred from Polyscience (USA). The commercial inhibitor of PLD is a product of Cayman Chemical (USA). ECL reagents were from Amersham Biosciences (Austria), molecular weights standards were from Fermentas International (Canada), plasmid midi kit was from Qiagen (Germany) and nitrocellulose membranes were from BioRad (USA).

b. Antibodies

The antibodies, their types, host, dilution and suppliers are indicated in the following table (Table 1).

Primary antibodies	Туре	Host	Dilution	Supplier
Anti-AnxA6	Monoclonal	Mouse	WB 1:1000 ICC 1: 200	Abcam, Cambridge, UK
Anti-PLD1	Monoclonal	Rabbit	WB 1:1000 ICC 1:200	Novus Biologicals, Cambridge, UK
Secondary antibodies	Туре	Host	Dilution	Supplier
Anti-mouse IgG	Peroxydase- conjugated	Sheep	WB 1:2000	Amersham, GE Healthcare, Piscataway, NJ, USA
Anti-mouse- Alexa488	Fluorescent dye- conjugated	Goat	ICC 1:1000	Molecular probes, Invitrogen, Carlsbad, CA, USA
Anti-rabbit- Alexa546	Fluorescent dye- conjugated	Donkey	ICC 1:1000	Molecular probes, Invitrogen, Carlsbad, CA, USA

Table 1. Primary antibodies and their characteristics

WB: Western blotting; ICC: Immunocytochemistry

2. Methods in Cell Biology

a. PC12 cell culture

PC12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose 4.5 g L⁻¹, 2 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, 5% (v:v) fetal bovine serum and 10% (v:v) horse serum. Cells were grown in adhesion in humidified atmosphere of 5% CO₂/95% (v:v) air at 37°C. When about 80% confluent, cells were split at a ratio of 1:5 into fresh media.

b. PC12 cell stimulation for secretion

To stimulate secretion, PC12 cells were incubated at room temperature in 10 mM NaHCO₃, 1 mM KH₂PO₄, 10 mM glucose, 20 mM HEPES, pH 7.4, 1 mM MgCl₂, 1.5 mM CaCl₂, with either 125 mM NaCl and 5 mM KCl (resting Locke's solution) or with 71 mM NaCl and 59 mM KCl (stimulating Locke's solution) or with 118 mM KCl and without NaCl (the K-Locke's solution).

c. Intracellular calcium measurement by Fura-2/AM

Time-dependent changes in $[Ca^{2+}]_i$ in PC12 cells were measured by means of Fura-2 (Molecular Probes) fluorescence using a Shimadzu RF5000 spectrofluorimeter. Prior to measurements, 5×10^6 PC12 cells were incubated with 1 mM Fura-2/AM in 3 mL of DMEM without serum at 37°C for 15 min. At the end of incubation cells were washed in a resting Locke's solution, suspended in 1.585 mL of the same solution and $[Ca^{2+}]_i$ was monitored. Cell membrane depolarization was induced by the addition of 1.415 mL of the K-Locke's solution to the 1.585 mL resting solution leading to extracellular $[K^+]$ elevation to 59 mM while $[Na^+]$ decreased to 66 mM. The fluorescence signal was calibrated in each run by the addition of 8 mM ionomycin followed by 100 mM EGTA, pH 7.5. For measurement of the relative calcium content of intracellular stores in PC12 cells the addition of 8 mM ionomycin was followed by the addition of 1 mM EGTA for calibration. $[Ca^{2+}]_i$ was calculated as in [Grynkiewicz *et al.*, 1985]. The excitation wavelengths for Fura-2 were 340 and 380 nm changing every second and the emission signal was collected at 510 nm.

d. Cell transfection

Cells were initially grown on \emptyset -100mm cell culture dishes covered with rat-tail collagen (50 µg/mL). Prior to transfection, 2×10⁶ PC12 cells were incubated with 100 µl room-temperature Nucleofector® Solution V (LONZA). After adding 2 µg of DNA (pmaxGFP, pN3-AnxA6-1-EGFP, pN3- AnxA6-2-EGFP, pN3-EGFP or pCDNA-PLD1-EGFP plasmids), the mixture was transferred into a certified cuvette. Once the cuvette was placed into the holder of the Nucleofector® device, transfection program U-29 was applied to allow transfection. Then cells were suspended in 500 µL of fresh cell culture medium and plated in \emptyset -35mm glass-bottom dishes (Mattek) initially covered with rat-tail collagen. Cells were grown for 24 h before imaging. The number of transfected cells was sorted and quantified by fluorescence-activated cell sorting (Cell sorter BD FACSAria- BD Science).

3. Methods in Biochemistry

a. Bradford assay

Total protein concentration of each sample was measured using the BioRad protein assay based on the Bradford assay (Bradford, 1976). 5 or 10 μ L of sample were diluted in water (volume completed to 800 μ L) then 200 μ L of Bradford Assay reagent was added. Absorbance was read at 595 nm. A standard curve made from BSA (1 to 10 μ g,) was used to quantify the protein concentration of samples.

b. Western blotting

 5×10^{6} PC12 cells were incubated in 100 µL of cell lysis solution containing 0.5% Triton X-100 (m:v), 50 mM Tris–HCl, pH 7.5, 80 mM NaCl, 20 mM EGTA and 10 µg mL⁻¹ protein inhibitor cocktail (PIC) for 30 min on ice. Then, the lysates were centrifuged at 1000g, for 5 min. Protein concentration in the supernatant was determined by the Bradford assay. Appropriate volume of sample corresponding to a total of 30 µg of protein was mixed with 5-times concentrated Laemmli buffer (Laemmli, 1970) containing 60 mM Tris-HCl, pH 6.8, 25% glycerol (v:v), 2% SDS (m:v), 0.1% bromophenol blue (m:v), 14.4 mM β-mercaptoethanol and the mixture was boiled at 100°C for 5 min. Proteins were separated by SDS-PAGE on 10% polyacrylamide gel of 1.5 mm thickness in a buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS, under stacking gel voltage: 90V, separating

gel voltage: 190V. Then proteins were transferred onto nitrocellulose membranes during 1.5 h at 4°C at 0.5 A, in a buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM Glycine, 0.05% SDS, 20% methanol (v:v). Membranes were washed 3 x 10 min with Tris buffer saline (TBS; 20 mM Tris-HCl, pH 7.5 and 500 mM NaCl). Then they were incubated in blocking solution containing 5% (m:v) non-fat milk in TBS during 1h at room temperature. After washing three times for ten minutes with TBST (TBS + 0.05% (v:v) Tween 20), membranes were probed overnight at 4°C with primary antibodies against AnxA6 (mouse) or PLD1 (rabbit) (1:1000 dilution in TBST + 3% non-fat milk (m:v). After washing three times for ten minutes with TBST, the immunoblots were incubated separately with HRP-conjugated secondary antibodies (1:2000 dilution in TBST + 3% non-fat milk (m:v) (3 g of non-fat milk in 100 mL of TBST) during 1 h at room temperature. After washing three times during ten minutes with TBST and 5 min with TBS, immunoreactive bands were visualized using ECL reagents. The molecular weights of proteins were determined based on their relative mobility compared to that of prestained molecular weights standards.

4. Methods in Molecular Biology

a. Plasmid amplification and purification

Competent *E. coli* Top10 cells were transformed with 1µl of recombinant plasmid DNA of interest (pN3-EGFP, pN3-AnxA6-1-EGFP, pN3-AnxA6-2-EGFP, pCDNA3-PLD1-EGFP, NPY-mRFP plasmids) and incubated on ice for 30 min. Bacteria were heat shocked at 42°C for 45 s and then placed on ice during two minutes. Then 900 µL of LB (without antibiotics) was added and bacteria were incubated for 1 h at 37°C with shaking (250 rpm). After centrifuging at 10 000g during 5 min at room temperature, the supernatant was discarded and the pellet was dissolved in 100 µL of LB. Bacteria were plated onto LB agar plates supplemented with 100 µg mL⁻¹ of appropriate antibiotics. The plates were incubated at 37°C overnight. Colonies were picked from the plates, transferred into 10 mL of LB supplemented with 100 µg mL⁻¹ of antibiotic and incubated during 8h at 37°C with shaking (250 rpm). One milliliter of bacterial culture was added into 100 mL of LB containing antibiotics and culture was maintained overnight at 37°C with shaking. Plasmids were purified using the Qiagen plasmid purification midi kit.

b. Human annexin A6 production

Human recombinant AnxA6-1 and AnxA6-2 were expressed in Escherichia coli strain B121-(DE3) and purified as described for AnxA5 (Buger et al., 1993) with slight modifications (Bandorowicz-Pikula et al., 2003). Briefly, an overnight culture of E. coli transformed with pRSET5D containing human AnxA6 or its W343F mutant cDNA, inserted between the NcoI and HindIII restriction sites, was grown at 37°C in 400 mL of LB medium containing 100 μ g mL⁻¹ ampicillin. When the absorbance at 600 nm reached a value of 1.0, IPTG (isopropyl-B-D-thiogalactopyranoside) was added to a final concentration of 1 mM. After 4 h of growth, the bacterial cells were harvested by centrifugation (5,000 x g, 15 min, 4°C). The bacterial cells were resuspended in 30 mL of the spheroblast buffer (0.5 mM EGTA, 750 mM sucrose, 200 mM Tris-HCl, pH 8.0) and 30 mg of lysozyme in 40 mL of solution containing 0.25 mM EGTA, 375 mM sucrose, 100 mM Tris-HCl, pH 8.0, were added in order to digest the cell wall. The suspension was incubated for 30 min at 4 °C. The spheroblasts were collected by centrifugation (14,000 x g, 30 min, 4°C) and resuspended in 40 mL of buffer containing 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 0.1 mg mL⁻¹ RNase, 0.1 mg/mL DNaseI, 2 mM PMSF, 1000 K.I.U/mL traskolan, 0.5 μ g mL⁻¹ pepstatin A, 0.1% (m:v) Triton X-100. The spheroblasts were disrupted by osmotic shock. To remove the cell debris the suspension was centrifuged at 120,000 x g for 4 hours at 4°C.

During the centrifugation step, liposomes were freshly prepared as follows. Asolectin from soya bean was dissolved in 50 μ l of chloroform and dried under a stream of nitrogen. The phospholipid mixture was resuspended by vortexing in the liposome buffer (100 mM NaCl, 3 mM MgCl₂, 20 mM Tris-HCl, pH 8.0) and sonicated 3 x 30 sec (Branson, USA). The supernatant after ultracentrifugation was combined with the liposomes, CaCl₂ was added to the final concentration of 10 mM, and the mixture was incubated for 30 min at 4 °C. The mixture was then centrifuged at 120,000 x g for 45 min at 4°C. The pellet was resuspended in a buffer containing 100 mM NaCl, 3 mM MgCl₂, 10 mM EDTA, 20 mM EGTA, 20 mM Tris-HCl, pH 8.0 in order to detach the protein from liposomes were removed by centrifugation (120,000 x g, 45 min, 4°C) and the supernatant containing AnxA6 was dialyzed overnight against a buffer containing 20 mM Tris-HCl, pH 8.3, 0.1 mM EGTA and 10 mM NaCl.

The protein solution was loaded on a Q-Sepharose column (diameter 1 cm, bed height 30 cm, flow rate 0.5 mL/min) and washed with 30 mL of solution containing 20 mM Tris-HCl, pH 8.3, 0.1 mM EGTA (flow rate 1 mL/min). The protein was eluted with a linear NaCl gradient (0-500 mM) at a flow rate of about 0.7 mL/min. Fractions were collected for 2 min and their protein content was analyzed by SDS-PAGE on 12% acrylamide gels followed by staining with Coomassie Brilliant Blue R-250.

AnxA6-1 and AnxA6-2 eluted as a single peak in about 350 mM NaCl. Fractions containing AnxA6 or its mutant were dialyzed overnight in 10 mM phosphate buffer pH 7.4. Final purification of proteins was achieved by hydroxyapatite column chromatography. Fractions containing the respective peptides were dialyzed against 10 mM phosphate buffer, pH 7.4, 0.1 mM EGTA, loaded on a hydroxyapatite column (diameter 1 cm, bed height 50 cm, flow rate 0.5 mL/min) and eluted at pH 7.4 using a linear gradient of phosphate buffer concentrations (20-250 mM) at a flow rate of 0.5 mL/min. AnxA6 was recovered in 0.7 mL fractions eluted with 230-250 mM phosphate buffer. Protein content was analyzed by SDS-PAGE on 12% gels followed by staining with Coomassie Brilliant Blue R-250 and destaining with H₂O/methanol/acetic acid 72.5:20:7.5 (v:v:v) Next, fractions containing the protein were combined, dialyzed against 10 mM Tris-HCl, pH 7.5 (2 h, 4°C), against 5 mM Tris-HCl, pH 7.5 (2 h, 4°C), and against 1 mM Tris-HCl, pH 7.5 (2 h, 4°C), lyophilized and stored at -80°C.

c. Subcloning of hPld1 coding sequence into pGAPza-A plasmid

29	GGAAACCACCAGAATCG
55	GGCAAATGGCATTCTGACAT
79	act att gcc agc att gct gct
105	ATGCGTCGAC AAAAGA ATGTCACTGAAAAACGAGCC
106	TGCTGCGGCCGCAGTCCAAACCTCCATGGG
107	AATCCTTGAGTGTTATAAATAGCA
108	TGATGAAGTACACAATTTAATTCAG
109	AGACAGCGGTGCCATTG
110	CGTAAGCGGCGTGGAT
111	ATTTACCCGTGGCTCGTT
112	AATACTTGGTACCCTTGTTGTAG
113	CCTGAATCACATCCTTGTAC
114	CATG GAATTC TCTAGA ATGGAGATGACACTGTTTGG
115	AGCTGCTAGCGCGGCCGCCATAGGAGTATAC

Table 1: PCR primers list and their schematic position on the recombinant plasmid



To subclone *h*Pld1 coding sequence from pcDNA-3 plasmid into pGAPZ α -A plasmid, we proceeded PCR with 105/106 primers. PCR mixture containing 2.5µL of each primers, 2.5µL of 10X buffer, 2.5µL of dNTP 2mM, 1µL of MgCl₂ 2mM, 1µL of cDNA (600 µg.µL⁻¹) was completed with 12.8µL of water. 0.2 µL of KOD-DNA polymerase was added to trigger the PCR reaction. The thermocycler was programmed as following:

 Step 1: 98°C
 5min

 Step 2: 98°C
 15s

 Step 3: 40°C
 30s

 Step 4: 68°C
 60s

 Step 5: Go to step 2 (x30)

 Step 6: 68°C
 40s

After having been purified from agarose gel, *h*Pld1 coding sequence was ligated into pGAPZ α -A plasmid. The ligation mixture was used to transfected E.*coli*. 1 mL of competent E.*coli* (in LB medium) were incubated with appropriate plasmid during 30min on ice prior to thermoshock at 42°C during 90s. Then they were put again on ice during 1 min followed by incubation at 37°C during 1h. Bacteria were pelleted by centrifugation at 14 000g duing 30s. Supernatant was discarded and cell pellet was completed with 50uL of fresh LB. Bacteria in LB containing Zeocine 200 ug.mL⁻¹ were grown on agar plate overnight. Single colonies were picked by using sterile tips and put into steril eppendorf for clone PCR experiment. The same tip was used to make replicate on a new agar plate in LB containing Zeocine 200ug.mL⁻¹. Eppendorf containing bacteria were filled with sterile water then boiled and used for clone PCR to screen for the presence of recombinant plasmid. The PCR mixture containing 2.5µL of each primers (79 with 107 primer or 79 with 55 primer), 2.5µL of 10X buffer, 2µL of dNTP 2mM, 1µL of MgCl₂ 2mM, 0,5-1µL of boiled bacteria was completed with 15.2µL of water. 0.1 µL of Taq polymerase was added to trigger the PCR reaction. The thermocycler was programmed as following:

Step 1: 5 min 95°C Step 2: 30s 95°C
Step 3: 30s 40 °C Step 4: 1min30s 72°C Step 5: Go to step 2 x 30 Step 6: 3min 72°C

After analyzing PCR result, appropriate clones were then identified and cultered in 200mL of LB supplemented with Zeocine 200ug.mL⁻¹ for 18h at 37°C. Plasmids were then purified by using Qiagen plasmid midi preparation kit by following manufactor's instruction. Plasmid was sequenced prior to transformation of Pichia *Pastoris* (c.f. Annexe 1 for result of sequencing).

The P. *pastoris* X33 strain (Invitrogen) was transformed by electroporation with 5 ug of linearized plasmid under a charging voltage of 1500V, a capacitance of 40μ F, and a resistance of 150Ω . Immediately after the cell transformation, 1ml of ice-cold 1M sorbitol was added into the cuvette. Then aliquots were taken and plated onto YPD agar plates in LB containing 200 ug.mL⁻¹ Zeocin. In negative control assays, P. *pastoris* cells were transformed with pGAPZ α -A not carrying the *h*Pld1-b gene. After a 3-day incubation period at 30 °C, colonies of transformed P. *pastoris* developed on the plates and transformants containing *h*Pld1-b gene were identified by direct PCR analysis: single colonies isolated from the plates were picked from culture plate and put into eppendorf containing 1mL of sterile water. The tubes were then heated in microwave 5x1min at maximal power to allow cell lysis. Then the lysat was used for PCR with the couple of primers 79 and 107. The PCR mixture was prepared as the same way as for E. coli screening and the same thermocycler program was used.

The presence of PLD in cell culture media or within cell body was detected by using transphosphatidylation assay with radiolabelled phospholipids. Appropriate quantity of phospholipids was dissolved in chloroform to obtain desired concentration. 180 μ L PE 8mM was mixed with 52.5 PIP₂ 2.4mM, 55 μ L PC 1.635 mM and 50 μ L [¹⁴C]-PC. The reactive mixture was then dried with nitrogen, and 1000 μ L of buffer (HEPES-Na 50mM, EGTA 3mM, KCl 80 mM, DTT 1mM) was added. The solution was sonicated during 10min to form phospholipid vesicles. 40 μ L lysat from the P.*pastoris* transformation was mixed with 30 μ L of reaction buffer (HEPES-NA 150mM, EGTA 9mM, KCl 240mM, DTT 3mM, MgCl₂ 10mM) CaCl₂ 7mM, 10 μ L of GTP γ S 500 μ M, 10 μ L of Phospholipid vesicle and 1 μ L of Arf (1 μ g/ μ L). The mixture was incubated during 2h at 37°C. At the end, phospholipids were extracted using the Blye et Dyer method (Blye and Dyer, 1959), and then separated by TLC. The separating eluant was Ethyacetate/Isooctane/Acetic acid (90/50/20, v/v/v). After drying,

Coomasie blue was used to reveal phospholipid spot. Silica zones corresponding to PA, PBut and PC were separately scratched off. Radioactivity was measured by liquid scintillation counting. The radioactivity associated with phosphatidylbutanol was expressed in percentage of total phospholipid radioactivity.

5. Cell imaging techniques

a. Electron microscopy

PC12 cells were incubated in resting Locke's solution containing 10 mM NaHCO₃, 1 mM KH₂PO₄, 10 mM glucose, 20 mM HEPES, pH 7.4, 1 mM MgCl₂, 1.5 mM CaCl₂, 125 mM NaCl, and 5 mM KCl. After centrifugation (500 g during five minutes at room temperature), they were fixed with 3% paraformaldehyde (m:v) and 1% glutaraldehyde (m:v) in 100 mM sodium phosphate buffer (pH 7.2) and embedded in LR White resin/propylene oxide. The probes were polymerized at 55°C for 48 h. Ultrathin sections were placed on formvar-covered nickel grids. The samples were counterstained with 2.5% uranyl acetate (m:v) in ethanol for 20 min at room temperature in the dark, washed in 50% ethanol (v:v in water), then three times in distilled water and kept for 24h before use. Samples were observed under a JEOL (Peabody, MA) JEM-1200EX electron microscope.

b. Ratiometric calcium imaging

i. Principle

Fura-2 is an indicator of intracellular calcium concentration. The maximal excitation peak is shifted from 380 nm (Ca^{2+} -free) to 340 nm (Ca^{2+} -bound) (Grynkiewicz *et al.*, 1985), while the maximal emission is measured at 510nm. The ratio of the 510-nm-emission intensities excited at 340 and at 380 nm allows to determine intracellular calcium concentration without knowing the concentration of Fura-2 in the cells. Cells which have been initially loaded with Fura-2, are excited alternatively with 340 nm and 380 nm laser pulses. A CCD camera records the respective emission signals at 510 nm. The fluorescence ratio



 F_{340}/F_{380} is calculated. Any change in intracellular calcium concentration is reflected by the corresponding change in F_{340}/F_{380} (Fig.III.1).

Figure III.1: Principle of ratiometric calcium imaging. PC12 cells plated on glass bottom culture dishes were alternatively illuminated at 340 nm (left column) and 380 nm (middle column). The camera recorded the respective images at 510 nm and the image ratios were calculated (right column). The intensity ratio reflects the intracellular calcium concentration. In resting conditions (1), the image ratio is low (blue-violet) showing basal calcium concentration. When PC12 cells are stimulated (2), there is an influx of calcium into the cytoplasm, which is showed by the increase of the image ratio (yellow-green). After stimulation, calcium is cleared from the cytoplasm, the image ratio goes back to a blue-green color (3).

ii. Sample preparation and data recording

After transfection by electroporation (LONZA) with either pN3-EGFP, pN3-AnxA6-1-EGFP, pN3-AnxA6-2-EGFP or pCDNA3-PLD1-EGFP plasmid and with the NPY-mRFP plasmid by using Cell Line Nucleofector® KitV (LONZA), 2 x 10⁶ cells were divided and plated onto four glass bottom cell culture dishes. Cells were grown for 24 h in Dulbecco's Modified Eagle's Medium (DMEM), high glucose (4.5 g L⁻¹) with 2 mM L-glutamine, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ of streptomycin, 5% (v:v) fetal bovine serum and 10% (v:v) horse serum, in humidified atmosphere of 5% CO₂/95% air at 37°C. Prior to calcium imaging cells were incubated in 1 mM Fura-2/AM in 3 mL of DMEM without serum at 37°C for 15 min, washed in a resting Locke's solution and then suspended in 1.585 mL of the same solution. The cells were stimulated for secretion by addition of 1.415 mL of the K-Locke's solution. Cells were visualized under Inverted Leica DMI6000 microscope (Leica Gmbh, Germany) mounted with 40x/0.45 dry CORA objective. Images were recorded by an integrated Leica DFC 360FX camera (Leica Gmbh, Germany) at the rate of one image per 100 ms. After the calcium imaging recording, an image of the working area was taken using the GFP filter to identify the position of positively transfected cells.

iii. Data analysis

Images were evaluated by Leica AF software (Leica Gmbh, Germany) and numerical data were analyzed by Excel (Microsoft, USA). By distinguishing between non-GFP cells, i.e. non transfected cells, GFP-positive ones, i.e. transfected cells, the 340/380 fluorescence ratio of each cell population could be plotted, as shown in Fig.III.2.



Figure III.2: Ratiometric calcium imaging of GFP and non-GFP PC12 cells. A) and B) are the images of the same working area indicating erespectively transfected (GFP) or non-transfected (non-GFP) cells. Cells of each population were selected randomly (circles) and the 340/380 fluorescence ratios were calculated and pooled. C) The averaged value of each population of PC12 cells.

c. Immunocytochemistry and confocal microscopy

 5×10^4 cells were seeded onto acid-washed glass coverslips precoated with rat-tail collagen-1 (50 µg mL⁻¹). Coverslips were placed in cell culture dishes and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 h. Cells were incubated in resting or stimulating Locke's solution for 10 min, then they were washed with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature. After washing with PBS, they were incubated in 50 mM NH₄Cl (in PBS) for 10 min at room temperature and permeabilized with 0.08% (m:v) Triton X-100 (in PBS) for 5 min at 4°C. The cells were incubated in blocking solution containing 5% (v:v) FBS in PBS during 45 min at room temperature. To detect AnxA6 and PLD1, the samples were incubated respectively with primary antibody against

AnxA6 (mouse) and with that against PLD1 (rabbit) (1:200 dilution in PBS containing 0.5% (v:v) FBS and 0.05% (m:v) (Tween 20) for 1h at room temperature. The primary antibodies were detected by goat anti-mouse and donkey anti-rabbit secondary antibodies labeled, respectively, with Alexa 488 and Alexa 546 (1:1000 dilution in PBS containing 0.5% (v:v) FBS and 0.05% (v:v) Tween 20, Molecular Probes). To stain the nuclei, after washing with PBS cells were incubated with 0.1 μ g mL⁻¹ DAPI in PBS during 30 min at room temperature, in the darkness. After additional washing with PBS, samples were mounted with Morwiol 4-88 (Calbiochem, Germany). Samples were then observed under Leica laser scanning confocal microscope SP5: Inverted Leica DMI6000 with HCX PL APO 63x/1.4 Oil-immersion objective, with a pixel size = 96 x 96 nm, and the optical scan thickness = 0,5 μ m.

The fluorescence intensity was measured along a $10-\mu m$ bar across the cell and centered on the cell nucleus by using ploting tool embedded in the Leica's software. The data were then exported and analyzed with R software.

d. Total internal reflection fluorescence microscopy - TIRFM

i. Principle

TIRF microscopy has been used to observe several types of biological processes including those occurring at or near the cell membrane. For example, endocytosis, exocytosis, membrane-bound protein dynamics, and viral particle formation have been monitored by TIRF microscopy. TIRFM is based on the excitation of fluorophores by an evanescent field of totally internally reflected light. When light passes from a high to a low refractive index medium, for example from glass, $n_1 = 1.53$, into cell, $n_2 = 1.38$, it becomes refracted (Fig.III.3.A).



Figure III.3: Illustration of refraction of light between media of different refractive index. Three different incident angles are shown: (A) below the critical angle, (B) at the critical angle, and (C) above the critical angle.

The Snell's law (Equation1) describes the behavior of the light at the interface between two media having different refractive indexes:

$$n_1 \sin \theta_1 = n_2 \sin \theta_2$$
 (Equation 1)

where n_1 and n_2 are refractive indexes of two media, $n_1 > n_2$; θ_1 and θ_2 are respective incident angles in medium 1 and medium 2, $\theta_2 > \theta_1$. As θ_1 increases θ_2 increases too, and when it reaches 90° the light propagates parallel to the interface. This incident angle is termed the critical angle or θ_c . When θ_1 comes higher than θ_c , the light will be totally reflected. This is called total internal reflection. However, at the interface there is a slight penetration of the light, the so-called evanescent light. The light intensity decreases exponentially with the distance z from the interface, as described by equation 2:

$$I_z = I_0 e^{\frac{-z}{d}}$$
 (Equation 2)

Where z is the distance from the interface; I_0 is the intensity of fluorescence at the interface; d is the decay constant of the evanescence field as described by equation 3:

$$d = \frac{\lambda}{4\pi \sqrt{n_1^2 \sin^2 \theta_1 - n_2^2}} \quad \text{(Equation 3)}$$

In TIRFM, only the fluorophores which are in the vicinity of the cell-glass interface will be excited.

ii. Experimental setup

In this work, we used a "through-objective" setup. The laser light for excitation comes through the objective, into the glass and then in the cell. We used the Inverted Axio Observer Z.1 microscope with an incubator, mounted with a "Plan-Apochromat" 100x/oil-immersion objective, having a numerical aperture of 1.46. With this objective, the maximal incident angle can reach 73°(Zeiss Microscopy GmbH).

Since n_1 (glass) = 1.53 and n_2 (cell)= 1.38, the critical angle will be is

$$\theta_c = \arcsin \frac{n_2}{n_1}$$

 $\theta_c = \arcsin \frac{1.38}{1.53} = 65^\circ$

The incident angle must be comprised between 65° and 73° to obtain a-total internal reflection.

A manual TIRF slider was introduced to modulate the incident angle of the laser. To excite the EGFP and mRFP fluorophores, two types of lasers were used: one Ar laser emitting at 488 nm, with a 100 mW power, and one HeNe laser emitting at 532nm, with 74 mW power. Using equation 3, with $64^{\circ} < \theta_1 < 73^{\circ}$, the depth of the evanescence fields will be:

 $\lambda = 488,\,80 <\!\! d_{488} < 280$ nm $\lambda = 532,\,87 <\!\! d_{532} < 312$ nm

iii. Sample preparation and data recording

Cells were co-transfected with the pN3-EGFP, pN3-AnxA6-1-EGFP, pN3-AnxA6-2-EGFP, pCDNA3-PLD1-EGFP plasmids and with the NPY-mRFP plasmid by using Cell Line Nucleofector® KitV (LONZA). Then they were plated in 35mm glass bottom dishes (Mattek) for 24 hours. The cells were washed with resting Locke's solution and then incubated in 792,5 μ L Locke's solution at 37°C. After manual setting of the appropriate angle for TIRFM, the samples were alternatively excited for EGFP emission (excitation: Ar at 488nm with a GFP filter) and mRFP emission (excitation: HeNe at 532nm, with a CFP/Cy3 filter). Then, about 750 μ L of K-Locke's solution was added into the dishes to stimulate cells. Images were recorded by means of a microscope with an integrated AxioVision software and then analyzed by ImageJ. The pixel size was 160 x 160 nm.

ImageJ software was used to calculate the mean intensity based on a circular area bordering the cell. The value of each time point was reported to the value of resting condition (-1 min). These relative values were ploted against the time.

Pearson's coefficient of correlation was used to evaluate the colocalization rate between red and green channels. PCC was calculated based on the overall image after the formular: $PCC = \frac{\sum_{i=1}^{i} (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum_{i=1}^{i} (R_i - \bar{R})^2 \times \sum_{i=1}^{i} (G_i - \bar{G})^2}}$ (Dunn *et al.*, 2011) where R_i and G_i correspond

respectively to the intensity of the red and the green channel of the pixel *i*, \overline{R} and \overline{G} correspond respectively to the mean intensity of red and green channel.

6. Infrared spectroscopy

a. Sample preparation

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE) lysophosphatidylserine from egg (lysoPS), lysophosphatidylglycerol from egg (lysoPG) and dimyristoylphosphatidate (DMPA) were prepared as follows: phospholipids (10 to 35 mg) were first dissolved in 1 mL of chloroform. After evaporation of chloroform, 300 to 1000 µL of a buffer containing 100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 2.5 mg mL⁻¹ Triton X-100, was added to obtain 50 mM phospholipid stock solution. The mixture was vortexed during 5 min at room temperature and then incubated at 45°C during 5 min. Multilamellar lipid vesicles were then kept at 4°C for further manipulation. PLD from S. chromofucus was prepared in a stock solution containing: 100 mM Tris HCl, 10 mM CaCl₂, 2.5 mg mL⁻¹ Triton X-100, pH 8.0. Protein concentration was 0.63-0.70 μ g μ L⁻¹ as determined by the Bradford method corresponding to a total activity of 0.22-0.25 M min⁻¹ (or 35 nmol µg⁻¹ mn⁻¹) as determined by infrared assay using phosphatidylcholine as substrate. For each experiment, a reaction mixture was prepared by adding 1-8 µL of the initial or 10 or 100-times diluted PLD solution and 5-50 µL of 50 mM phospholipids from the respective stock solutions so that a series of mixtures containing 1 to 50 μ g mL⁻¹ PLD and 5 to 40 mM phospholipids in buffer (Tris HCl 100mM, 10 mM CaCl₂, 2.5 mg mL⁻¹ X-100, pH 8.0) was obtained. After vortexing the mixtures were ready to be used for infrared assays. A stock solution of 2.5 mM halopemide (Cayman Chemical) was solubilized in DMSO. The final concentration of halopemide in buffer (100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 2.5 mg mL⁻¹ Triton X-100 with 5 to 40 mM phospholipids and with 1 to 50 μ g mL⁻¹ PLD) never exceeded 2.5 μ M (corresponding to a maximum 0.1 % v:v DMSO).

b. Data recording

IR data were acquired with a Thermo Scientific Nicolet iS10 spectrometer equipped with a DTGS detector. The IR spectra were recorded at 20°C with 256 interferograms at 4 cm⁻¹ resolution each and Fourier transformed. During data acquisition, the spectrometer was

continuously purged with dry filtered air (Balston regenerating desiccant dryer, model 75-45 12 VDC). At least three independent measurements have been performed to obtain kinetics parameters. For infrared measurements, an aliquot of 10 μ L of the reaction mixture containing 2 to 50 μ g mL⁻¹ PLD and 5 to 40 mM phospholipids in 100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 2.5 mg mL⁻¹ Triton X-100, pH 8 was deposed on CaF2 windows separated by 12 μ m spacers. Then it was sealed in a temperature controlled flow through cell (model TFC M13-3 Harrick Scientific Corp.). The hydrolytic activity of PLD is expressed as the amount of nanomoles of DMPC hydrolyzed per minute by 1 mg of PLD (nmol min⁻¹ mg⁻¹).

c. Colorimetric PLD assay

Alternatively, PLD activity was assayed spectrophotometrically by measuring the free choline released upon phosphatidylcholine hydrolysis, using a continuous method with some modifications as previously described (Takrama et al, 1991). Choline was continuously transformed into betaine in a reaction catalyzed by choline oxidase, which simultaneously vielded H₂O₂. In the presence of 4-aminoantipyrine and sodium 2-hydroxy-3,5dichlorobenzenesulfonate, H₂O₂ was used instantaneously by the added peroxidase to form a colored product absorbing light at 500 nm. Absorbance measurements were performed using a microplate scanning spectrophotometer (INFINITE M200 TECAN). The assay mixture (150 µL per well) contained 50 mM Tris-HCl, pH 8.0, 20 mM CaCl₂, 1.7 mM 4-aminoantipyrine, 9 mM sodium 2-hydroxy-3,5-dichlorobenzenesulfonate, 0.5 U choline oxidase and 0.5 U peroxidase. After a 10-min period of stabilization, the reaction was initiated by adding 1-1000 ng S. chromofucus PLD (corresponding to 0.007 to 7 µg mL⁻¹ final concentration) and the substrate (DMPC, 0.26 mM final concentration) and absorbance measurements were carried out continuously during 10 min. Control assays were performed simultaneously without PLD. The amounts of free choline released were quantified, based on a standard curve obtained with pure choline. The hydrolytic activity of PLD is expressed as the amount of nanomol of choline produced per minute by 1 mg of PLD.

CHAPTER IV: RESULTS

IV. Results

1. PC12 cells characterization

PC12 cells derived from a transplantable rat pheochromocytoma, have been used as an experimental model of a neurobiological system (Greene and Tischler, 1976). In the experiments described in this chapter we also used PC12 cells purchased from ATCC (American Type Culture Collection). Typical low-passage PC12 cells have a rounded or irregular shape about 10 μ m in diameter (Fig.IV.1 left panel) and large nuclei (50-70% of cell volume) as evidenced by DAPI staining (Fig.IV.1 middle and right panels). They have tendency to form clusters (Fig.IV.1).

PC12 cells contain various amounts of secretory vesicles also known as large densecore vesicles or LDCVs (Fig.IV.2 left panel) which are better visible on the enlarged cell images (Fig.IV.2 right panel, white arrows). LDCVs store mostly dopamine and a small amount of norepinephrine (Greene and Tischler, 1976). Upon cell depolarization, they release these catecholamines in a calcium-dependent manner (Greene and Rein, 1977).

The average diameter of vesicles in PC12 was estimated to be 166 ± 43 nm (mean \pm standard deviation) (Fig.IV.3). This finding is in accordance with previous observations (Greene & Tischler 1976, Schubert et al. 1980, Travis & Wightman 1998) which determined vesicle diameter to range from 150 to 240 nm.



Figure VI.1: Morphology of PC12 cells. Left panel: Phase contrast micrograph of living PC12 cells. Cells were plated on cell culture dishes covered with collagen (100 μ g/mL). Middle panel: DAPI staining shows large nuclei covering (Right panel: Merged phase contrast and DAPI-colored cells. Cells were observed under the inverted AxioObserver Z.1 microscope (Zeiss, Germany), 40x magnification. Scale bare = 10 μ m.

To verify the excitability of PC12 cells, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored by the addition of FURA2-AM (Fig.IV.4). In resting conditions, $[Ca^{2+}]_i = 273\pm27$

nM, upon membrane depolarization by 59 mM KCl, $[Ca^{2+}]_i$ increased up to 592 ± 110 nM (n = 5). To calibrate calcium concentration, 8 μ M Ionomycin was used as calcium ionophore, corresponding to saturated calcium concentration of approximately 1 mM, and 100 mM EGTA, corresponding to null calcium concentration.



Figure IV.2: Transmission electron micrograph of a PC12 cell. Cells were incubated in resting Locke's solution containing 10 mM NaHCO₃, 1 mM KH₂PO₄, 10 mM glucose, 20 mM HEPES, pH 7.4, 1 mM MgCl₂, 1.5 mM CaCl₂, 125 mM NaCl, and 5 mM KCl. Then they were fixed with 3% paraformaldehyde/1% glutaraldehyde in 100 mM

sodium phosphate buffer (pH 7.2) and embedded in LR White resin/propylene oxide (Polysciences, Warrington, PA). Ultrathin sections were placed on formvar-covered nickel grids and observed under JEOL (Peabody, MA) JEM-1200EX electron microscope. The left panel shows the entire cell while the right panel indicates the same cell at higher magnification. White arrows indicate secretory vesicles. Scale bar = $1\mu m$.



Figure IV.3: Vesicle size distribution in **PC12** cells. Electron micrograph of three PC12 cells was analyzed using the ImageJ software. Relative distribution of vesicles as function of vesicle а diameter is plotted. It was averaged from all vesicles to obtain a mean diameter that amounted to 166 ± 43 nm, (mean \pm standard deviation) n=123.



Figure IV.4: Intracellular calcium concentration of PC12. 5×10^{6} PC12 cells were incubated with 1 µM Fura-2/AM in 3 ml of DMEM without serum at 37^{0} C for 15 min. At the end of incubation cells were washed in a resting Locke's solution, suspended in 1.585 ml of the same solution and $[Ca^{2+}]_{I}$ was monitored. Cell membrane depolarization was induced by the addition of 1.415 ml of the K-Locke's solution leading to extracellular $[K^{+}]$ elevation to 59 mM while $[Na^{+}]$ decreased to 66 mM. The fluorescence signal was calibrated in each run by the addition of 8µM ionomycin followed by 100 mM EGTA, pH 7.5.

2. Translocation of AnxA6 and PLD1 in PC12 cells upon stimulation

Overexpression of two isoforms of AnxA6 lead to a decrease in Ca^{2+} uptake upon cell stimulation and to inhibition of dopamine secretion in PC12 cells suggesting that AnxA6 may play an important role in the Ca^{2+} -dependent secretion of dopamine in PC12 cells (Podszywalow-Bartnicka *et al.*, 2010). The effect of isoform 2 (AnxA6-2) overexpression was stronger than that of isoform 1 (AnxA6-1) (Podszywalow-Bartnicka *et al.*, 2010). The aim of the experiments, the results of which are described below, was to unveil the mechanism of this phenomenon by identifying potential partners of Anx6.

a. Immunocytochemistry

First, AnxA6 distribution in PC12 cells was characterized. Immunofluorescence experiments were performed to detect endogenous AnxA6 by using a specific antibody conjugated with the Alexa 488 fluorophore, before and after stimulation of the cells for dopamine secretion. Labeling of nuclei was achieved using DAPI and the obtained images were merged.



Figure IV.5: Annexin A6 localization in PC12 cells under resting conditions and upon stimulation. Cells were incubated in resting (upper panels) or stimulating Locke's solutions for 10 min (lower panels), as described in Materials and Methods. They were fixed by 3% paraformaldehyde for 20 min. AnxA6 was identified by using specific anti-AnxA6 antibody conjugated with Alexa488 (green color, left panels) while nuclei were stained with DAPI (blue color, middle panels). Merged fluorescence pictures, depicting localization of AnxA6 and nuclei, are shown in the right panels. Cells were observed under confocal microscope Leica SP5 DFC 360FX with a 63x oil immersion objective, the voxel size was 96 x 96 x 500 nm. Scale bar = 10 μ m.

Under resting conditions (upper panel), AnxA6 is distributed evenly in the cell but upon stimulation (lower panel) it relocates, concentrating at some foci in the cytoplasm and at the plasma membrane (Fig.IV.5). To evaluate the distribution of AnxA6 across the cell, standardized fluorescence intensity of each channel along a straight bar was traced (see Materials and Methods) (Fig.IV.6). Then AnxA6 signal intensities on the periphery of resting and stimulated cells were compared (Fig.IV.7). Such analysis confirmed that under resting conditions, AnxA6 is evenly distributed in the cell cytoplasm. However, upon stimulation, a small but significant population of AnxA6 shifted from the whole cytoplasm to the cell periphery, distinctly apart from the nucleus (p-value < 0.05). Therefore, a small population of AnxA6 relocated upon cell stimulation suggesting that this protein may interact with its membranous partners.



Figure IV.6: Distribution profile of AnxA6 in a PC12 cell. The normalized fluorescence intensity of each point is plotted along 10- μ m bars drawn across the cell and centered on the cell nucleus. Green and blue curves display AnxA6 distribution within the cells and localization of the nuclei, respectively. (Number of cells in resting condition = 30, number of stimulated cells = 29).



Figure IV.7: Comparison of green fluorescence intensity (AnxA6) on the of resting periphery versus stimulated cells. From cross-cell distribution profile of green channel plotted above (Fig. IV.6), fluorescence intensity of points having abscissa below 1 μm or above 9 μm were collected. Boxplots were drawn to depict the distribution of those values in resting and stimulated cells. Bv applying a Student's test, we obtained a p-value = 0.037 meaning a significant difference (at the 0.05 level) in AnxA6 localization in resting versus stimulated cell.

In the second step, to probe any possible relationship between AnxA6 and PLD1, we used immunohistochemistry to reveal their respective localization in PC12 cells (Fig.IV.8).



Figure IV.8: Localization of AnxA6 and PLD1 in PC12 cells under resting conditions and upon stimulation. Cells were incubated in resting (upper panels) or stimulating Locke's solutions for 10 min (lower panels), as described in Materials and Methods. Then, the cells were fixed by 3% paraformaldehyde for 20 min. Double labeling of AnxA6 and PLD1 was performed with specific antibodies conjugated, respectively, with Alexa488 (green color) and Alexa594 (red color). The nuclei were revealed by DAPI (blue color). The images of 3 channels were merged. Cells were observed under confocal microscope Leica SP5 DFC 360FX with a 63 x oil immersion objective, the voxel size was 96 x 96 x 500 nm. Scale bar = $10 \mu m$.

While the staining of AnxA6 revealed its translocation from cytoplasm to plasma membrane in stimulated PC12 cells (Fig.IV.8, green color) consistent with previous findings (Podszywalow-Bartnicka *et al.*, 2010), PLD1 didn't translocate toward plasma membrane in contrast to previous observations by Vital *et al.* (2001). PLD1 is characterized by an even distribution in PC12 cells (Fig.IV.8, red color). To explain the contradictory results of localization of PLD1, we checked the specificity of the anti-PLD1 antibodies. The whole cell lysate was subjected to Western blotting with antibodies against AnxA6 and PLD1 (Fig.IV.9).



Figure IV.9: Western blot analysis of anti-AnxA6 the and anti-PLD1 antibodies quality. The cellular lysates $(2 \times 10^8$ cells) were analyzed on a 12% SDS polyacrylamide gel and immunoblotted with anti-AnxA6 (A) or anti-PLD1 monoclonal antibodies (B). Secondary antibodies conjugated to horseradish peroxidase were visualized with the enhanced chemiluminescence reagent.

Molecular weights of AnxA6 and PLD1 are 67 kDa and 120 kDa, respectively. Antibody against AnxA6 revealed mostly the band at 67kDa whereas the one against PLD1 recognized not only the specific PLD1 but also few minor bands. This may explain why immunocytochemistry indicated a whole-cell distribution of PLD1 instead of a membranous localization. To overcome this problem, we transfected PC12 cells with plasmid containing recombinant hPLD1 labeled with EGFP. This alternative will also allow us to work on living cells.

b. EGFP labeling of PLD1 in PC12 cells

Due to the low specificity of the antibody against phospholipase D1 used in immunocytochemistry experiments, we labeled this protein with EGFP. The PLD1 coding sequence fused with EGFP was cloned into pCDNA-3 plasmid. The empty plasmid carrying only EGFP was used as control. PC12 cells were transfected using the Cell Line Nucleofector® KitV (LONZA) as described in the Materials and Methods (c.f.Chap.III.2.b.). Fig.IV.10 shows a representative result of transfection analyzed by FACs. The average transfection efficiency was $3.05 \pm 1.62 \%$ (n = 4).



Figure IV.10: Analysis of the efficiency of PC12 cells transfection using Cell Line Nucleofector® KitV (LONZA). Twenty four hours after transfection, the population of transfected cells was counted and sorted by using FACS (Cell sorter BD FACSAria). Control: non-transfected PC12 cells, pmaxGFP: cells transfected with pmaxGFP plasmid (included in the kit), EGFP: cells transfected with pCDNA-3-EGFP plasmid, PLD1-EGFP: cells transfected with pCDNA-3-PLD1-EGFP plasmid. In each panel, the number of cells with their respective fluorescence intensity is shown. Non-transfected cells emitted a basal signal due to auto-fluorescence (control) while transfected cells had a stronger fluorescence signal (pmaxGFP) thanks to EGFP. P2 gate was manually set to select highly fluorescent cells.

The pmaxGFP plasmid was included in the transfection kit and it was used as a positive control. The transfection with the pCDNA-3-PLD1-EGFP plasmid was less efficient than that with the empty plasmid pCDNA-3-EGFP ($10.80 \pm 2.26\%$). This might be due the fact that PLD1 overexpression could produce unwanted effects for the cell, including cell cytotoxicity. Therefore, it was necessary to verify whether or not overexpression of PLD1-EGFP could affect cell morphology and cell excitability. The cell morphology and the localization of PLD1-EGFP inside PC12 cells were determined (Fig.IV.11A).

PLD1 is characterized by both cytoplasmic and membranous localization (Fig.IV.11A) in contrast to the immunohistochemical staining which showed also nuclear localization (Fig.IV.8, red color). The control cells overexpressing EGFP alone showed an even distribution of the fusion protein (Fig.IV.11B). Upon cell stimulation, we observed an accumulation of PLD1 at some foci at the plasma membrane indicating that PLD1 behaves similarly to AnxA6 upon stimulation.

Figure IV.11A)



Figure IV.11B)



Figure IV.11: Localization of PLD1-GFP in PC12 cells. Cells were transfected with pCDNA3-PLD1-EGFP plasmid (A) or with pCDNA-3-EGFP plasmid as a control (B). Cells were grown in 35 mm glass-bottom dishes. 24h after transfection, they were observed under confocal microscope Leica SP5 DFC 360FX with a 63x oil immersion objective and the voxel size was 96x96x500 nm. Under resting conditions cells were incubated in 792.5 μ L of resting Locke's solution. To stimulate 707.5 μ L of K-Locke's solution was added. Left panels indicate localization of the green GFP (A Panels) and PLD1-GFP (B Panels). Middle panels show the morphology of the PC12 cells while the right panels indicate the merge images. White arrows indicate accumulation of PLD1-EGFP at the plasma membrane. Scale bar = 10 μ m.

c. EGFP labeling of AnxA6 in PC12 cells

As it was already mentioned, AnxA6 exists in PC12 cells in 2 isoforms due to the alternative splicing of mRNA (Moss and Crumpton, 1990). The full-length protein which has 673 residues is termed isoform 1 or AnxA6-1. The second isoform which lacks the 524-VAAEIL-529 fragment is called isoform 2 or AnxA6-2 (Crompton *et al.*, 1988; Moss and Crumpton, 1990). So far there is no specific antibody which can distinguish between these two isoforms. This is why it is difficult to assess the effect of the splicing on AnxA6 function. Previously, it has been proved (Podszywalow-Bartnicka *et al.*, 2010) that in PC12 cells, long-term overexpression of two isofroms of AnxA6 reduced calcium uptake upon cell stimulation and lowered the quantity of dopamine secreted. In addition it has been shown that the effect of AnxA6-2 is stronger than that of AnxA6-1 (Podszywalow-Bartnicka *et al.*, 2010). Here, by labeling separately each isoform, we aimed to visualize the behavior of each isoform under resting conditions and upon cell stimulation.

Both isoforms of AnxA6 (Fig.IV.12B and C) are characterized by cytosolic and membrane localization in contrast to EGFP alone (Fig.IV.12A). However, unlike the result obtained previously by immunocytochemistry (Fig.IV.8, green color): firstly, no AnxA6-1/2-EGFP signal was found in the nucleus of resting or stimulated cell; secondly upon cell stimulation, re-localization of AnxA6 was not clearly observed. These can be explained as follows:

- 1. The nuclear localization of AnxA6 observed in immunocytochemistry experiment could be due to non-specific labeling.
- Upon cell stimulation, there could be several distinct sub-populations of AnxA6 which are localized differently (Podszywalow-Bartnicka *et al.*, unpublished observation). It is be possible that overexpression of AnxA6-1-EGFP or AnxA6-2-EGFP can mask the subtle translocation of a small fraction of the protein.
- 3. Podszywalow-Bartnicka (Podszywalow-Bartnicka *et al.*, 2010) proved that long-term overexpression of AnxA6 altered calcium homeostasis in PC12 cells. Since AnxA6 to binds to phospholipidic membranes in a Ca²⁺-dependent manner, no translocalization of AnxA6 can be a consequence of abolished calcium homeostasis. Therefore, in the next chapter we show the results of verification of the effect of AnxA6 overexpression on calcium homeostasis in cells transiently overexpressing AnxA6.

Figure IV.12A)



Figure IV.12B)



Figure IV.12C)



Figure IV.12: Localization of AnxA6 isoforms in PC12 cells. The cells were transfected with pCDNA-3- EGFP plasmid (A panels), pN-3-AnxA6-1-EGFP plasmid (B panels) or with pN-3-AnxA6-2-EGFP plasmid (C panels). Cells were grown in 35mm glass-bottom dishes (Mattek). After transfection (24 h), the cells were observed under confocal microscope Leica SP5 DFC 360FX with a 63 x oil immersion objective; the voxel size was 96 x 96 x 500 nm. The localization of the AnxA6-1

and AnxA6-2 isoforms is evidenced by green color due to their attached GFP (left panels). Cell morphology is visualized in the middle panels, while merge images are shown in the right panels. In the resting conditions (indicated by resting in the panels), cells were incubated in 792.5 μ L of resting Locke's solution. To stimulate cells, 707.5 μ L of K-Locke's solution was added. Scale bar = 10 μ m.

d. Excitability of transfected cells

Due to the fact that a very low number of cells were transfected, the spectrofluorometer couldn't be used to measure intracellular calcium concentration as previously applied for native PC12 cells (as described in the Fig.IV.4). Ratiometric imaging should be considered, as described in the Materials and Methods section. The principle of this method has been fully explained in Chap.II.1.f. Briefly, cells were loaded with 1 μ M Fura-2AM (Molecular Probes) in 3 ml of DMEM without serum at 37°C for 15 min; then they were incubated with resting Locke's solution. To stimulate Ca²⁺ entrance inside the cell, 59 mM KCl was added. Upon Ca²⁺ binding, the absorption peaks of Fura-2 shifted from 363 nm to 335 nm; however for the ratiometric measurement, the excitation at 340 nm and that at 380 nm were widely used (Grynkiewicz *et al.*, 1985). By using a fluorescent microscope (Leica DFC 360F, with 40x/0.45 dry CORA objective), every second we recorded 2 images corresponding to fluorescence emission at 510 nm when exciting Fura-2 alternatively at 340 nm and 380 nm. Then the image quotient (340/380) was calculated. Any change of intracellular Ca²⁺ concentration ([Ca²⁺]_i) was reflected by variation of this quotient.

We observed that in cells overexpressing EGFP had lower 340/380 fluorescence ratio values than the non-GFP ones, either in resting condition or during cells stimulation (Fig.IV.13).





So far, it is not known that EGFP can induce any effect on intracellular Ca^{2+} homeostasis. However, in rat basophile leukemia cell EGFP expression distorted 340/380 fluorescence ratio leading to an underestimation of $[Ca^{2+}]_{I}$ (Bolsover *at al.*, 2001). The

explanations are that EGFP, by quenching emission at 510nm of Fura-2, lowered the recorded signal and/ emission band of FURA-2 overlaps with excitation band of EGFP (Figure IV.14).



IV.14: Excitation and emission spectra of Fura-2 and EGFP. Ca²⁺-free Fura-2 has excitation pick at 340nm (blue dashed line) which shifts to 380nm when binding Ca²⁺ (orange dashed line). Both Ca^{2+} -free and Ca^{2+} bounded form has emission maxima at 510nm (blue and

orange full lines). Maximal excitation pick of EGFP is located at 488nm (green dashed line) while emission pick is found at 520nm. Data was obtained at https://www.lifetechnologies.com.

For this reason, standardization was needed. Non-transfected cells was used as internal control: the 340/380 fluorescence ratio of transfected cells was divided by the ratio of non transfected cells.



Ratiometric imaging Figure IV.15: measurement of **PC12** cells overexpressing EGFP (blue), AnxA6-1-EGFP (green) and AnxA6-2-EGFP (dark green). Cells were plated in glassbottom dishes (Mattek). 24h after transfection, they were observed under a fluorescent microscope Leica DFC 360F, with 40x/0.45 dry CORA objective. Cells were initially incubated in 792.5 µL of resting Locke's solution. To stimulate cells 707.5 µL of K-Locke's

solution was added. For each experiment, 340/380 fluorescence ratio of transfected cells was normalized with the value of non-transfected one. GFP n = 29, AnxA6-1-EGFP n = 13, AnxA6-2-EGFP n = 19. All p-values were higher than 0.1 indicating that there wasn't any significant difference between these values.

It has been reported that long term overexpression of AnxA6 isoform altered Ca²⁺ uptake upon PC12 cells stimulation (Podszywalow-Bartnicka *et al.*, 2010). However we didn't observe any significant changes (Fig.IV.15). In AnxA6-EGFP cells, there was a significant rise of $[Ca^{2+}]_i$ upon cell depolarization which didn't differ from EGFP expressing cells (p-values > 0.5). Transient overexpression of AnxA6-EGFP didn't alter calcium uptake as observed in a cell line stably overexpressing this protein.

3. Co-localization of phospholipase D1 and AnxA6 with secretory vesicles

a. Co-localization of PLD1 with secretory vesicles at the plasma membrane

Using confocal microscopy, we observed that PLD1 accumulated at the plasma membrane upon cell depolarization (Fig.IV.11). It has been suggested also that PLD1 is co-localized with SNAP-25, which is a component of the SNARE-complex, and facilitates the fusion between secretory vesicles and the plasma membrane by producing phosphatidic acid, a fusogenic phospholipid, at the secretion site (Vitale *et al.*, 2001; Zeniou-Meyer *et al.*, 2007; Vitale, 2010).

So far, there was no direct evidence indicating the relative position of PLD1 and secretory vesicle at the site of exocytosis. Total internal reflection fluorescence microscopy, by offering a more restricted optical sectioning than confocal microscopy, can track vesicles undergoing exocytosis (Tsuboi *et al.*, 2006; Serulle *et al.*, 2007) and monitor protein recruitment (An *et al.*, 2010) at the plasma membrane. By labeling secretory vesicle with neuropeptide Y fused with mRFP (NPY-mRFP) and PLD1 with EGFP, we wanted to verify whether or not they co-localized at the plasma membrane (Fig.IV.16).



Figure IV.16: Total internal reflection fluorescence microscopy of PC12 cells double-labeled with PLD1-EGFP (green color) and with NPY-mRFP (red color). The first row displays representative cells before stimulation. Images in The 2^{nd} to the 6^{th} row were recorded at different times during stimulation (0 min, 1 min, 2 min, 5 min, 10 min). Green color (first column) corresponds to PLD1-EGFP signal. Red color (second column) corresponds to NPY-mRFP signal. The third column displays the merge of both colors. Scale bar = $10\mu m$. The last column displays magnification of areas of the merged picture where colocalization was found. Scale bar = $1 \mu m$.

The incident angle of the laser beam was set in such a way that it created an evanescent wave of totally internally reflected light; the intensity of the evanescent light decreased exponentially in space and it could reach 200 nm in depth (Oheim et al., 1998). Only fluorophores contained within this depth range were excited. PLD1-EGFP at the plasma membrane was not evenly distributed but was concentrated in distinct plasma membrane regions (Fig.IV.16, first column). To evaluate the movements of PLD1-EGFP and NPYmRFP, we measured average relative fluorescence intensity at different time as compared with the intensity of the resting state (UA=1.0 at time -1 min before stimulation) (Fig.IV.17.A). Just after the addition of KCl 59mM to stimulate the secretion, we observed a small decrease of both PLD-EGFP and NPY-mRFP (Fig.IV.17.A,) indicating movements of PLD-EGFP and NPY-mRFP. After one minute of stimulation, their quantities increased slightly followed by a slow decrease at longer incubation time. Pearson's coefficient of correlation between red and green channel was used to assess the colocalization between PLD1-EGFP and NPY-mRFP. PLD1-EGFP and NPY-mRFP co-localized strongly despite larger variations of the population of NPY-mRPF after 10 min stimulation (Fig.IV.17.B). This observation confirmed the participation of PLD1 in exocytosis.



Figure 17: A) Evolution of fluorescence intensity of PLD1-EGFP (green triangles) and NPY-mRFP (red triangles). Average fluorescence intensity (red or green channel) of a circular zone centered on the cell was recorded at different time points: before stimulation (-1min), at the moment when KCl solution was added (0 min), at +1, +2, +5 and +10 min after stimulation. After correction with the background, the value of each point was reported to value of resting condition. B) Pearson's coefficient of correlation between green and red channel at indicated time points. (n=3).

b. Co-localization of AnxA6 with secretory vesicles at the plasma membrane

AnxA6 decreased the secretion of dopamine in PC12 (Podszywalow -Bartnicka et al., 2010). However, transient overexpression of AnxA6-EGFP did not affect Ca²⁺ homeostasis as demonstrated in the paragraph 3.b.iv, suggesting that AnxA6 could affect other steps of exocytosis. As mentioned above, AnxA6 and PLD1 translocated toward plasma membranes upon stimulation of PC12 cells. We checked whether or not AnxA6 was also recruited at sites where the secretory vesicles accumulate as did PLD1 to consolidate the hypothesis of an interaction between AnxA6 and PLD1. Similarly to PLD1, AnxA6-1-EGFP (Fig.IV.18) colocalized with secretory NPY-mRFP- labeled-vesicles in the vicinity of the plasma membrane while this phenomenon was not observed for AnxA6-2-EGFP (Fig.V.19). Fluorescence intensity quantification confirmed that two isoforms of AnxA6 behave differently: AnxA6-2-EGFP intensity decreased already after one min of stimulation (Fig.20.A, green squares) suggesting its translocation into deeper region of cytoplasm, while AnxA6-1-EGFP remained at the plasma membrane and it started to be translocated only after 5 min after cell stimulation (Fig.20.A, green circles). The relative proportion of colocalization of both isoform with the secretory vesicles (NPY-mRFP) remained quite stable during the stimulation. However, a larger population of AnxA6-1 than that of AnxA6-2 was collocalized with NPY-mRPF (Fig.20.B).



Figure IV.18: Total internal reflection fluorescence microscopy of PC12 cells double-labeled with AnxA6-1-EGFP (green color) and NPY-mRFP (red color). NPY-mRFP labeling of specific vesicles is shown in red color. The first row displays representative cells before stimulation. Images presented in The 2^{nd} to the 6^{th} row were recorded at different times during stimulation (0 min, 1 min, 2 min, 5 min, 10 min). Green color (first column) corresponds to AnxA6-1-EGFP signal. Red color (second column) corresponds to NPY-mRFP signal. The third column displays the merge of both colors. Scale bar = 10 μ m. The last column displays magnification of areas of the merged picture where co-localization was found. Scale bar = 1 μ m.



Figure IV.19: Total internal reflection fluorescence microscopy of PC12 cells double-labeled with AnxA6-2-EGFP (green color) and NPY-mRFP (red color). The first row displays representative cells before stimulation. The 2^{nd} to the 6^{th} row images were recorded at different times during stimulation (0 min, 1 min, 2 min, 5 min, 10 min). Green color (first column) corresponds to AnxA6-1-EGFP signal. Red color (second column) corresponds to NPY-mRFP signal. The third column displays the merge of both colors. Scale bar = 10 µm. The last column displays magnification of areas of the merged picture where co-localization was found. Scale bar = 1 µm.



Figure 20: A) Evolution of fluorescence intensity of AnxA6-1-EGFP (green circles) and AnxA6-2-EGFP (green squares). Average fluorescence intensity of a circular zone centered on the cell was recorded at different time points: before stimulation (-1min), at the moment when KCl solution was added (0 min), at +1, +2, +5 and +10 min after stimulation. After correction with the background, the value of each point was reported to value of resting condition. B) Pearson's coefficient of correlation between green (AnxA6-1-EGFP or AnxA6-2-EGFP) and red channel (NPY-mRFP) at indicated time points. (n=3).

By using TIRFM, we observed the recruitment of PLD1 and AnxA6-1 to the secretory vesicles before cell stimulation which was maintained after cell stimulation. In contrast, AnxA6-2 didn't co-localize with the secretory vesicles. This is consistent with the observation by Podszywalow-Bartnicka (Podszywalow-Bartnicka et al., 2010), that alternative splicing of the Anx6 transcript affected distribution and functionality of the protein.

Many questions emerge: if AnxA6-1 and PLD1 are both found in the vesicles, how do they interact and what are the effects of these interactions on the secreted vesicles? Since PLD1 is crucial for exocytosis, could the presence of AnxA6-1 affect its enzymatic activity? Therefore, in the next chapter we verified whether or not AnxA6 could affect the PLD1 activity.

4. Overexpression of human PLD1-b in Pichia pastoris

a. Subcloning of hPLD1-b coding sequence

To verify the effect of AnxA6 on PLD1 activity, we aimed to produce the human recombinant enzyme. Human phospholipase D1 exists under two isoform due to alternative splicing: *h*PLD1-a (1074aa) and *h*PLD1-b (1036aa). The later is the most known variant. We aimed to produce pure *h*PLD1-b by using Pichia *pastoris* expression system to obtain a functional enzyme possessing complete post-translational modification. For that, pGAPZ α -A plasmid was used, where the α -factor at the N-terminus is the signal for secretion of recombined protein. pcDNA-3 plasmid containing *h*Pld1-b coding sequence flanked by Sal I and Not I restrictive sites was employed as a template for the subcloning into pGAPZ α -A plasmid (Fig. IV.21) by using PCR.



Figure IV.21: Schema of subcloning of *h*Pld1-b coding sequence from pcDNA3 plasmid into pGAPZ α -A plasmid. XhoI and SaII are compatible. α -factor corresponds to a secretion signal thanks to which *h*PLD1-b will be secreted into the culture medium. The sequence 6xHis tag is necessary for protein purification.

The 105 and 106 primer (c.f. table of primers) which contain respectively SalI and NotI restrictive site were exploited to isolate *h*Pld1-b coding sequence from pcDNA3 plasmid by PCR. The 3kb-fragment which corresponds to *h*Pld1-b cDNA (3225 bp) was excised and then purified (Fig.IV.22.A). The second analysis on 1% agarose gel showed the purity of DNA fragment (Fig.IV.22.B), white arrow). After digestion with SalI and NotI, the DNA fragment was cloned into pGAPZ α -A plasmid.



Figure IV.22: A) DNA Amplification by PCR with 105 and 106 primers. The 3kb-DNA fragment (white arrow) corresponding to *h*PLD-b sequence was then excised, purified and analyzed on 1% agarose gel. B) Analysis on 1% agarose gel after DNA purification. Right side: enlarged view of the molecular weighs of markers (MW) with the indicated MW.

Simultaneously pGAPZα-A plasmid was linearized by using XhoI and NotI (Fig.IV.23). Linearized plasmids showed only one band corresponding to their size (3kb) (Fig.IV.23, lane 1, 2 and 3) while undigested plasmid displayed, beside the original band, another band corresponding to supercoiled form (Fig.IV.23.lane 4).



Figure IV.23: Electrophoresis on 1% agarose gel of linearized pGAPZ α -A. Lane 1: pGAPZ α -A (1 μ g) + XhoI + NotI. Lane 2: pGAPZ α -A (1 μ g) + XhoI. Lane 3 : pGAPZ α -A (1 μ g) + NotI. Lane 4: native pGAPZ α -A (0.5 μ g). Right side: enlarged view of the molecular weighs of markers (MW) with the indicated MW.

The ligation reaction was done by mixing *h*Pld1-b insert with linearized pGAPZ α -A at different ratio as indicated below (Table 1). The mixture was incubated at room temperature for 1h.

Table 1: Ligation reaction at different pGAPZ α -A/*h*Pld1-b insert ratios. Volumes of the components of ligation medium in μ L are indicated.

Components	Ratio 1:0	Ratio 1:1	Ratio 1 :3
pGAPZ α -A (22 ng/ μ L) (μ L)	1	1	1
<i>h</i> Pld1-b (22 ng/µL) (µL)	0	1	3
Ligation buffer 5x (µL)	4	4	4
T4 DNA ligase (400U/µL)	1	1	1
(µL)			
Water (µL)	14	13	11

After 1-h incubation time, 0.5μ L of the ligation mixture was taken to transform E. *coli*. The selection was performed on agar plate containing Zeocine 200 µg.mL⁻¹. The resistant clones were screened for the presence of *h*Pld1-b insert by doing PCR with two pairs of primers: 79/107 and 79/55. The primers 79 and 55 correspond to pGAPZ α -A sequences at both sides of the cloning site (c.f. schema of primer sites, Material and Methods). PCR of empty plasmid will result in 550 bp fragment while recombinant plasmid generates 6000 bp fragment (Fig.IV.24.B). The 107 primer recognizes *h*Pld1-b sequence starting at position +200 (c.f. schema of primer sites, Material and Methods). The resulting PCR product of recombined plasmid with 79/107 primers pair will measure 300 bp while the empty plasmid will not give any PCR product (Fig.IV.24.A)



Figure IV.24: PCR product analysis on 1% agarose gel. A) 79 and 55 primers were used to detect the insertion of DNA fragment into to pGAPZ α -A plasmid. Lane 1: empty plasmid; Lane 2: recombinant plasmid). B) The presence of *h*Pld1-b sequence was revealed by 107 primers. Lane 1: Empty plasmid; Lane 2: recombinant plasmid. . Right side: enlarged view of the molecular weighs of markers (MW) with the indicated MW.

The resistant clone which was positively screened for hPld-1 sequence was isolated and cultured to amplify the plasmid. The recombined plasmid was then purified by using Qiagen plasmid midi kit (Qiagen, France) and then sequenced prior use for Pichia *pastoris* transfection (Annex 01).

b. Transformation of Pichia pastoris

The plasmid needed to be linearized by the BspHI restrictive enzyme prior to transfection (Fig.IV.25).



Figure IV.25: Analysis of product of plasmid linearization by electrophoresis on 1% agarose gel. Lane 1: pGAPZ α -A. Lane 2: pGAPZ α -A + BspHI. Lane 3: pGAPZ α -A-*h*Pld1-b. Lane 4: pGAPZ α -A-*h*Pld1-b + BspHI. Right side: enlarged view of the molecular weighs of markers (MW) with the indicated MW.

The transformation was done by electroporation. Transformed P. *Pastoris* were then grown on YPG agar plate containing Zeocine 200 μ g.mL⁻¹. Single resistant clones were isolated and PCR was performed with 105/107 primer pair to verify the presence of recombined plasmid with P.*pastoris* genome. Positive band of 200 bp indicated the presence of *h*Pld1-b sequence (Fig.IV.26) enabling to isolate the 10 *h*Pld1-b positive clones.



Figure IV.26: Clone PCR analysis on 1% agarose gel. Lane 1: Positive P. *pastoris* clone transfected with pGAPZ α -A-*h*Pld1-b. Lane 2: Positive P. *pastoris* clone transfected with pGAPZ α -A. Lane 3: positive controle, pGAPZ α -A-*h*Pld1-b plasmid. Lane 4: Negative control, water. Right side: enlarged view of the molecular weighs of markers (MW) with the indicated MW.

c. Protein purification and PLD assay by radioactivity

Selected *h*Pld1-b positive clones were grown on YPG agar plate containing Zeocine 200 μ g.mL⁻¹ and Zeocine 2mg.mL⁻¹ (Fig.IV.27, position 1 to 10), together with two control clones transfected with empty pGAPza A plasmid (Fig.IV.27, position 14 and 15).



Figure IV.27: Culture of P. *pastoris* on YPG agar plate containing Zeocine 200ug.mL⁻¹ (left) or 2mg.mL⁻¹ (right).

We distinguish 2 types of P.*pastoris*: super-resistant clones which grow on both media, and resistant clones which can only grow on low antibiotic concentration medium. We chose two *h*Pld1-b positive (1 and 4) clones and two empty-pGAPZ positive clone (14,15) to
grow in 200mL of YPG medium containing Zeocine 200 μ g.mL⁻¹. The recombinant *h*PLD1-b was expected to be secreted into media and to contain 6xHis tag. P.*pastoris* culture media was separated from cell pellets. Recombinant *h*PLD1-b was purified by Ni-NTA column. The presence of PLD was verified by using transphosphatidylation assay with radiolabelled phospholipids. PLD activity was not detected in any sample (Fig.V.28).



Figure V.28: Transphosphatidylation assay of *P.pastoris* **culture medium and cell lysates by radiolabelled phospholipids.** Medium 1, 4, 14 and 15 corresponded to transformed *P.pastoris* 1, 4, 14 and 15 while lysate 1 and 14 corresponded to lysates of transformed *P.pastoris* 1 and 14 respectively. Ctrl- and Ctrl+ corresponded respectively to control samples without and with commercial PLD.

We have successfully subcloned *h*Pld1 coding sequence into pGAPz α A plasmid. P. *pastoris* carrying this sequence was also obtained. However the protein was not expressed or the expressed protein was not active. Alternate cloning strategies by using other gene expression system need to be examined.

5. Phospholipase activity assays based on infrared spectroscopy

One of the means to verify if AnxA6 can interact with PLD1 is to determine PLD1 activity. So far, as reviewed (Selvy *et al.*, 2011; Morris *et al.*, 1997), there is no enzymatic assay to directly determine PLD activities using natural substrates. Therefore, we developed a direct label free assay to monitor PLD activity

a. Development of PLD assay based on infrared spectroscopy

The phosphate region of the infrared spectra of dimyristoylphosphatidylcholine (DMPC), lysophosphatidylglycerol (lysoPG), dipalmitoylphosphatidylethanolamine (DPPE), lysophosphatidylserine (lysoPS) and dimyristoylphosphatidate (DMPA), each at 50 mM, in 100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂ and 2.5 mg/mL Triton X-100, present sufficient differences in band shapes and positions to be used for analytical application (Fig. IV.29).



Figure IV.29: Infrared spectra of dimyristoylphosphatidylcholine (DMPC), lysophosphatidylglycerol (lysoPG), dipalmitoylphosphatidylethanolamine (DPPE), lysophosphatidylserine (lysoPS) and dimyristoylphosphatidate (DMPA). Each trace was recorded at 50 mM phospholipid, in 100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 2.5 mg/mL Triton X-100. As clearly shown, the DMPC spectrum has a specific absorption peak at 1230 cm⁻¹ while that of DMPA has one at 1024 cm^{-1} .

As reported earlier the disappearance of the 1230-cm⁻¹ band associated with the asymmetric phosphate stretching vibration (vPO₂⁻) of

DMPC (top trace in Fig.IV.29) and the concomitant increase in the 1115-cm⁻¹ band associated with the symmetric phosphate stretching vibration (vPO₂⁻) of DMPA (bottom trace of Fig.IV.29) could serve to monitor *S. chromofucus* PLD activity (Estrela-Lopis *et al.*, 2001). The position of the phosphate bands is not only sensitive to the nature of phosphoester-bonded alcohols but also to their ionic environment such as the presence of calcium ions and pH (Estrela-Lopis *et al.*, 2001). This analytical application could be extended for example to

other species/types of phospholipids such as lysoPG (1226 cm⁻¹), DPPE (1221 cm⁻¹), lysoPS (1218 cm⁻¹) as shown in Fig.IV.29.

To illustrate the potential of the enzymatic assay, we determined the apparent hydrolytic activity of *S. chromofucus* PLD towards DMPC in a buffer containing 10 mM CaCl₂, 100 mM Tris-HCl, pH 8.0 and 2.5 mg/mL Triton X-100, ensuring optimal activity under controlled environment. A series of IR spectra has been recorded over a 180 min time period (Fig.IV.30A). The disappearance of the 1230 cm⁻¹ band (i.e., disappearance of the substrate), which was better observed in the difference infrared spectra (Fig. 30B), served to estimate the activity.



Figure IV.30: Hydrolysis of 25 mM DMPC by S. chromofucus PLD. A) A series of infrared spectra were recorded at indicated time intervals. The decrease in intensity of the 1230-cm⁻¹ band signaled the disappearance of DMPC while the increase in the 1020-cm⁻¹ band indicated the appearance of DMPA. B) Difference spectra were calculated by subtracting the spectrum measured at time t from the one measured at 5 min (i.e., minimum incubation time from addition of substrate and measuring infrared spectrum). Significant change in the intensity of absorption bands at 1230 cm⁻¹ and 1020 cm⁻¹ was observed while other variations were also noticeable.



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60

60

By using a molar absorption coefficient of 311 \pm 8 M⁻¹ cm⁻¹ for the DMPC band located at 1230 cm⁻¹, we obtained an apparent activity hydrolytic of S. chromofucus PLD ranging from $39 \pm 5 \text{ nmol min}^{-1} \mu \text{g}^{-1}$ with 5 mM DMPC, to 33 ± 8 nmol min⁻¹ μ g⁻¹ with 20 mM DMPC or to $30 \pm 3 \text{ nmol min}^-$

¹ μ g⁻¹ with 40 mM DMPC (Fig.IV.31A). Change from 5 mM to 40 mM DMPC concentration did no significantly alter the apparent specific activity of *S. chromofucus* PLD, which remained around 35 ± 5 nmol min⁻¹ μ g⁻¹ (pooled averaged values) indicating saturated conditions. The slight overestimation of the apparent specific activity of PLD in the presence of 5 mM DMPC (39 ± 5 nmol min⁻¹ μ g⁻¹) as compared with that at 40 mM DMPC (30 ± 3 nmol min⁻¹ μ g⁻¹) was due to experimental errors and was not considered significant. Indeed, the initial slopes (Fig IV.31A) were the same within experimental errors (35 ± 5 nmol min⁻¹ μ g⁻¹) confirming that 5-40 mM DMPC concentrations corresponded to saturated conditions. A minimal concentration of DMPC of about 2.5 mM (corresponding to 0.001 Absorbance unit at 1230 cm⁻¹) was considered to be the limit for optimal infrared measurements. Although the 1024 cm⁻¹ and 1115-1130 cm⁻¹ bands associated with DMPA increased (Fig.IV.30A and IV.30B) with time confirming the PLD hydrolytic activity towards DMPC, these bands were

А

Absorbance at 1230 cm⁻¹

в

Absorbance at 1230 cm⁻¹

0,005

0,004

0,003

0,002

0,001

0

0,008

0,006

0,004

0,002

0

0

0

10

◆PLD 5µg/mL

■PLD10µg/mL

▲ PLD20 µg/ml

10

DMPC 5mM

DMPC20mM

▲ DMPC40mM

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20

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20

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30

Time (min)

Ì

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30

Time (min)

40

40

50

50

not used due to either weak intensity (1024-cm⁻¹ band) or to a slight overlapping with other bands (1115-1130 cm⁻¹ band) (Fig.IV.29), that could increase experimental errors for the determination of kinetic parameters. To assess the minimal amount of *S. chromofucus* PLD for the determination of activity by IR spectroscopy, a concentration range from 0.1-20 μ g PLD mL⁻¹ corresponding to a total amount of 1-200 ng of *S. chromofucus* PLD (3.5-750 pmol hydrolyzed-DMPC min⁻¹) was used. Reproducible apparent activities were obtained with 5-20 μ g PLD mL⁻¹ (Fig.IV.31B).

The IR difference spectra indicated the disappearance of the 1230-cm⁻¹-DMPC band and the concomitant apparition of the DMPA or PA band located at 1130 cm⁻¹ in a sample containing 10 ng of *S. chromofucus* PLD or 35 pmol hydrolyzed DMPC min⁻¹ after 20 min (Fig.IV.32A). However, the infrared assay was not able to detect any PLD activity in a sample with 1 ng of *S. chromofucus* PLD or 3.5 pmol hydrolyzed DMPC min⁻¹ (Fig.IV.32B). Therefore, the sensitivity of IR spectroscopy was estimated to be around 10 ng of *S. chromofucus* PLD or 35 pmol hydrolyzed DMPC min⁻¹.



Figure IV.32: Limit of detection of the IR spectroscopy method. Hydrolysis of 25 mM DMPC by S. chromofucus PLD in 100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 2.5 mg mL⁻¹ Triton X-100. A) Two difference spectra of 25 mM DMPC and 1 $\mu g S. chromofucus PLD mL^{-1}$. They were calculated by subtracting the spectrum measured at 4 min from the one measured at 2 min (i.e., minimal incubation time separating addition of the substrate and infrared spectrum measurement) (Dashed line) or by subtracting the spectrum measured at 20 min from the one measured at 2 min (Full line). Significant differences appeared after 20min. B) Two difference spectra of 25 mM DMPC and 0.1 μ g S. chromofucus PLD mL⁻¹. They were calculated by subtracting the spectrum measured at 4 min from the one measured at 2 min (Dashed line) or by subtracting the spectrum measured at 20 min from the one measured at 2 min (Full line). No differences were detected after 20 min.

Comparison of the infrared assay with a coupled enzyme assay (Fig.IV.33) indicated that the sensitivity of the coupled assay was better than that of the infrared assay. However the apparent specific activity of 1 to 1000 pg *S. chromofucus* PLD, ranging from 3 ± 0.2 nmol min⁻¹ μ g⁻¹ to 91 \pm 29 nmol min⁻¹ μ g⁻¹ determined by the coupled enzyme assay was highly

dependent on *S. chromofucus* PLD concentration (0.007 to 7 μ g mL⁻¹). Although DMPC concentration was varied from 0.267 to 1 mM in the coupled enzyme assay, similar results were obtained indicating the difficulty to reach optimal conditions for reliable apparent activity measurements. IR spectroscopy was more reliable than the coupled assay in the determination of the apparent specific activity that amounted to 35 ± 5 hydrolysed DMPC (in nmol min⁻¹ μ g⁻¹) and remained stable over 50-200 ng of *S. chromofucus* PLD.



Figure IV.33: Comparison of apparent activities of S. chromofucus PLD determined by an infrared assay and by a colorimetric coupled enzyme assay as a function of the amount of S. chromofucus PLD. For colorimetric assays (squares), S. chromofucus PLD ranging from 1 to 1000 ng and DMPC from 0.267 to 1 mМ (final concentration) was mixed with the reaction buffer containing 50 mM Tris-HCl, pH 8.0, 20 mM CaCl2, 1.7 mM 4aminoantipyrine, 9 mM sodium 2hydroxy-3,5-dichlorobenzenesulfonate, 0.5 U choline oxidase and 0.5 U peroxidase. Absorbance measurements

at 500 nm were carried out continuously during 10 min. A standard curve obtained with pure choline was used to calculate the quantity of released choline. The hydrolytic activity of PLD is expressed as nmol of choline min-1 mg-1 of PLD. For IR assays (rhombuses), 50 to 200 ng S. chromofucus PLD were mixed with 20 mM DMPC to initiate the reaction. The hydrolytic activity of PLD was calculated as mentioned above and expressed as nmol of hydrolyzed DMPC min-1 mg-1 of PLD. n = 3.

To examine the feasibility of the infrared assay to screen inhibitors for PLD, halopemide at 2.4 μ M, a known mammalian PLD2 inhibitor (Monovich *et al.*, 2007; Su *et al.*, 2009), has been tested on *S. chromofucus* PLD by means of the IR assay (around 50 ng of *S. chromofucus* PLD) and by the coupled enzyme assay (1-1000 ng *S. chromofucus* PLD). Both assays indicated that halopemide at 2.4 μ M did not inhibit *S. chromofucus* PLD. The lack of effect of halopemide could originate from the poor homology of *S. chromofucus* PLD (does not contain a characteristic HKD motif) to members (of the PLD superfamily containg an HKD motif (Exton, 2002). Cobalt, a known *S. chromofucus* PLD inhibitor (Imamura and Horiuti , 1979), has been tested and the IC₅₀ value of 0.8 ± 0.2 mM has been found from the IR assay (Fig. IV.34).



Figure IV.34: The effect of cobalt concentration on the PLD activity from *S. chromofucus*. Apparent activities of 20 μ g of *S. chromofucus* PLD mL⁻¹in 100 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 2.5 mg mL⁻¹Triton X-100 and of 20 mM DMPC as determined by the infrared assay as a function

of cobalt concentration. IC_{50} value of 0.8 ± 0.2 mM (the cobalt concentration necessary to inhibit half the enzyme activity) was obtained from these data. n = 3.

These results showed that our direct method of measuring PLD activity based on IR spectroscopy was reliable and that it can be used to identify PLD inhibitor.

b. Effect of AnxA6 on PLD activity

To evaluate the effect of AnxA6 on PLD activity, we incubated the enzyme with equimolar quantity of annexinA6-1 and *S. chromofucus* PLD. Since we were not able to obtain human recombinant PLD, we used *S. chromofucus* PLD. The IR method served to monitor the enzymatic activity (Fig.IV.35).



Figure IV.35: AnxA6 inhibits PLD activity in *vitro*. Enzymatic activity of PLD from S. *chromofuscus* in the absence or in the presence of annexin A6-1 (equimolar) was monitored by IR spectroscopy. In the presence of AnxA6-1, the enzymatic activity of PLD was significantly reduced to $72\pm11\%$ comparing to normal condition. n=3, (*) p-value < 0.05.

We observed that AnxA6-1 isoform inhibited significantly S. *chromofuscus* PLD activity. Although the sequence homology of enzyme used in this experiment is distinct from that of the mammalian isoform, this is the first time that the ability of AnxA6 to inhibit enzyme activity has been evidenced. As PLD1 is key activator of exocytosis, one can speculate that in the cell AnxA6 could decrease PLD1 activity which in turn will decrease exocytosis.

CHAPTER V: DISCUSSION

V. Discussion

1. PC12 as a model of neurosecretion

PC12 cells have been widely used as a model of neurosecretion besides chromaffin cells. While chromafin cells are isolated from material obtained from a slaughterhouse (bovine cells) or laboratory animals (murine cells), PC12 cells are derived from pheochromocytoma of the rat adrenal medulla and they can undergo 70 cell divisions without major alteration of morphology and functionality (Greene and Tischler, 1976). The advantages of PC12 are the following: firstly, the use of PC12 avoids ethical issues, secondly, these cells are very easy to culture, thirdly, a large amount of knowledge about proliferation and differentiation of PC12 cells has been accumulated. Furthermore, PC12 cells can be induced to acquire neuron-like morphology and they contain dense-core vesicles which are smaller and more similar to neuronal vesicles than the vesicles of chromaffin cells (Greene and Tischler, 1976; Westerink and Ewing, 2008). PC12 cells synthesize, store and secrete catecholamines, mostly dopamine and a smaller amount of norepinephrine but not epinephrine, upon cell stimulation (Greene and Tischler, 1976). The main disadvantage of using PC12 cells lies in the fact that they originate from tumoral cell lines and not from primary cells. Therefore the main findings obtained with PC12 cells need to be substantiated by further experiments on primary cells or on animal models.

Nevertheless, in this work, we used PC12 cells as a cellular model of regulated exocytosis since we aimed to elucidate the inhibitory effect of AnxA6 observed in this cell line (Podszywalow-Bartnicka *et al.*, 2010). To obtain a good homogeneity of data, we worked with cells at low passages (5th to 10th passage). Firstly, we verified their cell morphology and cell excitability. The cells were round and irregular in shape, with a large nucleus. They had a tendency to aggregate into clumps (Fig.IV.1). Electron micrographs of PC12 cells confirmed the cell size, the large area occupied by the nucleus and the presence of dense-core vesicles (Fig.IV.2). Cell excitability was verified by measuring the intracellular calcium concentration ($[Ca^{2+}]_i$). Fura2-AM served as an intracellular calcium indicator (Grynkiewicz *et al.*,1985). Although basal ($[Ca^{2+}]_i$) was higher than usual, cells did respond to stimulation as expected (Fig.IV.4). These results confirmed that PC12 cells had the typical characteristics as proved by their morphologies and their excitability. They were suited for our investigations concerning the actions of AnxA6 and PLD1 during exocytosis.

2. AnxA6 and PLD1 localization in PC12 cells revealed by immunohistochemistry

It is established that upon cell stimulation, phospholipase D1 (PLD1) is translocated to the plasma membrane in a Ca^{2+} -dependent manner (Vitale *et al.*, 2001). While AnxA6 inhibits secretion of catecholamines (Podszywalow-Bartnicka et al., 2010), PLD1 acts more like an activator of secretion (Vitale et al., 2001). It hydrolyzes phosphatidylcholine to form phosphatidic acid and through that inducing a negative curvature in the plasma membrane and facilitating vesicle-plasma membrane fusion (Vitale et al., 2001, 2002). Phosphatidic acid has a cone-shape hydrophobic head in contrast to the cylindric shape of phosphatidylcholine. Thus, hydrolysis of phosphatidylcholine will destabilize the structure of the plasma membrane and induce vesicle fusion (Vitale et al., 2001, 2002). In PC12 cells, PLD1 colocalized with SNAP-25, a membrane marker (Vitale et al., 2001), which was also found partially colocalized with AnxA6 upon cell stimulation (Podszywalow-Bartnicka et al., unpublished observation). Our results of immunocytochemistry experiments confirmed the translocation of AnxA6 but not that of the PLD1. Indeed, a significant accumulation of AnxA6 has been observed at the plasma membrane upon cell stimulation (Fig.IV.5 and IV.8, green color, and Fig.IV.7). In contrast, immunostaining of PLD1 revealed homogenous distribution and no recruitment of PLD1 at the plasma membrane has been detected (Fig.IV.8, red color). This finding was unexpected and we suspected that the commercial antibody against PLD1 was unspecific as indicated in Fig.IV.9.B.

3. EGFP labeling of AnxA6 and PLD1 in PC12 cells

a. Confocal microscopy

To check whether PLD1 can translocate during cell stimulation, we performed EGFP labeling of PLD1 as well as that of AnxA6. There are three main advantages of using EGFP labeling: firstly no antibodies are needed and we can avoid non-specific signals; secondly, we can work with living cells and investigate fast and dynamic events during exocytosis. Thirdly, we can label separately two isoforms of AnxA6, to explore their functional difference. PC12 cells were transfected with a plasmid allowing transient overexpression of EGFP-AnxA6 or EGFP-PLD1 in PC12 cells. As expected, PLD1 was translocated upon cell stimulation as revealed by the EGFP-PLD1 labeling and confocal microscopy (Fig.IV.11A). However translocation of EGFP-AnxA6 (both isoforms) was

not detected (Fig.IV.12 B and C). We propose three explanations: 1) it is known that translocation of AnxA6 is a Ca²⁺-dependent process. Stable AnxA6 overexpression in PC12 inhibited calcium uptake (Podszywalow-Bartnicka *et al.*, 2010), while transient overexpression of EGFP-AnxA6 may have less effect on the calcium entry subsequently reducing the extent of AnxA6-EGFP translocation. 2) There are two populations of AnxA6 which behave differently: one translocates to the plasma membrane in a Ca²⁺-dependent manner, the other associates with endosome membrane in a Ca²⁺-dependent but also Ca²⁺-independent manner. 3) Overexpression of AnxA6-EGFP may create a saturation of the fluorescence signal masking the translocation process. It was then necessary to verify the effect of transfection on the excitability of the cell.

b. Ratiometric calcium imaging: the excitability of transfected cells

Cell excitability is defined by the capacity to respond to external stimulation. For example in the case of PC12 cells, it is measured by the elevation of intracellular calcium concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ of native PC12 was monitored by fluorometric measurement of Fura-2 fluorescence (Grynkiewicz *et al.*, 1985). This method allows quantifying an average value of $[Ca^{2+}]_i$ of a homogenous cell population (Fig.IV.4). Since the transfection efficiency was very low (Fig.IV.10, only measurements performed on individual cells could distinguish calcium response due to transfected and non-transfected cells. Ratiometric calcium imaging is the method of choice. It indicated that the transient overexpression of AnxA6-1-EGFP, AnxA6-2-EGFP and PLD1-EGFP did not affect calcium entrance in transfected PC12 cells (Fig.IV.15).

Calcium entrance is required to trigger exocytosis. A stable overexpression of AnxA6 affected calcium homeostasis in cardiomyocytes and in PC12 cells (Gunteski-Hamblin *et al.*, 1996). In HEK293 cells AnxA6 inhibited store-operated calcium entrance (SOCE) by stabilizing actin cortex (Monastyrskaya *et al.*, 2009). However, SOCE is not the major calcium entrance gate in excitable cells. In PC12 cells, the main calcium entrances are the L-type voltage-dependent calcium channel (L-VDCC) and the Na⁺/Ca²⁺ exchanger (NCX) operating in the reverse mode (Podszywalow-Bartnicka *et al.*, 2010). In PC12 cells, stable overexpression of AnxA6 modulated VDCC activity rather than affected its expression. However, an increase in NCX contents has been observed (Podszywalow-Bartnicka *et al.*, 2010). This finding is consistent with VDCC modulation in rat sensory neurons involving a Ras-mitogen-activated protein kinase.

Taken together, these reports suggest that AnxA6-p120GAP interactions can regulate VDCC activity through Ras inhibition. Therefore, it is expected that either transient or stable overexpression of AnxA6 will alter calcium homeostasis. However we observed that transient overexpression of AnxA6 did not affect calcium entrance (Fig.III.15). There was not any significant difference between cells overexpressing AnxA6 and the control cells. This is apparently contradictory and we suspect that a long term AnxA6 overexpression might involve different targets and consequences for cell physiology than temporary overexpression.

c. TIRF microscopy: recruitment of AnxA6-1 and PLD1 to secretory vesicles

Although the increase in intracellular Ca^{2+} promotes association of AnxA6 with the plasma membrane, the proteins quickly resume their cytoplasmic localization upon restoration of the basal $[Ca^{2+}]_i$ (Monastyrskaya *et al.*, 2007). A method allowing fast monitoring of cellular localization should be helpful in examining high mobility of these proteins.

TIRFM has been used to monitor single vesicle fusion in living cells with high spatiotemporal resolution (Oheim *et al.*, 1998; Ravier et *al.*, 2008, An et *al.*, 2010). In this work, we used this technique to show recruitment of AnxA6 and PLD1 on the surface of the secretory vesicles. PLD1 strongly colocalized with secretory vesicles at the plasma membrane (Fig.IV.16). This confirmed the participation of PLD1 in exocytosis (Vitale *et al.*, 2001). Interestingly AnxA6-1 (Fig.IV.18) but not AnxA6-2 (Fig.IV.19) was recruited at sites of exocytosis. Previously it has been suggested that stable overexpression of AnxA6-2 had stronger inhibitory effects on calcium homeostasis and dopamine secretion than AnxA6-1 overexpression (Podszywalow-Bartnicka *et al.*, 2010). Our data confirmed a difference of the localization of the two isoforms. AnxA6-1 colocalized more strongly with secretory vesicles than AnxA6-2 (Fig.IV.20). We propose that each AnxA6 isoform interacts with its own partners and/or acts at separate steps of exocytosis.

4. Production of human recombinant PLD

To check whether AnxA6 can act with PLD, it was necessary to produce human recombinant PLD, especially the isoform PLD1 which was shown to be colocalized with AnxA6 on the surface of vesicles. For this purpose, we selected Pichia *pastoris* and using a

pGAPZ α -A plasmid to over express the human recombinant *h*PLD1-B possessing complete post-translational modification. We obtained the subcloned *h*Pld1 coding sequence into pGAPz α A plasmid. P. *pastoris*. However the protein was not expressed or the expressed protein was not active. There were no detectable *h*PLD1-B activity. Alternate cloning strategies by using other gene expression system need to be examined. Therefore, we selected *S. chromofucus* PLD which is commercially available, to evaluate the possible interactions between PLD and AnxA6.

5. Inhibitory effect of AnxA6 on PLD activity as revealed by IRbased assay

a. Development of PLD assay based on infrared spectroscopy

One possible experiment to monitor interaction between the AnxA6 isoforms and their partners, especially PLD, is to determine their influence on PLD activity. Since there is no direct method to determine PLD activity, we developed and optimized a PLD enzymatic assay. It was reported earlier that IR spectroscopy could be used to monitor PLD activity (Estrela-Lopis et al., 2001; Yamamoto et al., 1995). Here, we described the conditions to obtain the kinetic parameters of PLD-catalyzed activity, such as the apparent specific activity, and to calculate IC₅₀ values from the IR assay. Phospholipases act on a substrate present in an insoluble aggregate and therefore many phospholipases exhibit interfacial kinetics that do not follow Michaelis-Menten kinetics (Selvy et al., 2011). To optimize the conditions in terms of sensitivity and reproducibility as well as to minimize the interfacial impact, Triton X-100 has been used to solubilize the phospholipid samples. Most PLDs are sensitive to calcium and to magnesium concentrations (Morris et al., 1997), therefore the PLD assay was performed at 10 mM calcium concentration. The optimal conditions to measure the apparent specific activity within 20 minutes were the following: 1-20 µg S. chromofucus PLD mL⁻¹ corresponding to a total amount of 10-200 ng of S. chromofucus PLD should be used for an assay and the substrate concentration should range between 2-40 mM DMPC at pH 8.0. The 20 min assay was sensitive enough to detect the activity of as little as 5-10 ng of S. chromofucus PLD at 2-40 mM DMPC concentration. Under these conditions, reliable apparent specific activity could be determined. Much lower S. chromofucus PLD concentration could be used at longer incubation times. The only limitation concerns the phospholipid concentrations used for the infrared measurements, which should be greater than 2 mM

for optimal results (molar absorption coefficient was $311 \pm 8 \text{ M}^{-1} \text{ cm}^{-1}$ for the DMPC band located at 1230 cm⁻¹). In this respect, IR is insensitive to light scattering effects caused by the turbidity of the samples and relatively large concentrations of lipids can be employed. For the IR assay, substrate concentration (≥ 2 mM) corresponded to saturated conditions consistent with the apparent K_M value of around 0.625 mM for egg-yolk phosphatidylcholine and PLD from S. chromofucus as determined by using a choline oxidase-oxygen electrode. We could not determine the apparent K_M due to higher concentrations of DMPC (2-40 mM) that have to be used in this assay. However reliable and reproducible apparent specific activity of S. chromofucus PLD, which amounted to 35 ± 5 nmol hydrolysed DMPC min⁻¹ μ g⁻¹, could be obtained. This activity remained stable over 50-200 ng of S. chromofucus PLD and over 5-40 mM of DMPC. Despite its limitation, IR spectroscopy is more reliable than the colorimetric assay in determining apparent specific activity, which remained constant over 1-20 µg S. chromofucus PLD mL^{-1} , making it a method of choice for searching PLD inhibitors. Reliable IC₅₀ values could be obtained from the IR assay as exemplified with a value of IC₅₀ for cobalt, a known S. chromofucus PLD inhibitor (Imamura et al., 1979). A value amounting to $0.8 \pm$ 0.2 mM has been calculated from the IR assay consistent with the reported value of 1 mM for a complete inhibition (Imamura et al., 1979). To conclude, the main advantages of the IR assay lie in the possibility to directly determine the hydrolytic activity of PLD in situ and to obtain IC₅₀ of inhibitors using natural substrates in an almost continuous manner. This is in contrast with other assays based on transphosphatidylation, where phospholipids and products need to be extracted to be analyzed. Furthermore, the feasibility to determine PLD activity by using not only DMPC but also other natural substrates such as lysoPG, DPPE, lysoPS was demonstrated. The results clearly show that the infrared assay is well suited to determine an overall PLD activity toward distinct types of phospholipids.

b. Inhibitory effect of AnxA6-1 on PLD activity

According to our TIRFM findings, both AnxA6-1 and PLD1 are recruited by secretory vesicles. However this method is unable to detect functional relationship between proteins. AnxA6 has been suggested to be a scaffolding protein that facilitates the recruitment of PKC α (Grewal *et al.*, 2010), fodrin (Watanabe *et al.*, 1994), p120GAP (Grewal and Enrich, 2006) to the plasma membrane. Alternatively, we propose that AnxA6-1 interacts with PLD1. Although the inhibition induced by AnxA6-1 on S. 76

chromofuscus PLD activity was significant, we are well aware that S. *chromofuscus* PLD is different from human PLD and that the interaction between human AnxA6-1 and human PLD1 need to be ascertained by other experimental approaches.

CHAPTER VI: CONCLUSIONS

VI. Conclusions

The roles of AnxA6 and of PLD1 in exocytosis in PC12 cells: coupling exocytosis and endocytosis

The interaction network of AnxA6 comprises structural proteins and also signaling molecules indicating that the function of AnxA6 in the cell is very broad. 1) AnxA6 as an organizer of the site of exocytosis: Cholesterol is a key component of the membrane rafts. Small rafts are sometimes stabilized to form larger platforms through protein-protein and protein-lipid interactions" (Pike, 2006). These membrane microdomains regulate certain signal transduction and membrane traffic pathways. AnxA6 associates with cholesterolenriched membrane microdomain of rat brain (Orito et al., 2001). In PC12, it has been shown that syntaxin 1 and SNAP-25, two t-SNARE proteins, were enriched in lipids rafts (Chamberlaine et al., 2001). In parallel, by using high resolution imaging, Lang and coworkers showed that at the plasma membrane, syntaxin 1 and SNAP-25 were concentrated into distinct cholesterol-dependent clusters which partially co-localized; syntaxin clusters were the docking and fusion sites of vesicles (Lang et al., 2001). AnxA6, by binding cholesterol-enriched membrane domains might determine the site of exocytosis at the plasma membrane. It is tempting to correlate these findings with ours. We propose that the specific recruitment of AnxA6-1 to the vesicles, as demonstrated by our work, may serve to direct the vesicles to the cholesterol-enriched regions of plasma membranes. Indeed, thanks to the capacity to transiently and reversibly bind to membrane lipids, AnxA6 as a scaffolding protein, allows a specific recruitment of AnxA6-binding proteins, for example p120GAP, to the plasma membrane, as discussed above. Many other regulators of exocytosis but also endocytosis have been shown to interact with AnxA6. AnxA6 co-localized with actin, in a Ca^{2+} -dependent manner, along stress fibers as well as membrane ruffles in bovine liver cells (Hosoya et al., 1992), in secretory ameloblasts and odontoblasts of the rat incisor (Golberg et al., 1991). In HEK293 cells, stable plasma-membrane-localization of AnxA6 induced reorganization and accumulation of F-actin which inhibited the store-operated calcium entry (Monastyrskaya et al., 2009). This inhibitory effect was due to the stabilization cortical cytoskeleton because latrunculin A which disrupted actin cytoskeleton abolished the inhibitory effect of membrane-anchored AnxA6 on SOCE (Monastyrskaya et al., 2009). However, further investigation should be conducted to demonstrate the AnxA6-induced stabilization of cortical actin of neurons or endocrine cells. A dynamic reorganization of the actin cortex is mandatory to allow penetration of secretory vesicles. There are very little

information of the effects of AnxA6-1 and AnxA6-2 isoforms on the mechanisms of exocytosis. Recently it has been reported that stable overexpression of AnxA6-2 had stronger inhibitory effects on calcium homeostasis and dopamine secretion than AnxA6-1 overexpression (Podszywalow-Bartnicka et al., 2010) indicating a distinct effect for each isoform that need to be substantiated. We confirmed the distinct effect and we showed that there is a concomitant recruitment of PLD1 and AnxA6-1 to the membranes, while AnxA6-2 behaved differently. In addition, we revealed for the first time that AnxA6-1 inhibited PLD activity. Efforts should be done to purify mammalian PLD1 to confirm the inhibition by AnxA6. Since high PLD1 activity will favor exocytosis, the inhibitory properties of AnxA6 may provide a possible mechanism leading to the Anx-A6-induced decrease in exocytosis. AnxA6 participates actively in endocytosis. For example, AnxA6, but not other annexins, was required for budding of clathrin-coated vesicles, the first step of macromolecule internalization during receptor-induced endocytosis (Lin et al., 1992). AnxA6 interacted with calspectin (spectrin) in a Ca^{2+} -dependent manner in the rat brain (Watanabe *et al.*, 1994). This interaction has been shown later to be responsible for the recruitment of calpain 1 which cleaves the spectrin-actin cytoskeleton to allow the entry of endocytic vesicles (Kamal et al., 1998). In vitro experiments showed that AnxA6, but also AnxA1 and A2, bound to the µ subunit of the clathrin assembly protein complex AP-2 (Creutz and Snyder, 2005).

Growing evidence suggests that during regulated exocytosis vesicle membrane does not intermix with the plasma membrane and that exocytosis is rapidly followed by a compensatory endocytosis to maintain membrane integrity and cell homeostasis (Opazo *et al.*, 2010; Opazo and Rizzoli, 2010; Ceridono *et al.*, 2011; Haucke *et al.*, 2011; Houy *et al.*, 2013). This hypothesis is supported by the fact that clathrin is rapidly recruited to the granule membrane right after merging with the plasma membrane (Ceridono *et al.*, 2011). Taken together these findings and ours, we propose that AnxA6 and PLD1 can regulate exocytosis as well as endocytosis. Due to its high affinity for cholesterol enriched membranes, AnxA6 is selectively recruited to vesicles and may serve to direct the vesicles to plasma membrane. On the other hand, overexpression of AnxA6 decreases exocytosis probably by inhibiting PLD1 activity which can promote exocytosis. It is still unclear which AnxA6 isoform and by what mechanism regulates endocytosis and this issue needs to be further examined.

VII. References

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Interplay between AnnexinA6 and Phospholipase D1 during the process of exocytosis in PC12 cells

Regulated exocytosis is a key process allowing cell-cell communication through the release of hormones and neurotransmitters. In neurons and neuroendocrine cells, it is strictly controlled by extracellular signals such as transmembrane potential and ligand binding to receptors. Substantial progress has been made to understand the molecular mechanism of exocytosis. Major components of the secretory machinery have been brought to light. Now, the emergent question concerns the role of scaffolding proteins that are thought to coordinate the action of each other. In the case of the annexin family, well known to be involved in exocytosis, their modes of sequential or concerted interactions with other proteins, and their regulatory effects on exocytosis, are not very well established. Annexin A6 (AnxA6) which contains two annexin domains instead of one, exists in two isoforms which differs by 6 residues due to alternative splicing. Previous findings indicated that annexin A6 (AnxA6) affected calcium homeostasis and dopamine secretion from PC12 cells, which are used as a cellular model of neurosecretion and that two isoforms exert their inhibitory effect with different extent (Podszywalow-Bartnicka et al., 2010). To determine role of AnxA6 during the secretion of dopamine, we were looking for molecular partners of AnxA6 in PC12 cells. We hypothesized that AnxA6 could interact with phospholipase D1 (PLD1), an enzyme involved in the membrane fusion step which occurs prior to secretion of catecholamines. We labeled two isoforms of AnxA6 with EGFP; then using confocal microscopy and total internal reflection fluorescence microscopy, we found that isoform 1 of AnxA6 and phospholipase D1 are both recruited on the surface of secretory vesicles upon stimulation of PC12 cells for secretion. The second isoform of AnxA6 which lacks 6 residues due to alternative splicing of AnxA6 mRNA, did not show this behavior confirming the hypothesis of functional difference between these two isoform. To investigate an eventual effect of AnxA6 on the PLD activity, we developed an enzymatic assay based on infrared spectroscopy. By using this method, we showed that AnxA6-1 inhibited activity of PLD (S. chromofuscus) in vitro. To conclude, we propose that AnxA6 acts not only in membrane organization by its capacity to bind to negatively charged phospholipids and to cholesterol, but AnxA6 also affects PLD1 activity inducing changes in membrane lipid composition and membrane morphology.

Key words: Exocytosis, PC12 cell, Annexin A6, Phospholipase D1, Total internal reflection fluorescence microscopy, Laser scanning confocal microscopy, Calcium imaging, Infrared spectroscopy

Relation entre l'annexine A6 et la phospholipase D1 pendant le processus d'exocytose dans les cellules PC12

L'exocytose est un processus permetant la communication entre les cellules à travers la sécrétion des hormones et des neurotransmetteurs. Dans les neurones et les cellules neuroendocrines, elle est strictement contrôlée par des signaux extracellulaires tels que le potentiel trans-membranaire et la fixation des ligands sur des récepteurs. Des progrès substantiels ont été effectués afin de comprendre le mécanisme moléculaire de l'exocytose. Les composants majeurs de la machinerie de sécrétion ont été dévoilés. Maintenant, la question qui émerge concerne le rôle de la plateforme de protéines qui semble avoir une action coordonnée entre chaque protéine. Dans le cas de la famille des annexines, qui est bien connue pour son action au cours de l'exocytose, leurs modes d'interactions séquentielles ou concertées avec d'autres protéines ainsi que leurs effets régulateurs sur l'exocytose ne sont pas encore bien établis. L'annexine A6 (AnxA6), possédant deux domaines d'annexine au lieu d'un, existe dans les cellules sous deux isoformes issus d'un épissage alternatif. Des résultats précédents ont indiqué que l'AnxA6 affectait l'homéostasie du calcium et la sécrétion de la dopamine à partir des cellules PC12, utilisées comme un modèle cellulaire de neurosécrétion et que les deux isofomes exerce leur effet avec de différentes degrés (Podszywalow-Bartnicka et al., 2010). Afin de déterminer l'effet inhibiteur de l'AnxA6 sur l'exocytose de la dopamine, nous cherchons des partenaires moléculaires de l'AnxA6 dans les cellules PC12. Nous faisons l'hypothèse que l'AnxA6 interagit avec la PLD1, une enzyme active dans l'étape de la fusion des vésicules avec la membrane plasmique. Nous avons marqué les deux isoformes de l'AnxA6 et la PLD1 par de l'EGFP. En utilisant la microscopie confocale et la microscopie à onde évanescente, nous avons observé que l'isoforme 1 de l'AnxA6 et la PLD1 sont tous les deux recrutés sur la surface des vésicules au cours de la stimulation des cellules PC12. Cependant l'isoforme 2 de l'AnxA6 ne présente pas ce comportement. Cela confirme l'hypothèse de la différence fonctionnelle entre eux. Afin de chercher un éventuel effet de l'AnxA6 sur l'activité de la PLD, nous avons développé une méthode de dosage d'activité enzymatique de la PLD par spectroscopie d'infrarouge. En utilisant cette méthode, nous avons montré que l'isoforme 1 de l'AnxA6 inhibait l'activité de la PLD (S. chromofuscus) in vitro. En conclusion, nous proposons que l'AnxA6 n'agisse pas seulement dans la réorganisation des membranes par ses capacités à se lier avec des phospholipides négativement chargés et avec le cholestérol, mais elle influence également l'activité de la PLD1, changeant la composition lipidique des membranes.

Mots-clés : Exocytose, Cellule PC12, Annexin A6, Phospholipase D1, Microscopie à onde

évanescence, Microscopie confocale, Imagerie calcique, Spectroscopie infrarouge.
	10	20	30	40	50	60	70	80
nCanz_bD1d1_a	.		. Teetetetete		. GTD GCCD TCT:	. Стерортотст		
pGapz-hFld1-h	AGATCTTTTT	GTAGAAAIGICI	TGGTGTCCTCC	TCCAATCAG	GTAGCCATCT	CTGAAATATCT	GGCTCCGTT	CAACTCCGAACGA
Construct	AGATCTTTTT	GTAGAAATGTCT	TGGTGTCCTCG	TCCAATCAG	GTAGCCATCT	CTGAAATATCT	GGCTCCGTT	CAACTCCGAACGA
	120	120	140	1.50	160	120	100	190
			.	1	.	.	1	. .
pGapz-hPld1-a	CGGGGTAAAAC	TTAAATGTGGAG	TAATGGAACCA	GAAACGTCT	CTTCCCTTCT	CTCTCCTTCCA	CCGCCCGTT	ACCGTCCCTAGGAA
pGAPZ-hPld1-b	CGGGGGTAAAAC	TTAAAT GT GGAG	TAATGGAACCA	GAAACGTCT	CTTCCCTTCT	CTCTCCTTCCA	CCGCCCGTT	ACCGTCCCTAGGAA
Construct	CGGGGGTAAAAC	TTAAATGTGGAG	TAATGGAACCA	GAAACGTCT	CTTCCCTTCT	CTCTCCTTCCA	CCGCCCGTT	ACCGTCCCTAGGAA
	230	240	250	260	270	280	290	300
			.	1	.	.	1	.
pGapz-hPld1-a	TTCTACGGCCC	CCTTGCAGCAAT	GCTCTTCCCAL	CATTACETT	GCGGGGTAAAA	CGGAGGTCGTG	TACCCBACC	AGCAGCCCAGGGA
pGAPZ-NPIGI-D	TTCTACGGCCC	COTTECASCAAT	GOTOTTCCCAG	CATTACGTT	GCCCCCTAAAAA	CGGAGGTCGTG	TACCOBACCI	AGCAGCCCAGGGA
construct	IICIACOOCCC	CULIBCAGCAAI	GUICITUUA	CATIACETT	GC GGG TAAAA	C664661C616	TACCOBACCI	AGCAGCCCAGGGA
	340	350	360	370	380	390	400	410
nGanz-hP1d1-a	GCAATAATAGC	GGGCGGACGCAT	GTCATGAGATI	ATTGGAAAC	CACCARAATC	GAATATAAAAG	FCGAACACC	TTCCCAATTTTGG
pGAPZ-hPld1-b	GCAATAATAGC	GGGCGGACGCAT	GTCATGAGATI	ATTGGAAAC	CACCAGAATC	GAATATAAAAG	GCGAACACC	TTCCCAATTTTGG
Construct	GCAATAATAGC	GGGCGGACGCAT	GTCATGAGATI	ATTGGAAAC	CACCAGAATC	GAATATAAAAG	GCGAACACC	TTCCCAATTTTGG
	450	460	470	400	490	500	510	F20
		460		400	4.50	.	1	.
pGapz-hPld1-a	AATTTAATTTA	TTTGTCCCTATT	TCAATCAATTG	AACAACTAT	TTCGAAACGA	FGAGATTTCCT	TCAATTTTT	ACTECTETTTATT
pGAPZ-hPld1-b	AATTTAATTTA	TTTGTCCCTATT	TCAATCAATTG	AACAACTAT	TTCGAAACGA	FGAGATTTCCT	TCAATTTTI	ACTGCTGTTTTATT
Construct	AATTTAATTTA	TTTGTCCCTATT	TCAATCAATTG	AACAACTAT	TTCGAAACGA	FGAGATTTCCT	TCAATTTTI	ACTGCTGTTTTATT
	560	570	580	590	600	610	620	630
				1	-	.	1	
pGapz-hPld1-a	CTCCAGTCAAC	ACTACAACAGAA	GATGAAACGGC	ACAAATTCC	GGCTGAAGCT	GTCATCGGTTA	CTCAGATTT	AGAAGGGGATTTCG
pGAPZ-hPld1-b	CTCCAGTCAAC	ACTACAACAGAA	GATGAAACGGC	ACAAATTCC	GGCTGAAGCT	GTCATCGGTTA	CTCAGATTTA	AGAAGGGGGATTTCG
Construct	CTCCAGTCAAC	ACTACAACAGAA	GATGAAACGGG	ACAAATTCC	GUCTGAAUCT	GTCATCGGTTA	CTCAGATTI	AGAAGGGGGATTTCG
	670	680	690	700	710	720	730	740
nGanz-bP1d1-a	AACAGCACAAA		. GTTTATAAATA	СТАСТАТТС	CCAGCATTGC	. Гесталаелае	 A A C C C C T A T (
nGAPZ-hPld1-h	AACAGCACAAA	TAACGGGTTATT	GTTTATAAATA	СТАСТАТТС	CCAGCATTGC	TGCTAAAGAAG	AAGGGGTAT	TCTCGAGAAAAGA
Construct	AACAGCACAAA	TAACGGGTTATT	GTTTATAAATA	CTACTATTG	CCAGCATTGC	IGCTAAAGAAG	AAGGGGGTAT	TCTCGAGAAAAGA
	700	790	900	010	920	020	040	950
			.	1	.	.		.
pGapz-hPld1-a	GGTAAATACCT	CTGCACTGCAGA	AAATTGCTGCT	GACATGAGT	AATAT CATAG	AAAATCTGGAC	ACGCGGGGAA	CTCCACTTTGAGGG
pGAPZ-hPld1-b	GGTAAATACCT	CTGCACTGCAGA	AAATTGCTGCI	GACATGAGT	AATAT CATAG	AAAA <mark>TCT</mark> GGAC	ACGCGGGGAA(CTCCACTTTGAGGG
Construct	GGTAAATACCT	CTGCACTGCAGA	AAATTGCTGCI	GACATGAGT	AATATCATAG	AAAATCTGGAC	ACGCGGGGAA	CTCCACTTTGAGGG
	890	900	910	920	930	940	950	960
					.	.	1	
pGapz-hPld1-a	CTCCCAGCGAT	CCCAAGATACAA	GAAGTGTATAT	CCCTTTCTC	TGCTATTTAT	AACACTCAAGG	ATTTAAGGA	GCCTAATATACAGA
pGAPZ-hPld1-b	CTCCCAGCGAT	CCCAAGATACAA	GAAGTGTATAT	CCCTTTCTC	TECTATTTAT	AACACTCAAGG	ATTTAAGGA	CCTAATATACAGA
Construct	CTCCCAGCGAT	CCCAAGATACAA	GAAGTGTATAT	CCCTTTCTC	IGCTATTIAN	AACACTCAAGG	ATTTAAGGA	CC TAA TA TA CAGA
	1000) 1010	1020	1030	1040	1050	1060	1070
nGanz-bPld1-a	AAAGCACAAGT	TCTCCALCTCCA	ACGCTTCACAT	CTACAACAA	GEETACCAAE'		ACACTATTO	
nGAPZ-hPld1-h	AAAGCACAAGT	TCTGGAAGTGGA	ACGCTTCACAT	СТАСААСАА	GGGTACCAAG'	TATTAATCTTT	ACACTATTG	ATTAACACATGGG
Construct	AAAGCACAAGT	TCTGGAAGTGGA	ACGCTTCACAT	CTACAACAA	GGGTACCAAG'	TATTAATCTTT	ACACTATTG	ATTAACACATGGG
	1110		1100	1140	1150	1160	1100	1100
		, 1120 	.	1140	.	.	1	1180
pGapz-hPld1-a	GAAATTCAAGC	ATTTTCAAGAAT	TTCACAGAGAG	CTGCTCAAG	TACAAAGCCT	TATCCCCATC	CCCATTCCC	ACTAGAAGACACAC
pGAPZ-hPld1-b	GAAATTCAAGC	ATTTTCAAGAAT	TTCACAGAGAG	CTGCTCAAG	TACAAAGCCT	TATCCGCATC	CCCATTCCC	ACTAGAAGACACAC
Construct	GAAATTCAAGC	ATTTTCAAGAAT	TTCACAGAGAG	CTGCTCAAG	TACAAAGCCT	TTATCCGCATC	CCCATTCCC	ACTAGAAGACACAC
	1220) 1230	1240	1250	1260	1270	1280	1290
				1	-		1	
pGapz-hPld1-a	AGGAGCCTCGA	GAGATGCCCAGT	TTGCCCCGTTC	ATCTGAAAA	CATGATAAGA	GAAGAACAATT	CCTTGGTAG	AGAAAACAAC <mark>T</mark> GG
pGAPZ-hPld1-b	AGGAGCCTCGA	GAGATGCCCAGT	TTGCCCCGTTC	ATCTGAAAA	CATGATAAGA	GAAGAACAATT	CCTTGGTAG	AGAAAACAAC <mark>T</mark> GG
Construct	AGGAGCCTCGA	GAGATGCCCAGT	TTGCCCCGTTC	ATCTGAAAA	CATGATAAGA	GAAGAACAATT	CCTTGGTAG	AGAAAACAAC <mark>T</mark> GG
	1000	1040	1950	1960	1070	1000	1000	1400

pGapz-hPld1-a pGAPZ-hPld1-b Construct	AAAATGCCCATGTAT AAAATGCCCATGTAT AAAATGCCCATGTAT	AGAAACTATC Agaaactatc Agaaactatc	ATGCCACAAC ATGCCACAAC ATGCCACAAC	AGAGTTTCT AGAGTTTCT AGAGTTTCT	GATATAAGCO GATATAAGCO GATATAAGCO	AGCTGTCTTT AGCTGTCTTT AGCTGTCTTT	CATCCATGAT CATCCATGAT CATCCATGAT	TTGGGACCAAAG TTGGGACCAAAG TTGGGACCAAAG
pGapz-hPld1-a pGAPZ-hPld1-b Construct	1440 AAGATCTGGAGGACA AAGATCTGGAGGACA AAGATCTGGAGGACA	1450 CAGAATACCA CAGAATACCA CAGAATACCA	1460 GGCTTGAATI GGCTTGAATI GGCTTGAATI	1470 GCTGTGGTCJ GCTGTGGTCJ GCTGTGGGTCJ	1480 AGGGAAGAGCC AGGGAAGAGCC AGGGAAGAGCC	1490 TGCTACAGAT TGCTACAGAT TGCTACAGAT	1500 GGT CAAAAAG GGT CAAAAAG GGT CAAAAAG	1510 ATGGTTAATAGT ATGGTTAATAGT ATGGTTAATAGT
pGapz-hPld1-a pGAPZ-hPld1-b Construct	1550 TGAAACCAGACAGCG4 TGAAACCAGACAGCG4 TGAAACCAGACAGCG4 TGAAACCAGACAGCG4	1560 GTGCCATTGC GTGCCATTGC GTGCCATTGC	1570 CTTCGTCCTG CTTCGTCCTG CTTCGTCCTG	1580 CTGGTAGACI CTGGTAGACI CTGGTAGACI	1590 AAGAATTCAA AAGAATTCAA AAGAATTCAA	1600 AATTAAGGTG AATTAAGGTG AATTAAGGTG	1610 GGGGAAGAAGG GGGAAGAAGG GGGAAGAAGG	1620 AGA CAGAAA CGA AGA CAGAAA CGA AGA CAGAAA CGA
pGapz-hPld1-a pGAPZ-hPld1-b Construct	1660 CTTTCAAGGACACTT CTTTCAAGGACACTT CTTTCAAGGACACTT CTTTCAAGGACACTT	1670 ••••• •••• • TTTTAAAAT • TTTTAAAAT • TTTTAAAAT	1680 GCAACAGCTA GCAACAGCTA GCAACAGCTA	1690 TAGACATGCI TAGACATGCI TAGACATGCI	1700 CGGTGGTGGG CGGTGGTGGG CGGTGGTGGG	1710 GAGGGGCTAT GAGGGGCTAT GAGGGGCCTAT	1720 AGAAGAATTC AGAAGAATTC AGAAGAATTC	1730 ATCCAGAAACAT ATCCAGAAACAT ATCCAGAAACAT
pGapz-hPld1-a pGAPZ-hPld1-b Construct	1770 TCGATTTGGGTCATA TCGATTTGGGTCATA TCGATTTGGGTCATA	1780 IGCTGCTATC IGCTGCTATC IGCTGCTATC	1790 CAAGAGAATG CAAGAGAATG CAAGAGAATG	1800 CTTTAGCTAA CTTTAGCTAA CTTTAGCTAA	1810 ATGGTATGTT ATGGTATGTT ATGGTATGTT	1820 AATGCCAAAG AATGCCAAAG AATGCCAAAG	1830 GATATTTTGA GATATTTTGA GATATTTTGA	1840 . AGATGTGGCAAA AGATGTGGCAAA AGATGTGGCAAA
pGapz-hPld1-a pGAPZ-hPld1-b Construct	1880 AGATTTTTATCACAG AGATTTTTATCACAG AGATTTTTATCACAG	1890 ACTGGTGGCT ACTGGTGGCT ACTGGTGGCT	1900 GAGTCCAGAA GAGTCCAGAA GAGTCCAGAA	1910 ATCTTCCTGA ATCTTCCTGA ATCTTCCTGA	1920 AACGCCCAGT AACGCCCAGT AACGCCCAGT	1930 GGTTGAGGGA GGTTGAGGGA GGTTGAGGGA	1940 AATCGTTGGA AATCGTTGGA AATCGTTGGA	1950 . .GGTTGGACTGCA .GGTTGGACTGCA .GGTTGGACTGCA
pGapz-hPld1-a pGAPZ-hPld1-b Construct	1990 GGAGTGAGGATCTTC/ GGAGTGAGGATCTTC/ GGAGTGAGGATCTTC/	2000 ATAATGCTCT. ATAATGCTCT. ATAATGCTCT.	2010 ACAAAGAGGT ACAAAGAGGT ACAAAGAGGT	2020 GGAACTCGCT GGAACTCGCT GGAACTCGCT	2030 CTTGGCATCA CTTGGCATCA CTTGGCATCA	2040 ATAGTGAATA ATAGTGAATA ATAGTGAATA	2050 CACCAAGAGG CACCAAGAGG CACCAAGAGG	2060 . ACTITGATGCGT ACTITGATGCGT ACTITGATGCGT
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2100 GAGACACCCGGATCA GAGACACCCGGATCA GAGACACCCGGATCA	2110 IGTGTCATCC IGTGTCATCC IGTGTCATCC	2120 ACCGTCTATI ACCGTCTATI ACCGTCTATI	2130 TGTGGGCTCJ TGTGGGCTCJ TGTGGGCTCJ	2140 ACCATGAGAAG ACCATGAGAAG	2150 CTTGTCATCA CTTGTCATCA CTTGTCATCA	2160 TTGACCAATC TTGACCAATC TTGACCAATC	2170 GGTGGCCTTTGT GGTGGCCCTTTGT GGTGGCCCTTTGT
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2210 GAAGGTGGGACGACAA GAAGGTGGGACGACAA GAAGGTGGGACGACAA	2220 ATGAGCACAG ATGAGCACAG ATGAGCACAG	2230 ACTCACAGAC ACTCACAGAC ACTCACAGAC	2240 GTGGGCAGT(GTGGGCAGT(GTGGGCAGT(GTGGGCAGT(2250 TGAAGCGGGT TGAAGCGGGT TGAAGCGGGT	2260 CACTTCAGGA CACTTCAGGA CACTTCAGGA	2270 .ccgtctctgg .ccgtctctgg .ccgtctctgg	2280 GTTCCCTCCCAC GTTCCCTCCCAC
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2320 TCCTTAAGACTCAAA TCCTTAAGACTCAAA TCCTTAAGACTCAAA TCCTTAAGACTCAAA	2330 GA TAAAAA TG GA TAAAAA TG GA TAAAAA TG	2340 AGCCTGTTCA AGCCTGTTCA AGCCTGTTCA	2350 AAACCTACCC AAACCTACCC AAACCTACCC	2360 ATCCAGAAGA ATCCAGAAGA ATCCAGAAGA	2370 GTATTGATGA GTATTGATGA GTATTGATGA	2380 ATGTGGATTCA ATGTGGATTCA ATGTGGATTCA	2390 . AAACTGAAAGGA AAACTGAAAGGA AAACTGAAAGGA
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2430 CAAATTTAGTCTCTA CAAATTTAGTCTCTA CAAATTTAGTCTCTA CAAATTTAGTCTCTA	2440 CAAGCAGCTC CAAGCAGCTC CAAGCAGCTC	2450 CACAGGCACC CACAGGCACC CACAGGCACC	2460 ACCTGCACGA ACCTGCACGA ACCTGCACGA	2470 ACGCAGATAGO ACGCAGATAGO ACGCAGATAGO	2480 ATCAGCAGCA ATCAGCAGCA ATCAGCAGCA ATCAGCAGCA	2490 TTGACAGCAC TTGACAGCAC TTGACAGCAC	2500 CTCCAGTTATTT CTCCA CTCCA
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2540 ATTTAATCCATGGTT	2550 FAAAACCCCA	2560 CTTCAAACTC	2570 	2580 CCAGTGAGTC	2590 TGAGCAAGGA	2600 ACTCACTAGAC	2610 CTCATGCTGATA ATA ATA
	2650	2660	2670	2680	2690	2700	2710	2720

pGapz-hPld1-a pGAPZ-hPld1-b Construct	A CAGGTGTGGGGAGAGCTGCATGGGGAAACCAGATTCTGGCATGGAAAGGACTACTGCAATTTCGTCTTCAAAGACTGGGTTCAACTT A CAGGTGTGGGGAGAGCTGCATGGGGAAACCAGATTCTGGCATGGAAAGGACTACTGCAATTTCGTCTTCAAAGACTGGGGTTCAACTT A CAGGTGTGGGGAGAGCTGCATGGGGAAACCAGATTCTGGCATGGAAAGGACTGCAATTTCGTCTTCAAAGACTGGGTTCAACTT							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2760 2770 2780 2790 2800 2810 2820 2830 TGACAGGTACTCCACGCCCGGATGCCCTGGCATGACATTGCCTCTGCAGTCCACGGGAAGGCGGCTCGTGATGTGGCACGTCACT TGACAGGTACTCCACGCCCCGGATGCCCTGGCATGACATTGCCTCTGCAGTCCACGGGAAGGCGGCTCGTGATGTGGCACGTCACT TGACAGGTACTCCACGCCCCGGATGCCCTGGCATGACATTGCCTCTGCAGTCCACGGGAAGGCGGCTCGTGATGTGGCACGTCACT							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2870 2880 2890 2900 2910 2920 2930 2940 							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	2980 2990 3000 3010 3020 3030 3040 3050 							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3090 3100 3110 3120 3130 3140 3150 3160 TATCGAAAACCAGTTTTTCATAAGCTGTGCTGATGACAAAGTTGTGTTCAACAAGATAGGCGATGCCATTGCCCAGAGGATCCTGA TATCGAAAACCAGTTTTTCATAAGCTGTGCTGATGACAAAGTTGTGTTCAACAAGATAGGCGATGCCATTGCCCAGAGGATCCTGA TATCGAAAACCAGTTTTTCATAAGCTGTGCTGATGACAAAGTTGTGTTCAACAAGATAGGCGATGCCATTGCCCAGAGGATCCTGA							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3200 3210 3220 3230 3240 3250 3260 3270 							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3310 3320 3330 3340 3350 3360 3370 3380 AGAGGAGAAAATTCCATCCTTGGACAGTTAAAAGCAGAGCTTGGTAATCAGTGGATAAATTACATATCATTCTGTGGGTCTTAGAAC AGAGGAGAAAATTCCATCCTTGGACAGTTAAAAGCAGAGCTTGGTAATCAGTGGATAAATTACATATCATTCTGTGGGTCTTAGAAC AGAGGAGAAAATTCCATCCTTGGACAGTTAAAAGCAGAGCTTGGTAATCAGTGGATAAATTACATATCATTCTGTGGGTCTTAGAAC							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3420 3430 3440 3450 3460 3470 3480 3490 AGTAACTGAGCTTATCTATGTCCACAGCAAGTTGTTAATTGCTGATGATAACACTGTTATTATTGGCTCTGCCAACATAAATGACC AGTAACTGAGCTTATCTATGTCCACAGCAAGTTGTTAATTGCTGATGATAACACTGTTATTATTGGCTCTGCCAACATAAATGACC AGTAACTGAGCTTATCTATGTCCACAGCAAGTTGTTAATTGCTGATGATAACACTGTTATTATTGGCTCTGCCAACATAAATGACC							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3530 3540 3550 3560 3570 3580 3590 3600 GTGAAATGGCTGTCATTGTGCAAGATACAGAGACTGTTCCTTCAGTAATGGATGG							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3640 3650 3660 3670 3680 3690 3700 3710 GTTGTCCTTGGCTATCTTGATGACCCAAGTGAGGACATTCAGGATCCAGTGAGTG							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3750 3760 3770 3780 3790 3800 3810 3820 TGA CAA GGTTTTCCGGTGCCTTCCCAA TGA TGA GA GTA CA CAA TTTA A TTCA GCTGA GA GA CTTTA TA A A CAA GCCC GTA TTA GCTA TGA CAA GGTTTTCCGGTGCCTTCCCAA TGA TGA A GTA CA CAA A TTTA A TTCA GCTGA GA GA CTTTA TA A A CAA GCCC GTA TTA GCTA TGA CAA GGTTTTCC GGTGCCTTCCCAA TGA TGA A GTA CA CA CA A TTTA A TTCA GCTGA GA GA CTTTA TA A A CAA GCCC GTA TTA GCTA TGA CAA GGTTTTCC GGTGCCTTCCCAA TGA TGA A GTA CA CA CA A TTTA A TTCA GCTGA GA GA CTTTA TA A A CAA GCCC GTA TTA GCTA							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	3860 3870 3880 3890 3900 3910 3920 3930 AGGAACTGAAGAAGATCCGTGGATTTTTGGTGCAATTCCCCTTTTATTTCTTGTCTGAAGAAAGCCTACTGCCTTCTGTTGGGACC AGGAACTGAAGAAGATCCGTGGATTTTTGGTGCAATTCCCCTTTTATTTCTTGTCTGAAGAAAGCCTACTGCCTTCTGTTGGGACC AGGAACTGAAGAAGATCCGTGGATTTTTGGTGCAATTCCCCTTTTATTTCTTGTCTGAAGAAAGCCTACTGCCTTCTGTTGGGACC							
pGapz-hPld1-a	3970 3980 3990 4000 4010 4020 4030 4040 							

pGAPZ-hPld1-b Construct	GTTTGGACTGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCAT GTTTGGACTGCGGCCGCCAGCTTTCTAGAACAAAAACTCATCTCAGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCAT CATCATCATCATCATCATCATGAAGAGGATCTGAATAGCGCCGTCGACCATCATCATCATCATCAT							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	4080 4090 4100 4110 4120 4130 4140 4150 GACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCT GACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCT GACTGTTCCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTAGATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCT							
pGapz-hPldl-a pGAPZ-hPldl-b Construct	4190 4200 4210 4220 4230 4240 4250 4260 							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	4300 4310 4320 4330 4340 4350 4360 4370 GGTAGGGGGTTTGGGAAAATCATTCGAGTTGATGTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACC GGTAGGGGTTTGGGAAAATCATTCGAGTTGATGTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACC GGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGACC GGTAGGGGTTTGGGAAAATCATTCGAGTTTGATGTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGAGCC							
pGapz-hPldl-a pGAPZ-hPldl-b Construct	4410 4420 4430 4440 4450 4460 4470 4480 CACCATAGCTTCAAAATGTTTCTACTCCTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC CACCATAGCTTCAAAATGTTTCTACTCCTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC CACCATAGCTTCAAAATGTTTCTACTCCTTTTTACTCTTCCAGATTTTCTCGGACTCCGCGCATCGCCGTACCACTTCAAAACACC							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	4520 4530 4540 4550 4560 4570 4580 4590 CCTCTTTCTTCCTCAGGGGTGTCGTTAATTACCCGGTACTAAAGGTTTGGAAAAGAGAAAAAGGGCCCCCGGTTTCTTTTTCTTCGT CCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCGGTACTAAAGGTTTGGAAAAGAAAAAGGGCCGCCCCGGTTTCTTTTTCTTCGT CCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCCGTACTAAAGGTTTGGAAAAGAAAAAGGACCGCCTCGTTTCTTTTCTTCGT CCTCTTTCTTCCTCTAGGGTGTCGTTAATTACCCCGTACTAAAGGTTTGGAAAAGAAAAAGGACCGCCTCGTTTCTTTTCTTCGT							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	4630 4640 4650 4660 4670 4680 4690 4700 							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	4740 4750 4760 4770 4780 4790 4800 4810 							
pGapz-hPldl-a pGAPZ-hPldl-b Construct	4850 4860 4870 4880 4890 4900 4910 4920 ATAGTATAATACGACAAGGTGAGGAACTAAACCATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCG							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	4960 4970 4980 4990 5000 5010 5020 5030 GCTCGGGGTTCTCCCGGGGACTTCGTGGAGGACGACTTCGCCGGGTGTGGTCCGGGACGACGTGGACCCTGTTCATCAGCGCGGGTCCAGGA GCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGA GCTCGGGTTCTCCCGGGACTTCGTGGAGGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGA							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5070 5080 5090 5100 5110 5120 5130 5140 TGGCCTGGGTGTGGGGTGCGCGGGCCTGGACGACGTGTACGCCGAGTGGTCGGGAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGC Image: Content of the content of th							
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5180 5190 5210 5220 5230 5240 5250 CAGCCGTGGGGGGGGGGGGGGGGGGGTCGCCCGGCGGGCG							
pGapz-hPld1-a pGAPZ-hPld1-b	5290 5300 5310 5320 5330 5340 5350 5360 TCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGCCTTACATTCACGCCCCCCCC							

Construct	TCGGAGATCCGTCCCCCTTTTCCTTTGTCGATATCATGTAATTAGTTATGTCACGCTTACATTCACGCCCTCCCCCCACATCCGCTC								
	5400	5410	5420	5430	5440	5450	5460	5470	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	CAACCTGAAGTCTAG CAACCTGAAGTCTAG CAACCTGAAGTCTAG	GTCCCTATTI GTCCCTATTI GTCCCTATTI	ATTTTTTTAT ATTTTTTTAT ATTTTTTTAT	AGTTATGTT/ AGTTATGTT/ AGTTATGTT/	AGTATTAAGA/ AGTATTAAGA/ AGTATTAAGA/	ACGTTATTTAT ACGTTATTTAT ACGTTATTTAT	ATTTCAAAT ATTTCAAAT ATTTCAAAT	TTTCTTTTTTT TTTCTTTTTTTT TTTCTTTTTTTT	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5510 TAACATTATACTGAA TAACATTATACTGAA TAACATTATACTGAA	5520 AACCTTGCT1 AACCTTGCT1 AACCTTGCT1	5530 Gagaaggtt1 Gagaaggtt1 Gagaaggtt1	5540 TGGGACGCT TGGGACGCT TGGGACGCT	5550 CGAAGGCTTT CGAAGGCTTT CGAAGGCTTT	5560 AATTTGCAAGO AATTTGCAAGO AATTTGCAAGO	5570 TGGAGACCAI TGGAGACCAI	5580 ACATGTGAGCAAA ACATGTGAGCAAA ACATGTGAGCAAA	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5620 GTAAAAAGGCCGCGT GTAAAAAGGCCGCGT GTAAAAAGGCCGCGT	5630 TGCTGGCGT1 TGCTGGCGT1 TGCTGGCGT1	5640 TTTTCCATAGG TTTTCCATAGG TTTTCCATAGG	5650 CTCCGCCCCC CTCCGCCCCC CTCCGCCCCC	5660 CCTGACGAGC/ CCTGACGAGC/ CCTGACGAGC/	5670 ••••••••••••••••••••••••••••••••••••	5680 CGACGCTCAI CGACGCTCAI	5690 AGTCAGAGGTGGC AGTCAGAGGTGGC AGTCAGAGGTGGC	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5730 TACCAGGCGTTTCCC TACCAGGCGTTTCCC TACCAGGCGTTTCCC	5740 CCTGGAAGCT CCTGGAAGCT CCTGGAAGCT	5750 CCCTCGTGCG CCCTCGTGCG CCCTCGTGCG	5760 CTCTCCTGT CTCTCCTGT CTCTCCTGT	5770 . CCGACCCTG(CCGACCCTG(CCGACCCTG(5780 CCGCTTACCGG CCGCTTACCGG CCGCTTACCGG	5790 ATACCTGTC ATACCTGTC ATACCTGTC	5800 CGCCTTTCTCCCT CGCCTTTCTCCCT CGCCTTTCTCCCT	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5840 ATGCTCACGCTGTAG ATGCTCACGCTGTAG ATGCTCACGCTGTAG	5850 GTATCTCAGT GTATCTCAGT GTATCTCAGT	5860 TTCGGTGTAGG TCCGGTGTAGG TCCGGTGTAGG	5870 TCGTTCGCT TCGTTCGCT TCGTTCGCT	5880 . 	5890 CTGTGTGCACG CTGTGTGCACG CTGTGTGCACG	5900 AACCCCCCG AACCCCCCG AACCCCCCG	5910 TCAGCCCGACCG TCAGCCCGACCG	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	5950 GTCTTGAGTCCAACC GTCTTGAGTCCAACC GTCTTGAGTCCAACC	5960 CGGTAAGACA CGGTAAGACA CGGTAAGACA	5970 CGACTTATCG CGACTTATCG	5980 CCACTGGCA CCACTGGCA CCACTGGCA	5990 . GCAGCCACTG GCAGCCACTG GCAGCCACTG	6000 GTAACAGGATI GTAACAGGATI GTAACAGGATI	6010 AGCAGAGCGA AGCAGAGCGA AGCAGAGCGA	6020 AGGTATGTAGGCG AGGTATGTAGGCG AGGTATGTAGGCG	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	6060 TGGCCTAACTACGGC TGGCCTAACTACGGC TGGCCTAACTACGGC	6070 TACACTAGAA TACACTAGAA TACACTAGAA	6080 AGGA CAGTATI AGGA CAGTATI	6090 TGGTATCTG TGGTATCTG TGGTATCTG	6100 . CGCTCTGCTG/ CGCTCTGCTG/ CGCTCTGCTG/	6110 MAGCCAGTTAC MAGCCAGTTAC MAGCCAGTTAC	6120 CTTCGGAAAA CTTCGGAAAA CTTCGGAAAA	6130 AGAGTTGGTAGC AGAGTTGGTAGC AGAGTTGGTAGC	
pGapz-hPld1-a pGAPZ-hPld1-b Construct	6170 CGCTGGTAGCGGTGG CGCTGGTAGCGGTGG CGCTGGTAGCGGTGG	6180 TTTTTTTGT TTTTTTTGT TTTTTTTGTT TTTTTTTGTT	6190 TGCAAGCAGC TGCAAGCAGC TGCAAGCAGC	6200 AGATTACGCO AGATTACGCO AGATTACGCO	6210 GCAGAAAAAA GCAGAAAAAAA GCAGAAAAAAA	6220 IIIIII AGGATCTCAAG AGGATCTCAAG	6230 AAGATCCTT AAGATCCTT AAGATCCTT	6240 FGATCTTTTCTAC FGATCTTTTCTAC	
pGapz-hPld1-a pGAPZ-hPld1-b	6280 AAAACTCACGTTAAG AAAACTCACGTTAAG	6290 GGATTTTGG GGATTTTGG	6300 CATGCATGAG	ATC ATC					

Construct AAAACTCACGTTAAGGGATTTTGGTCATGCATGAGATC

List of publications

[1] Chang L, **Do LD**, Mébarek S, Popowycz F, Pellet-Rostaing S, Lemaire M, Buchet R, (2011) Synthesis and evaluation of thiophenyl derivatives as inhibitors of alkaline phosphatase. Bioorg Med Chem Lett. 21: 2297-2301.

[2] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S, (2012) Direct determination of phospholipase D activity by infrared spectroscopy. Anal Biochem. 430: 32-38.

[3] Debray J, Chang L, Marqués S, Pellet-Rostaing S, **Do LD**, Mebarek S, Buchet R, Magne D, Lemaire M, Popowycz F (2013) Inhibitors of TNAP to prevent vascular calcification associated withchronic kidney disease. J Med Chem, doi: 10.1016/j.bmc.2013.09.053

[4] Mebarek S, Abousalham A, Magne D, **Do LD**, Bandorowicz-Pikula J, Pikula S, Buchet R (2013) Phospholipases of mineralization competent cells and matrix vesicles: roles in physiological and pathological mineralizations. Int J Mol Sci. 14: 5036-5129.

List of conferences

[1] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S, (2014) Interplay between Annexin A6 and Phospholipase D1 during the process of exocytosis in PC12 cells. Poster presented at "Membrane, Morphology and Function" meeting, 5-8 Mai, Abruzzo, Italie.

[2] Ren Z, **Do LD**, Bechkoff G, Mebarek S, Keloglu N, Ahamada S, Meena S, Magne D, Pikula S, Wu Y, Buchet R, (2014) Direct determination of phosphatatase activity from physiological substrates in cells. Poster presented at 41st European Calcified Tissue Society Congress, 17-20 Mai, Prague, Czech Republic.

[3] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S, (2013) Annexin A6 and phospholipase D1 in catecholamine exocytosis in PC12 cells: a plausible relationship? Oral communication at The First Nencki Conference, 16-18 Octobre, Warsaw, Poland.

[4] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S (2013) Direct determination of phospholipase D activity by infrared spectroscopy. Poster presented at 18^e Journée de l'EDISS, 03 Octobre, Lyon, France.

[5] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S, (2013) Direct determination of phospholipase D activity by infrared spectroscopy. Poster presented at 48th Meeting of Polish Society of Biochemistry, 2–5 Septembre, Torun, Poland.

[6] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S, (2012) Direct determination of phospholipase D activity by infrared spectroscopy. Poster presented at 10th Euro Fed Lipid Congress, 23–26 Septembre, Cracow, Poland. Book of abstract: 203.

[7] **Do LD**, Bechkoff G, Kelogu N, Mebarek S, Pikula S, Magne D, Buchet R, (2012) Hydrolyse du pyrophosphate par la phosphatase alcaline déterminée par spectroscopie infrarouge. Poster presented at Journées Françaises de Biologie des Tissus Minéralisés. 29-31 Mai, Bordeaux, France.

[8] **Do LD**, Buchet R, Pikula S, Abousalham K, Mebarek S, (2012) Direct determination of phospholipase D activity by infrared spectroscopy. Poster presented at 12th Journée Scientifique de l'IMBL, 14 Mai, Lyon, France.