The Crosstalk between the ERK and the cAMP Signalling Pathways in PC12 Cells

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DECLARATION

I declare that the work described in this thesis has been carried out by me unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

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ABSTRACT

The extracellular-regulated kinase (ERK) signalling pathway is involved in the control of different biological processes such as survival, proliferation and differentiation. In PC12 cells, the ERK signalling pathway integrates different external stimuli: epidermal growth factor (EGF) stimulates the ERK pathway transiently and induces cell proliferation, whereas nerve growth factor (NGF) induces sustained activation of ERK and promotes cell differentiation into sympathetic-like neurons. The second messenger 3',5'-cyclic adenosine monophosphate (cAMP) controls a plethora of cellular events from metabolic to cellular signalling pathways. Over the years, lines of evidence have shown that cAMP is also involved in the regulation of cell growth and cell differentiation, suggesting a possible crosstalk between these two pathways.

Selective phosphodiesterase (PDE) inhibitors were used to block the degradation of cAMP. These inhibitors increased the level of cAMP, but had different effects on the activation of ERK. Upon both NGF and EGF stimulations, cilostamide had the strongest effect and doubled the intensity of the phosphorylation of ERK, identifying PDE3 to control the level of cAMP relevant for the regulation of the ERK pathway. The treatment with cilostamide enhanced the differentiation of PC12 cells and the combination of both cilostamide and rolipram (a PDE4 inhibitor) turned the proliferative effect of EGF into a differentiation effect.

The route for cAMP in the regulation of the ERK pathway was decomposed by using the cAMP analogues 8-pCPT-2'-*O*-Me-cAMP and 6-Bnz-cAMP. They specifically activate the Exchange protein activated by cyclic AMP (Epac) and the cAMP regulated protein kinase (PKA) respectively, which were hypothesised to be the effectors of cAMP in the regulation of ERK. The Epac agonist mimicked the effects of cilostamide on the activation of ERK, but failed to enhance cell differentiation. The PKA agonist reduced the phosphorylation ERK upon EGF. It was suggested that the activation of ERK in response to cAMP was mainly mediated through Epac rather than PKA, and that the activation of both PKA and Epac are required to induce cellular differentiation.

To elucidate the differential regulation of the activation of ERK upon NGF and EGF stimulation and in response to cAMP, the activity of Ras and Rap1 were measured by affinity pulldown assays. Upon EGF the signal was transduced through Ras only, whereas upon NGF the signal was mediated through both Ras and Rap1. cAMP sensitised Rap1 that became activated upon EGF stimulation indicating that cAMP can switch on the Rap1/B-Raf pathway. This correlated with the increase in the

phosphorylation of ERK in response to high levels of cAMP upon EGF stimulation. Then, the role of Raf-1 and C3G, a guanine exchange factor for Rap1, were investigated using small interfering RNA. The depletion of Raf-1 showed that Raf-1 is not essential for transducing the mitogen signal upon NGF stimulation and suggested that Ras mediates the signal through B-Raf upon EGF stimulation to compensate for the loss of Raf-1. The depletion of C3G also confirmed that the activation of ERK in response to cAMP is mediated through the Rap1/B-Raf pathway.

Finally, the interaction between Raf-1 and AKAP79 was demonstrated for the first time suggesting the existence of a complex between Raf-1, AKAP and PKA and therefore a possible molecular mechanism for the inhibition of Raf-1 by cAMP through PKA.

The data presented in this thesis demonstrates that cAMP participates to finely tune the regulation of the ERK signalling pathway and can be use as a tool to elucidate the network comprising the ERK cascade.

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LIST OF ABBREVATIONS

AC	Adenylyl Cyclase
ADBI	Assay Dilution Buffer I
AKAP	A Kinase Anchoring Protein
AMP	Adenosine Monophosphate
AP	Alkaline Phosphatase
ATP	Adenosine Trisphosphate
BDNF	Brai-Derived Neurotrophic Factor
C3G	Crk SH3 domain-binding guanine nucleotide releasing factor
Ca^{2+}/CaM	calcium/calmodulin
cAMP	cyclic 3'5' Adenosine Monophosphate
CBD	cAMP Binding Domain
CDC37	Cell Division Cycle 37
cGMP	cyclic Guanosine Monophosphate
CHK	Crk Homologous Kinase
CNG	Cyclic Nucleotide-Gated ion channel
CR	Conserved region
CRD	Cysteine Rich Domain
CREB	cAMP Response Element Binding Protein
DEAE	Diethyl aminoethyl
DEP	Dishevelled, Egl-10, Pleckstrin
dH_2O	Deionised water
DMEM	Dulbecco's modification of Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Diaminoethanetetra-acetic acid
EGF	Epidermal Growth Factor
EGFR	EGF Receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
Epac	Exchange protein directly activated by cAMP
ERK	Extracellular signal-Regulated Kinase/MAPK
FBS	Foetal Bovine Serum
FRS2	Fibroblast Growth Factor Receptor Substrate 2
GAP	GTPase Activating Protein
GDP	Guanosine DiPhosphate
GEF	Guanine nucleotide Exchange Factor
Giα	Inhibitory G-protein α-subunit
GPCR	G-Protein Coupled Receptor
G-protein	Guanine nucleotide binding regulatory protein
GRB2	Growth Factor Receptor-Bound protein2
GST	Glutathione S-Transferase
Gsα	Stimulatory G-protein α-subunit
GTP	Guanosine TriPhosphate
HEK	Human Embryo Kidney
H-Ras	Harvey-Ras
HSP70	Heat Shock Protein 70

HSP90	Heat Shock Protein 90
IBMX	Isobutylmethylxanthine
IC_{50}	Concentration of inhibitor required to inhibit half the specific activity
Ig	Immunoglobulin-like domain
IMP	Impedes Mitogenic signal Propagation
IP	Immunoprecipitate
IPTG	Isopropyl-ß-D-thiogalactopyranoside
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton
K-Ras	Kirsten-Ras
KSR	Kinase Suppressor of Ras
LB	L-Broth
LRR	Leucine-Rich motif
MAPK	Mitogen Activated Protein Kinase/ERK
MAPKK	MAPK Kinase/MEK
MAPKKK	MAPK Kinase Kinase/Raf
MEF	Mouse Embryonic Fibroblast
MEK	MAPK Kinase/ERK Kinase
MEM	Minimum Essential Medium
NGF	Nerve Growth Factor
NHR	N-terminal Hydrophobic membrane association Region
NT	Neurotrophin
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PAGE	Polyacrylamide Gel Electrophoresis
PAK	p21 Activated Kinase
PBS	Phosphate Buffered Saline
PDE	phosphodiesterase
PDGF	Platelet Derived Growth Factor
PI3-Kinase	Phosphatidylinositol 3'-kinase
PKA	Protein Kinase A
PKB	Protein Kinase B/Akt
PKC	Protein Kinase C
PKI	Protein Kinase Inhibitor
PLC-γ	Phospholipase C-γ
PP2A	Protein Phosphatase 2A
PTB	Phosphotyrosine-Binding domain
RA	Ras Association Domain
RBD	Ras Binding Domain
REM	Ras Exchange Motif
RKIP	Raf Kinase Inhibitor Protein
RNA	Ribonucleic Acid
RTK	Receptor Tyrosine Kinase
SDS	Sodium Dodecyl Sulphate
SH2 domain	Src Homology 2 domain
SH3 domain	Src Homology 3 domain
siRNA	Small Interfering RNA
SoS	Son of Sevenless
TBS	Tris Buffered Saline
TBST	TBS plus Tween 20

Tris	Tris (hydroxymethyl) aminomethane
Trk	Tropomyosin receptor kinase, or tropomyosin-related kinase receptor kinase
Tween 20	Polyoxyethylene sorbitan monolaurate
UCR	Upstream Conserved Region
WT	Wild Type

Units

g	gram
h	hour
l	litre
M	molar
mg	milligram
min	minute
ml	millilitre
mM	millimolar (millimoles per litre)
nM	nanomolar
pM	picomolar
rpm	revolutions per minute
sec	second
V	Volt
µCi	microcurie
µg	microgram
µl	microlitre
µM	micromolar
°C	degrees centigrade

Single letter amino acid code

Alanine	Ala (A)	Leucine	Leu (L)
Arginine	Arg (R)	Lysine	Lys (K)
Asparagine	Asn (N)	Methionine	Met (M)
Aspartic Acid	Asp (D)	Phenylalanine	Phe (F)
Cysteine	Cys (C)	Proline	Pro (P)
Glutamic Acid	Glu (E)	Serine	Ser (S)
Glutamine	Gln (Q)	Threonine	Thr (T)
Glycine	Gly (G)	Tryptophan	Trp (W)
Histidine	His (H)	Tyrosine	Tyr (Y)
Isoleucine	Ile (I)	Valine	Val (V)

CHAPTER 1

GENERAL INTRODUCTION

1.1 MAPK SIGNALLING PATHWAY

1.1.1 Mitogen-activated protein kinase (MAPK) signalling pathways

Mitogen-activated protein kinase signalling pathways are responsible for the transduction of a wide range of extracellular signals from the plasma membrane to the nucleus to allow the appropriate cellular response to occur. They are involved in the regulation of gene expression as well as cytoplasmic activities. The MAPK signalling pathways are evolutionary conserved in all eukaryotes and the best characterised pathways can be classified into at least three distinguished MAPK subfamilies (Fig. 1.1): the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38-MAPKs (Davis, 2000; Schaeffer and Weber, 1999; Tibbles and Woodgett, 1999; Wada and Penninger, 2004). Each pathway responds to different external stimuli and exerts distinct biological functions such as cell proliferation, cell differentiation, cell cycle regulation or apoptosis. However, there are also overlapping activators and effectors, such as the transcription factor Elk, which is a substrate for both ERK and JNK, or Mnk2 and MAPKAP2/3 which are phosphorylated by both ERK and p38 (Pearson et al., 2001). The ERK signalling pathway is activated in response to growth factor stimuli and is known to be involved in cell proliferation, differentiation and apoptosis (Houslay and Kolch, 2000). JNKs and p38-MAPKs signalling pathways are stress responsive and seem to be involved in the control of cell cycle checkpoints, differentiation and apoptosis (Davis, 2000; Schaeffer and Weber, 1999; Tibbles and Woodgett, 1999).

The MAPK signalling pathways share common mechanisms and a conserved structure consisting of a module of three kinases. Basically, the three kinases activate each other in a cascade of phosphorylations that relays extracellular signals. The first kinase or MAPKK kinase (MAPKKK) is activated by a small G-protein that itself is activated by cell surface receptors in response to an extracellular stimulus. The MAPKKK phosphorylates and activates the MAPK kinase (MAPKK), which in turn phosphorylates and activates the MAP kinase (MAPK). The role of the small G-protein is to connect the MAPK module to the cell surface receptor. It does so by, when activated, physically binding the MAPKKK and recruiting it to the cell membrane where the activation of the MAPKKK takes place. The MAPKKKs integrate the input

signals and exhibit complex regulation. The MAPKKs, however, are very specific for their substrates and phosphorylate only a restricted subset of MAPKs. The MAPKs have multiple substrates located both in the nucleus and in the cytosol. MAPKs constitute the effector ends of the kinase cascade.

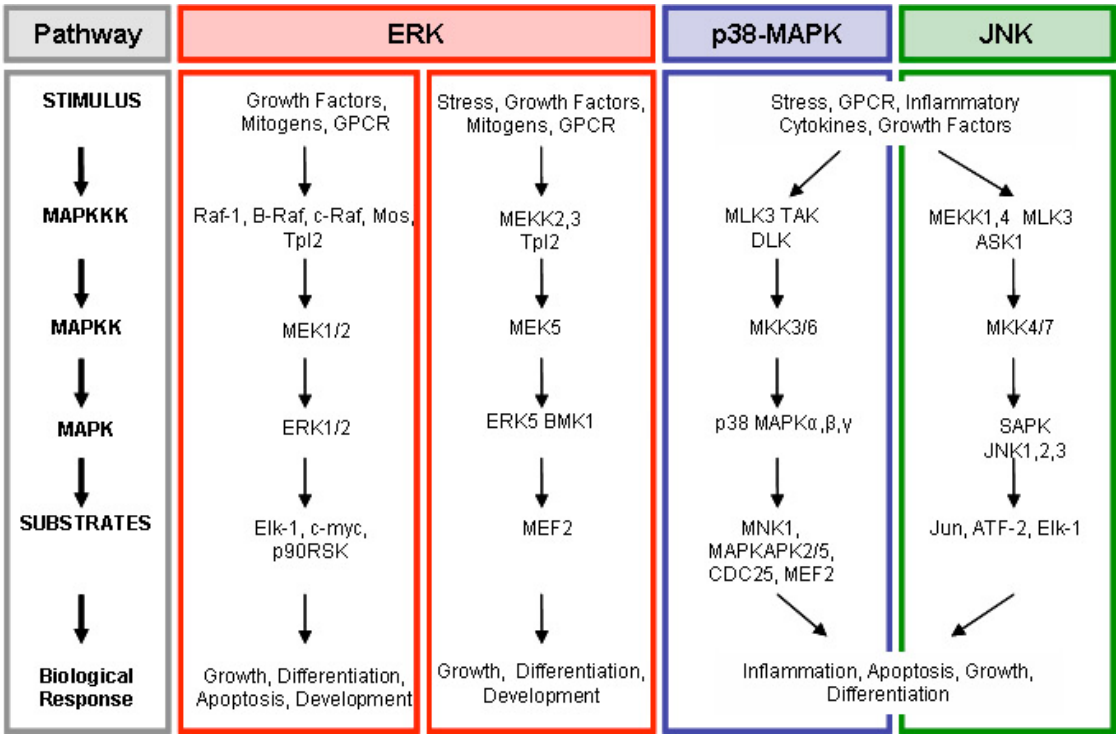


Figure 1. 1: Classification of the MAPK signalling pathways. The MAPK signalling pathways can be classified into three distinguished subfamilies: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38-MAPKs.

1.1.2 Extra-cellular-signal-regulated kinase pathway

The Extra-cellular-signal-regulated kinase (ERK) pathway was the first signalling pathway to be entirely mapped from the cell membrane to the nucleus. The MAP kinase module consists of Raf protein kinase as MAPKKK, MEK protein kinase as MAPKK and ERK protein kinase as MAPK (Fig.1.2). The ERK cascade is initiated by the small G-protein Ras that is activated in response to mitogen stimulations of receptor tyrosine kinases (RTKs) at the cell membrane. Upon activation Ras recruits Raf from the cytosol to the cell membrane where the activation of Raf takes place. At the plasma membrane, Raf is activated by phosphorylation. In its activated state, Raf phosphorylates and activates the dual specificity kinase MEK, which in turn phosphorylates and activates

ERK (Chong and Guan, 2003; Kolch, 2000). Phosphorylated ERK can enter the nucleus and can affect directly gene expression by phosphorylating transcription factors such as Elk1, Ets-2 or c-myc. ERK can also phosphorylate a wide range of cytoplasmic substrates. Ras and Raf are oncogenes, and the ERK pathway is hyperactivated in 30% of all cancers.

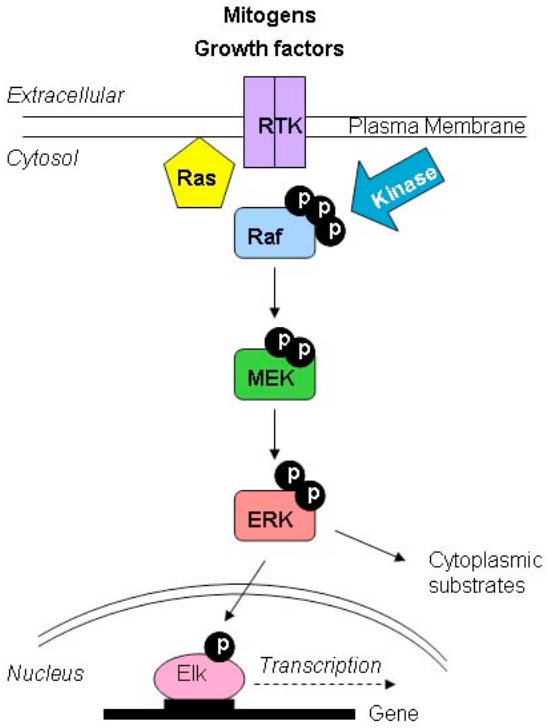


Figure 1. 2: The ERK Signalling pathway. Upon mitogen stimulation of the receptor tyrosine kinase (RTK), Raf is recruited by Ras to the plasma membrane where it is phosphorylated. Raf can phosphorylate and activate MEK, which in turn phosphorylates and activates ERK. Phosphorylated ERK can enter into the nucleus and activate transcription factors or activates cytoplasmic substrates.

1.1.3 Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are members of the large family of protein tyrosine kinases. The tyrosine kinases are enzymes that catalyze the transfer of phosphoryl group from ATP to tyrosine residues of protein substrates (Hubbard and Miller, 2007). 90 protein tyrosine kinases have been identified and 58 of them are RTKs (Robinson et al., 2000). The RTKs have in common a single transmembrane domain, a single cytoplasmic tyrosine kinase domain and are differentiated by their extracellular domain (Huang and Reichardt, 2003). Basically, the RTKs are activated by dimerisation initiated by ligand binding, which induces conformational changes of the receptor resulting in the auto-phosphorylation of tyrosine residues in the kinase activation loop

and other areas of the kinase domain. These phosphotyrosine residues serve as docking sites for adaptor and scaffolding proteins such as Grb2 and Crk through their Src homology-2 (SH2) or phosphotyrosine-binding (PTB) domains (Hubbard and Miller, 2007). These adaptor proteins recruit downstream signalling proteins such as the guanine nucleotide exchange factors SOS and C3G that activate respectively the small G-proteins Ras and Rap1 leading to the activation of downstream signalling pathways.

1.1.3.1 EGF Receptor

The epidermal growth factor receptor (EGFR or ErbB1) and its three homologues ErbB2, ErbB3 and ErbB4 constitute one of the 20 subfamilies of RTKs (Robinson et al., 2000). These receptors are involved in cell division, adhesion and proliferation. Overexpression or mutations of these receptors contribute to several cancers including breast, lung, colorectal and pancreatic cancers (Yarden and Sliwkowski, 2001). The overall structure of the EGFR consists of an extracellular domain, a single transmembrane domain, a juxtamembrane domain (JM) and a carboxy-terminal domain (Fig.1.3.a). The extracellular domain consists of two ligand binding domain (L1 and L2), and two cysteine rich domains (CR1 and CR2). The carboxy-terminal domain contains the kinase domain flanked by tyrosine autophosphorylation sites (Burgess et al., 2003; Jorissen et al., 2003). When EGF binds to the ligand binding domains of EGFR, receptor dimerisation is induced, which is required for the activation of the kinase domain (Burgess et al., 2003; Moriki et al., 2001). After dimerisation, the carboxy-terminus becomes rapidly phosphorylated through autophosphorylation by the tyrosine kinase domain. The phosphorylations of the tyrosine residues induce the recruitment of the Grb2 adaptor protein through its SH2 domain leading to the activation of the MAPK pathway (Nioche et al., 2002).

1.1.3.2 NGF Receptor: TrkA

Another RTK subfamily contains the Trk (tropomyosin-related kinase) receptor tyrosine kinases TrkA, TrkB and TrkC, also termed tropomyosin receptor kinases. These receptors were first identified as a colon-derived oncogenes in which tropomyocin was fused to a tyrosine kinase domain (Jing et al., 1992). These receptors can be activated by one or more of four neurotrophins, which are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT3 and NT4) (Bibel and Barde, 2000; Huang and Reichardt, 2003). These receptors are involved in cell survival,

proliferation and neuronal differentiation. These three receptors share a highly conserved tyrosine kinase domain, a less conserved extracellular domain and a single transmembrane domain (Fig.1.3.b). The extracellular domain consists of two cysteine clusters (C1, C2) separated by the ligand binding domain that contains multiple repeats of leucine-rich motifs (LRR1-3) and two immunoglobulin-like domains (Ig1, Ig2) (Schneider and Schweiger, 1991). The binding of NGF induces the dimerisation of the receptors (Jing et al., 1992) that results in the phosphorylation of tyrosine residues in the activation loop of the kinase domain. This induces autophosphorylation of tyrosines located outside of the loop. There are two specific phosphorylated tyrosine residues identified to be docking sites for adaptor molecules: Y490 and Y785 in TrkA (Friedman and Greene, 1999). Y785 resides in the C-terminus of the receptor and was identified to bind and phosphorylate phospholipase C-γ (PLC-γ) via its SH2 domain. Y785 is also a site for association of TrkA with Csk homologous kinase (CHK) that was identified to be involved in NGF-promoted out growth in PC12 cells, which was suppressed by injection of anti CHK antibodies (Yamashita et al., 1999). Y490 promotes the binding and phosphorylation of Shc via its phosphotyrosine binding domain (PTB). This induces the binding of Grb2-SOS complex to TrkA that participates in the activation of Ras (Basu et al., 1994). This site was identified to mediate the activation of the ERK pathway and to be involved in neurite outgrowth. Furthermore, it seems that phosphorylated Y490 recruits and phosphorylates the adaptor fibroblast growth factor receptor substrate-2 (FRS2) via its PTB domain, providing binding sites for additional signalling proteins such as Grb2 and Crk (Yan et al., 2002). The association of Crk to FRS2 induces the activation of the guanine exchange factor C3G leading to the activation of Rap1 (Nosaka et al., 1999).

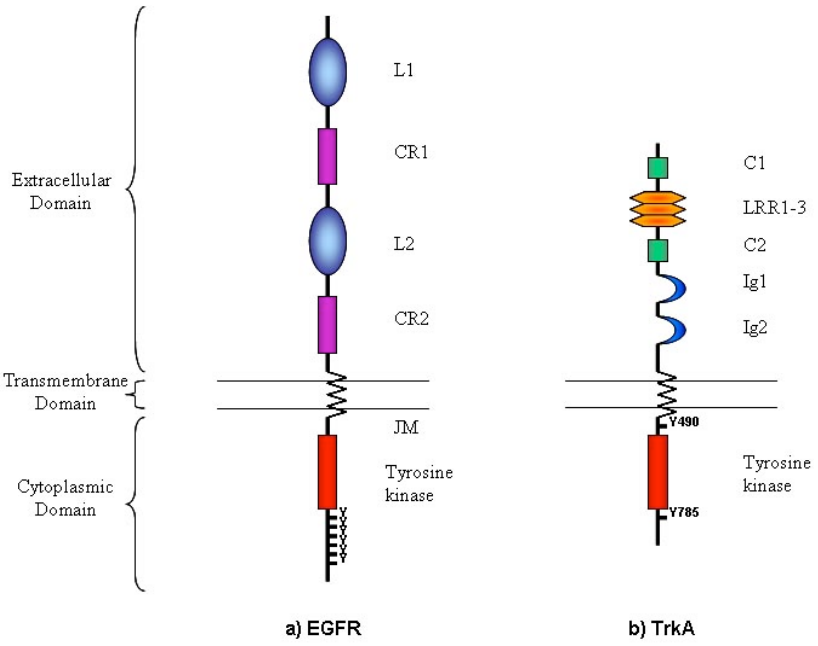


Figure 1. 3: Receptor Tyrosine Kinases. RTKs have in common a single transmembrane domain, a single cytoplamic tyrosine kinase domain and are differentiated by their extracellular domains. a) The extracellular domain of the EGF receptor consists of two ligand binding domain (L1 and L2), and two cysteine rich domains (CR1 and CR2). The carboxy-terminal domain contains the kinase domain flanked by tyrosine autophosphorylation sites. b) The extracellular domain of the NGF receptor (TrkA) consists of two cysteine clusters (C1, C2) separated by the ligand binding domain that contains multiple repeats of leucine-rich motifs (LRR1-3) and two immunoglobulin-like domains (Ig1, Ig2). There are two specific phosphorylated tyrosine residues identified to be docking sites for adaptor molecules: Y490 and Y785 in TrkA.

1.1.4 Small G-proteins Ras and Rap-1

The Ras proteins are a superfamily of small GTP-binding proteins that can hydrolyse GTP. These small GTPase proteins cycle between an inactive GDP-bound form and an active GTP-bound form. This family comprises Ras and its isoforms H-Ras, N-Ras and K-Ras, as well as the Ras-like small GTPases Rap1, Rap2, Ral, TC21, Rheb, R-Ras and M-Ras (Bos, 1998; Hancock, 2003). Ras proteins are localised at cellular membranes, although they are not intrinsic membrane proteins. Therefore, they are targeted to cellular membranes after undergoing a series of posttranslational modifications including prenylation, proteolysis to cleave their C-terminal CAAX motif, exposing the cysteine that is finally carboxymethylated. This creates a hydrophobic C-terminus allowing membrane association (Mor and Philips, 2006). The conversion of the inactive to the active form is mediated by guanine-nucleotide exchange factors (GEFs), such as SOS and C3G. Upon stimulation the cytosolic GEFs are recruited to RTKs via adaptor

proteins (section 1.1.3). GEFs catalyse the release of GDP from the guanine nucleotide binding pocket of Ras, allowing the binding of GTP that is tenfold more abundant in the cytosol than GDP (Hernandez-Alcoceba et al., 2000). Active Ras can interact and activate several effector proteins such as Raf kinases (Raf-1, B-Raf and A-Raf), phosphatidylinositol 3-kinase (PI3K), RalGEFs (RalGDS, Rlf and Rgl) (Bos, 1998; Hancock, 2003). The signalling is terminated by hydrolysis of GTP to GDP by the intrinsic GTPase activity of Ras in combination with the help of GTPase-activating proteins (GAPs), which accelerate the intrinsic GTPase activity of Ras proteins.

Ras is an oncogene that is implicated in up to 30% of all cancers (Bos, 1989; Hernandez-Alcoceba et al., 2000) and various types of cancers are due to mutations of the different isoforms. In particular, K-Ras is mutated in about 50% of adenocarcinomas of the colon and in 90% of pancreatic carcinomas (Bos, 1989), mutations in N-Ras are found in 30% of myelogenous leukaemias and in 13% of melanomas. In general, Ras becomes oncogenic due to mutations that make Ras insensitive to the action of GAPs, therefore preventing Ras inactivation.

Rap1 is the closest relative of Ras and they share 100% similarity in their effector domains (Bos et al., 2001; Mor and Philips, 2006). However, Rap1 has a tenfold lower intrinsic GTPase activity than Ras, suggesting that the nucleotide exchange reaction might be different. Rap1 has two isoforms Rap1a and Rap1b. Rap1 is activated through stimulation of various transmembrane receptors including RTKs, heterotrimeric G-Protein-coupled receptors, cytokine receptors and cell-adhesion molecules. The first GEF for Rap1 to be identified was the Crk SH3-domain-binding guanine-nucleotide releasing factor (C3G). C3G mediates the activation of Rap1 induced by RTKs (Wu et al., 2001). In addition to its catalytic region, C3G contains several proline-rich sequences that associate to the SH3 domain of Crk (Nosaka et al., 1999). Rap1 can also be activated by the exchange protein directly activated by cAMP (Epac) in response to elevated levels of cAMP (Bos, 2003; Enserink et al., 2002). Rap1 has been shown to bind to both Raf-1 and B-Raf, but Rap1 specifically activates B-Raf while it inhibits Raf-1 activation (Okada et al., 1999; Vossler et al., 1997).

1.1.5 Raf Kinases

The Raf family of serine/threonine-specific kinases regroups kinases highly conserved in structure. Raf kinases contains three conserved regions CR1, CR2 and CR3 (Chong and Guan, 2003). CR1 contains the Ras binding domain (RBD) and the cysteine-rich domains (CRD). These two domains are required for the binding to Ras and are involved in Raf activation. CR2 is serine/threonine rich and contains inhibiting phosphorylation sites. CR3 contains the catalytic domain where activating phosphorylation sites are located.

There are thee active raf genes in humans, A-raf, B-raf and c-raf-1 encoding for three Raf kinase isoforms, A-Raf, B-Raf and c-Raf (or Raf-1). Raf-1 is a 74 kDa protein and consists of 648 amino acids, while A-Raf is slightly smaller and is a 68 kDa protein (606 amino acids). B-Raf is subjected to alternative splicing which gives rise to proteins varying in length from 75-100 kDa. They are distinguished by their tissue-specific expression pattern (Storm et al., 1990). Raf-1 is ubiquitously expressed with highest expression in striated muscle, cerebellum and foetal brain. A-Raf is mainly expressed in urogenital tissues, and B-Raf is more highly expressed in neuronal tissues, testis and haematopoetic cells (Erhardt et al., 1995; O'Neill and Kolch, 2004). Raf kinases are the entry point to the ERK pathway and hence play a central role to control the signal flow through the ERK pathway (Dhillon and Kolch, 2002). They have distinct biological functions, which were studied using Raf knockout animal models (Hagemann and Rapp, 1999; O'Neill and Kolch, 2004). A-Raf knockout mice survied up to 21 days. They showed neurological and intestinal abnormalities and died from extensive bowel distension (Pritchard et al., 1996). B-Raf knockout mice died *in utero* from massive haemorrhage caused by apoptosis of endothelial cells (Wojnowski et al., 1997). However, more recent studies with conditional B-Raf knockout mice showed no endothelial phenotype, but a role for B-Raf in placenta development and neuronal development (Galabova-Kovacs et al., 2008; Galabova-Kovacs et al., 2006; Zhong et al., 2007). Raf-1 knockout mice are growth retarded and die *in utero* from widespread apoptosis with anomalies in the placenta and in the foetal liver (Mikula et al., 2001). The Raf kinases have a crucial role during embryonic development and organogenesis. A-Raf is involved in intestinal and neurological development, B-Raf suppresses apoptosis and Raf-1 is responsible for cell growth and the development of skin, lung and placenta. Interestingly, all three Raf isoforms serve distinct functions (Galabova-

Kovacs et al., 2006; Hagemann and Rapp, 1999) but they share Ras as a common upstream small G-protein activator and MEK as the only downstream substrate (Schaeffer and Weber, 1999). The regulation of Raf kinases is complex and still not completely understood.

1.1.5.1 Activation of Raf kinases

1.1.5.1.1 Initiation of Raf activation by Ras

The activation of Raf is a complex event that takes place at the plasma membrane where the small G-protein Ras is localised. It involves membrane relocalisation, phosphorylation and dephosphorylation of active residues and the presence of adaptor and scaffolding proteins.

In resting cells, Raf-1 is phosphorylated on S259 and S621 (corresponding to S365 and S729 in B-Raf), which are binding sites for the dimeric protein 14-3-3. It was proposed that 14-3-3 stabilises Raf kinases in a closed and inactive conformation by holding together the CR2 and CR3 regions masking the catalytic domain of Raf (Dhillon and Kolch, 2002). The first step in the activation of Raf is its binding to activated Ras (Ras-GTP) at the cell membrane (Traverse et al., 1993). The RBD of Raf has a higher affinity for Ras-GTP than for Ras-GDP. Therefore, in presence of active Ras, Raf translocates from the cytosol to the plasma membrane. The binding to Ras displaces 14-3-3 from phosphorylated S259 (Tzivion et al., 1998), which is dephosphorylated by protein phosphatase 2 A (PP2A) (Kubicek et al., 2002). Raf-1 can be activated only if S259 is dephosphorylated (Dhillon et al., 2002a; Morrison et al., 1993). Ras-GTP binds to the RBD with high affinity and to the CRD of Raf. Single mutations of specific amino acids (Gln66, Lys84 and Arg89) in the RBD can disrupt the interaction between Raf-1 and Ras (Fabian et al., 1993). The RBD alone is sufficient for the translocation of Raf-1 to the cell membrane, but it is not sufficient for Raf-1 to be fully activated (Kolch, 2000). The binding of Ras to the CRD is required for the activation of Raf and is thought to enhance the interaction between Raf-1 and Ras (Dhillon and Kolch, 2002). The interaction between Ras and Raf unmasks the catalytic site by dissociating the regulatory domain from the kinase domain of Raf. Now, Raf is in an open conformation exposing the MEK docking site and the kinase domain.

1.1.5.1.2 Full activation of Raf by phosphorylation

To be fully activated, Raf kinases require the phosphorylation of specific tyrosine and serine residues within the CR3 domain.

In Raf-1 (Fig. 1.4), mitogen stimulation results in the phosphorylation of S338 and Y341 that lie upstream the kinase domain in the N-region (negative charge in this region is essential for kinase activity), and the phosphorylation of T491 and S494 within the activation loop in the kinase domain (Chong et al., 2001; Fabian et al., 1993; King et al., 1998). The phosphorylation of S338 is induced by mitogens, integrin and Ras and is absolutely required for the activation of Raf-1. The mutation of serine to alanine blocks the activation of Raf-1 (Barnard et al., 1998; Diaz et al., 1997; King et al., 1998), whereas mutation of serine to phosphomimetic acid aspartic results in a slight activation of Raf-1 (Diaz et al., 1997). The substitution of Y341 to phenylalanine also blocks the activation of Raf-1 by mitogens, oncogenic Ras and activated Src (Diaz et al., 1997; Marais et al., 1995) and the replacement by an acid aspartic residue results in strong activation of Raf-1 (Fabian et al., 1993) indicating the crucial role of Y341 in the activation of Raf-1. PAK1 and PAK3 have been identified to be responsible for the phosphorylation of S338 in a PI3K dependent manner (King et al., 1998). However the phosphorylation of S338 by PAKs occurs in the cytosol and not at the plasma membrane. Therefore, PAKs phosphorylate S338 in a Ras- or growth factor-independent manner. More data suggest that PAKs are not the only kinases responsible for the phosphorylation of S338. For example, dominant-negative PAKs do not block the phosphorylation of S338 upon growth factor stimulation (Chiloeches et al., 2001; Wellbrock et al., 2004). It is more likely that other kinases are involved but they remain to be identified (Mason et al., 1999). It was demonstrated that a tyrosine kinase of the Src family is responsible for the phosphorylation of Y341 (Marais et al., 1995).

Within the activation loop, T491 and S494 have been identified to be phosphorylated upon mitogen stimulation and to participate to the activation of Raf-1. Substitutions of both sites with acidic residues enhance the kinase activity of Raf-1 (Chong et al., 2001). The kinases responsible for their phosphorylation have not been identified yet and Raf autophosphorylation might be a possibility.

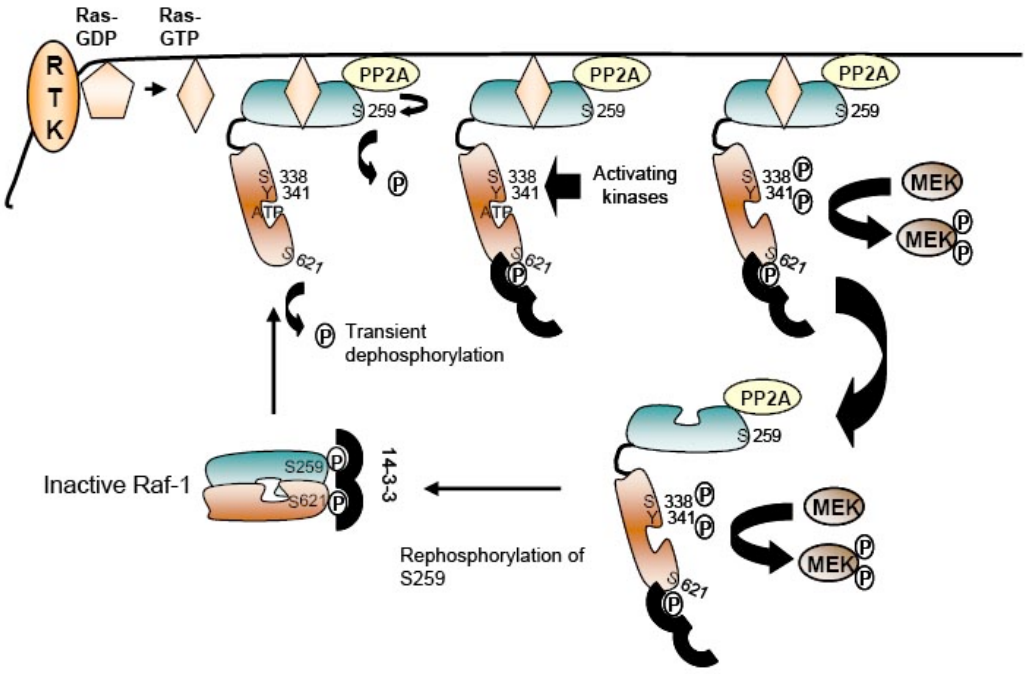


Figure 1. 4: Model for Raf-1 activation. In quiescent cells, Raf-1 is held in a stable and inactive conformation by 14-3-3 that binds to the phosphorylated S259 and S621. Mitogenic stimulation induces the activation of Ras and recruitment of Raf-1 to the plasma membrane. Active Ras displaces 14-3-3 from S259, which becomes available for dephosphorylation by PP2A. Raf-1 is in an open conformation exposing both catalytic domain and the MEK docking site. Raf-1 is now ready for activation. Activating kinases including Src and PAK phosphorylate other sites such as Ser338 and Tyr341. Activated Raf-1 can then phosphorylate MEK. Raf-1 is then released from Ras and the membrane, and begins to return to an inactive state by rephosphorylation of Ser259 allowing 14-3-3 to bind and stabilise the inactive conformation.

These phosphorylation sites required for the activation of Raf-1 are conserved in A-Raf (Fig. 1.5) and it seems that A-Raf is activated by similar mechanisms to Raf-1 (Marais et al., 1997).

However, the activation of B-Raf is different although Ras is essential for its activation. The main difference in the activation of B-Raf relies in the N-region (Fig. 1.5). The equivalent Y341 of Raf-1 is replaced by and aspartic acid (D449) and therefore is negatively charged (Mason et al., 1999). The substitution of D449 with phenylalanine reduces its basal activity but B-Raf can still be activated by Ras (Marais et al., 1997). In addition, S446 (equivalent to S338 of Raf-1) is constitutively phosphorylated (Mason et al., 1999). The consequence is that B-Raf has elevated basal kinase activity compare to Raf-1. It is highly responsive to Ras and does not required tyrosine phosphorylation by Src (Marais et al., 1997). B-Raf is fully activated by Ras and does not require secondary signal to be activated.

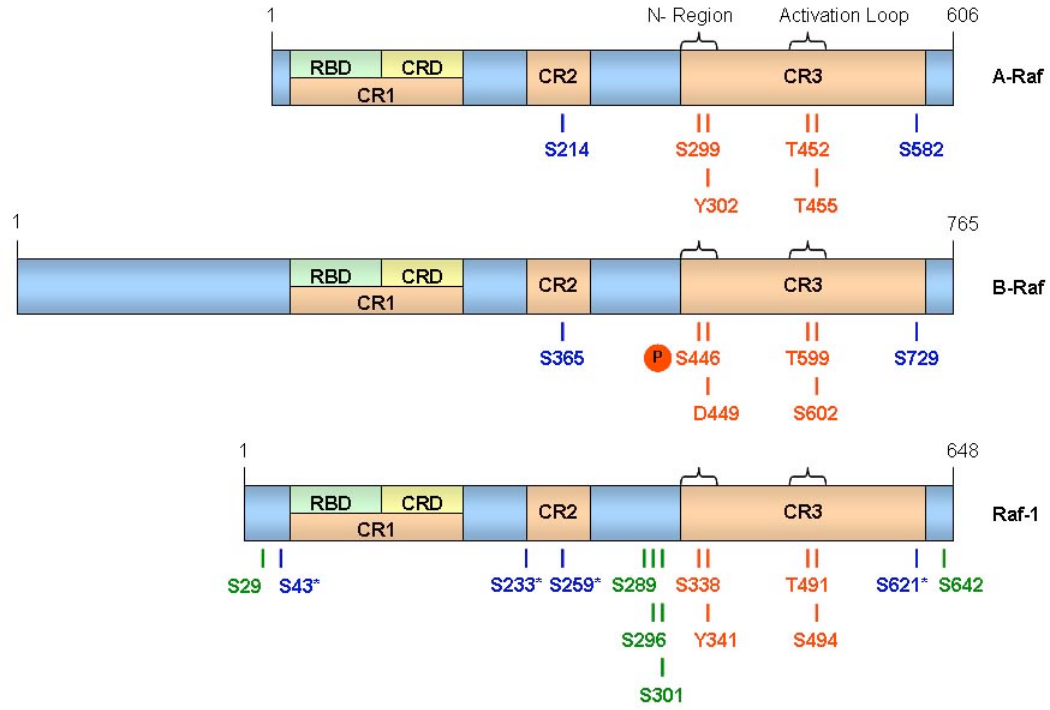


Figure 1. 5: Structure and phosphorylation sites of Raf kinases. The Raf isoforms have in common three conserved regions CR1, CR2 and CR3. The CR1 contains the Ras binding domain (RBD) and cysteine rich domain (CRD) involved in the interaction between Raf and active Ras at the plasma membrane. The activating phosphorylation sites are highlighted in red. The phosphorylation sites involved in the regulation of Raf are highlighted in blue. The asterix indicates the PKA phosphorylation sites (see section 1.3.1). The inhibitory ERK feedback phosphorylation sites are indicated in green (see section 1.1.5.2.4).

1.1.5.2 Regulation of Raf

1.1.5.2.1 14-3-3 Protein

It has been demonstrated that 14-3-3 and Raf-1 form a complex in resting cells (Li et al., 1995). It is thought that 14-3-3 stabilizes Raf-1 in an inactive conformation holding together CR2 and CR3 regions masking the catalytic domain of Raf-1 (Dhillon and Kolch, 2002; Muslin et al., 1996). The 14-3-3 proteins are members of a family of highly conserved and ubiquitously expressed 28-33 kDa acidic polypeptides. Their name is based on their fraction number on DEAE-cellulose chromatography and migration position on starch gel electrophoresis. There are at least seven genes that have been identified, which code for nine isotypes of 14-3-3 proteins (Yaffe et al., 1997). They spontaneously form homo or heterodimers. The 14-3-3 proteins bind to a large variety of proteins including protein kinases such as PKCs (Meller et al., 1996), or proteins involved in apoptosis such as BAD (Zha et al., 1996), but also proteins

involved in cell cycle control such as Cdc25 phosphatases (Peng et al., 1997). 14-3-3 binds to phosphoserine and phosphothreonine proteins containing the consensus motifs RSXpSXP or RXXXpSXP (Yaffe, 2002), where pS stand for phosphoserine and X stand for any amino acid. All Raf isoforms contain two consensus motifs located in the regulatory and the catalytic domains, but the role of 14-3-3 in the regulation of Raf remains unclear and 14-3-3 has been suggested to be involved in both positive and negative regulation of Raf. For Raf-1, one 14-3-3 dimer binds to phosphorylated S259 in the regulatory region and to phosphorylated S621 in the C-terminus (Tzivion et al., 1998). The phosphorylation on S259 has an inhibitory effect on the activation of Raf-1, which was demonstrated by mutation of S259 resulting in constitutively active kinase (Morrison et al., 1993). In contrast the phosphorylation of S621 is required for the kinase activity of Raf-1. This was demonstrated by mutation of S621 that resulted in an inactive Raf kinase activity (Morrison et al., 1993; Yip-Schneider et al., 2000). Three different models have been proposed to explain the role of 14-3-3 in the regulation of Raf-1. In the first model, Tzivion *et al* propose that the free arm of 14-3-3 that was displaced from phosphorylated S259 bind to a third, yet unidentified phosphoserine on Raf-1 (Tzivion et al., 1998). They hypothesised that this new bridge between the third binding site and S621 hold Raf-1 in an active conformation. The second model proposed by Thorson *et al* suggests that 14-3-3 stabilises the active conformation of Raf-1 and protect phosphoserine 621 from dephosphorylation by phosphatases (Thorson et al., 1998). The third model suggests that the interaction between 14-3-3 and Raf-1 is required for the recruitment to the plasma membrane of Raf-1 by Ras (Roy et al., 1998). At the plasma membrane, 14-3-3 is completely released from the active Raf-1 and will reassociate during inactivation of Raf-1.

It has been demonstrated that 14-3-3 interacts with B-Raf *in vivo* (Yamamori et al., 1995). The interaction sites seem to be located in the N- and C-terminus domains of B-Raf (Papin et al., 1996). One 14-3-3 binding consensus motif in the C-terminal catalytic domain of B-Raf is centred on S728 corresponding to S621 in Raf-1. The substitution of serine 728 to alanine disrupts the interaction between B-Raf and 14-3-3. The kinase activity of B-Raf was reduced by 50-60% *in vitro* and severely impaired *in vivo*. Furthermore, this mutation compromised dramatically the ability of B-Raf to induce PC12 cell differentiation (MacNicol et al., 2000). From their results, MacNicol *et al* suggested that the role of 14-3-3 binding to B-Raf is to scaffold the binding of B-Raf to its downstream effectors (MacNicol et al., 2000).

1.1.5.2.2 RKIP

Originally, the Raf kinase inhibitor protein (RKIP) was identified in a two-hybrid screen as a Raf-1 associated protein (Yeung et al., 1999). RKIP was shown to bind to Raf-1, MEK and ERK both *in vitro* and *in vivo*. In addition, confocal microscopy revealed that RKIP and Raf-1 co-localise in both quiescent and Ras-transformed cells (Yeung et al., 1999). But RKIP inhibits the activation and phosphorylation of MEK by Raf-1, without inhibiting the phosphorylation of ERK by MEK. The inhibition of RKIP by antibody microinjection or the downregulation of the expression of RKIP using antisense RNA, cause the activation of the ERK pathway. However overexpression of RKIP inhibits the ERK pathway and cellular transformation, which confirms that RKIP suppresses this pathway (Yeung et al., 1999). MEK and RKIP have been shown to bind to different binding sites on Raf-1, Raf-1 and RKIP bind to different binding sites on MEK, while MEK and Raf-1 bind to overlapping binding sites on RKIP, making their binding mutually exclusive. Thus, RKIP is a scaffold for MEK and ERK, but an inhibitor of the Raf-1/MEK interaction. RKIP dissociates the interaction between the two kinases in a competitive manner (Yeung et al., 2000). The binding of RKIP to Raf-1 is controlled by growth factor and upon stimulation the association between Raf-1 and RKIP is lost, allowing Raf-1 to activate MEK. When the activity of ERK declines, then RKIP reassociates with Raf-1 (Yeung et al., 2000). However the mechanism through which RKIP dissociates from Raf-1 is not fully known and remains to be established.

Trakul *et al.* demonstrated that mutation of S338 and Y341 to phosphomimetic residues in Raf-1 prevented its association with RKIP and therefore inhibition (Trakul et al., 2005). They demonstrated that also B-Raf co-precipitated with RKIP. However the depletion in RKIP did not enhance the kinase activity of B-Raf, while the activity of Raf-1 was increased. Since the S445 corresponding to S338 on Raf-1 is constitutively phosphorylated and that Y341 is replaced by an aspartic acid in B-Raf, it was then not surprising that the activity of B-Raf was not inhibited by RKIP.

In contrast to these findings, Park *et al* demonstrated that RKIP inhibits the activity of B-Raf in melanoma cancer cells (Park et al., 2005). They demonstrated that RKIP associates with B-Raf using yeast two hybrid and co-immunoprecipitation experiments. They also demonstrated that in Raf-1 knockout MEF cells, the phosphorylation of MEK was reduced in cells infected with retrovirus expressing RKIP, suggesting that RKIP

inhibits the kinase activity of B-Raf. In contrast, the downregulation of RKIP by siRNA in Raf-1 knockout MEF cells increased the phosphorylation of MEK. Then, they demonstrated that when RKIP was transfected in Cos-1 cells, the basal activity of B-Raf was inhibited and the activity of B-Raf activated by Ras was reduced. Furthermore, the activity of B-Raf was inhibited by bacterial recombinant RKIP *in vitro*. And finally they showed that overexpression of RKIP in PC12 cells inhibits the differentiation of the cells that is mediated by B-Raf.

1.1.5.2.3 Chaperones

Heat shock proteins are members of a group of highly conserved proteins. As molecular chaperones, they have diverse role such as assisting in the folding of newly synthesised proteins or helping to stabilise the tertiary structure of certain proteins (Helmbrecht et al., 2000). It has been shown that both Raf-1 and B-Raf exist in multiprotein complexes including Hsp90, Hsp70 and cdc37 (Jaiswal et al., 1996; Stancato et al., 1997). Geldanamycin is a drug that binds to Hsp90 and prevents Hsp90 from binding to its substrates. Treatment with geldanamycin reduces the levels of Raf-1 in the cell causing its aggregation, ubiquitination and further degradation (Stancato et al., 1997). It seems as well that chaperones are involved in signal regulation. Jaiswal *et al* demonstrated that in PC12 cells, the activation of the ERK pathway upon NGF stimulation is principally due to B-Raf activity over Raf-1 activity, which correlates with the association of B-Raf with Hsp90 (Jaiswal et al., 1996).

Bag-1 is another chaperone for Raf-1. Bag-1 has been shown to bind to Raf-1 and to redirect Raf-1 to mitochondrial membrane where Raf-1 binds to Bcl-2 (Wang et al., 1996a). It was also demonstrated that Bag-1 increases the kinase activity of Raf-1 *in vitro*, but this is likely to be due to its chaperone function to keep Raf-1 in an active conformation rather than direct activation (Wang et al., 1996b).

1.1.5.2.4 ERK-induced Feedback regulation of Raf-1

Previous observations have suggested that Raf-1 might be subject to feedback inhibition. For example, Alessi *et al* showed that Raf-1 activation is prolonged when MEK is pharmacologically inhibited (Alessi et al., 1995). It was also demonstrated that Raf-1 activity is elevated in cells overexpressing IMP (Impedes mitogenic signal propagation). IMP disrupts the interaction between Raf and MEK by inhibiting the

scaffold/adaptor protein KSR that scaffolds the interaction between Raf, MEK and ERK) (Matheny et al., 2004). In cells overexpressing IMP, MEK and ERK can not be activated. However Raf-1 remains active. Therefore it was hypothesized that MEK and/or ERK might be involved in the downregulation of Raf-1. Dougherty *et al* identified six residues (S29, S43, S289, S296, S301 and S642) that become hyperphosphorylated after mitogen stimulation (Fig. 1.5) correlating with the inactivation of Raf-1 (Dougherty et al., 2005). The mutation of these six sites to alanine abolishes the hyperphosphorylation of Raf-1, results in the prolonged mitogen-induced kinase activity and accumulation of Raf-1 at the plasma membrane (Dougherty et al., 2005). The treatment with U0126, a MEK inhibitor, revealed that the hyperphosphorylation and the downregulation of Raf-1 are dependent on the MEK/ERK pathway. They found that activated ERK2 is capable to phosphorylate these sites except S43 *in vitro*, suggesting that ERK is the most likely to be responsible for this feedback regulation of Raf-1. Furthermore they also found that the hyperphosphorylated Raf-1 is not degraded but recycled to an inactive form by dephosphorylation of S29, S289, S296, S301 and S642 by PP2A. This is consistent with the work of Hekman and colleagues who reported that the phosphorylation of S296 and S301 contribute to Raf-1 inhibition (Hekman et al., 2005). However, the direct feedback regulation of Raf-1 by ERK is subject to debate. It was also demonstrated that Raf-1 is phosphorylated by ERK on S289, S296 and S301, but this phosphorylation resulted in increasing the kinase activity of Raf-1 (Balan et al., 2006).

1.1.6 MEK and ERK

The Raf isoforms share MEK as a common substrate *in vivo* (Schaeffer and Weber, 1999). Two MEK isoforms are known: MEK1 (43 kDa) and MEK2 (46 kDa). They are encoded by two different genes located at different chromosomes (Brott et al., 1993). MEK is activated by phosphorylation of two serine residues within its activation loop, S217/218 and S221/222 (Dhanasekaran and Premkumar Reddy, 1998). The mutation of one of these sites to alanine can inhibit the activity of MEK, suggesting that both sites are necessary and sufficient for the activation of MEK (Zheng and Guan, 1994). The interaction of MEK with Raf is dependent on a proline-rich sequence unique to MEK kinases and deletion of this sequence impaired the ability of MEK to bind to Raf and reduces the ability of Raf to activate MEK (Catling et al., 1995). Although all Raf

isoforms can activate MEK *in vitro*, they interact differentially with MEK1 and MEK2. A-Raf activates preferentially MEK1, whereas Raf-1 seems to activate both MEK1 and MEK2 equally well. B-Raf has a higher MEK kinase activity and binds to MEK1 and MEK2 with a higher affinity than Raf-1 and A-Raf (Marais et al., 1997; Papin et al., 1996). Oncogenic B-Raf phosphorylates MEK1 10 times more efficiently than Raf-1 and 500 times more efficiently than A-Raf (Pritchard et al., 1995). MEKs are dual specificity kinases that can phosphorylate both threonine and tyrosine residues. MEK1 and MEK2 phosphorylate ERK1/2 on threonine 183 and tyrosine 185 in their activation loop (Dhanasekaran and Premkumar Reddy, 1998; Schaeffer and Weber, 1999).

ERK1 and ERK2 are serine/threonine kinases which have more than 160 known substrates including transcription factors, cytosolic proteins such as Rsk and cytoskeletal proteins (Yoon and Seger, 2006). Phosphorylated ERK can also translocate to the nucleus where it phosphorylates and activates transcription factors including Elk-1, Ets and c-Myc. Activated ERK can also phosphorylate SOS and Raf-1, inhibiting respectively Ras and Raf-1 activation and serving as a negative feedback mechanism of the ERK signalling pathway (Dhillon et al., 2007; Dougherty et al., 2005; Langlois et al., 1995).

1.1.7 PC12 cells: proliferation versus differentiation

The rat PC12 cell line is derived from a rat pheochromocytoma tumour found in the adrenal medullary glands (Greene and Tischler, 1976). PC12 cells are a commonly used model cell system to study cell proliferation and differentiation, which are regulated by the ERK signalling pathway (Marshall, 1995). These cells have the property to either proliferate or differentiate depending on the type of mitogen stimulation. Upon epidermal growth factor (EGF) stimulation cells proliferate. However, upon nerve growth factor (NGF) stimulation the cells differentiate into sympathetic-like neurons (Dichter et al., 1977; Greene and Tischler, 1976). The fate of the cell has been shown to depend on the longevity of the ERK signal (Kao et al., 2001; Marshall, 1995; Traverse et al., 1992). It is commonly accepted that EGF causes a transient activation of the ERK pathway allowing the cells to continue to divide (Kao et al., 2001). However, NGF causes a sustained activation of the pathway, allowing the translocation and the accumulation of phosphorylated ERK in the nucleus. In the nucleus phosphorylated ERK can activate specific transcription factors and can induce the expression of specific

genes causing the cells to withdraw from the cell cycle and therefore to differentiate (Traverse et al., 1992; Yao et al., 1998). This is supported by the observations that EGF can induce sustained activation of ERK and PC12 cell differentiation when EGFRs are overexpressed (Marshall, 1995). Indeed, overexpression of the EGF receptors slows down or prevents the inactivation of the receptors resulting in the prolonged activation of ERK and translocation in the nucleus.

1.1.8 Regulation of the differential activation of the ERK pathway in PC12 cells

As described above the fate of PC12 cells depends on the duration of the ERK signal. The question is how the signal is integrated through the same pathway to induce different biological responses? It seems that the differential activation of ERK is encoded at the receptor level and is dependent on the activation of the Ras family proteins as well as the Raf kinases involved. It is commonly accepted that both NGF and EGF can activate Ras, which can activate both Raf-1 and B-Raf. One hypothesis is that Ras is responsible for both transient and sustained activations of ERK (Lu et al., 2000; Zwartkruis et al., 1998). Another hypothesis is that only NGF, but not EGF can activate Rap1 and therefore B-Raf, causing the sustained activation of the ERK pathway (Marshall, 1995; York et al., 1998). This suggests that two distinct pathways are involved in the activation of ERK and that Rap1/B-Raf is responsible for the sustained activation of ERK upon NGF stimulation. In contrast to this hypothesis, Kao *et al* demonstrated that both NGF and EGF stimulations activate Rap1 and therefore B-Raf, suggesting that both growth factors mediate the signal through both Ras/Raf-1 and Rap1/B-Raf pathways. They were the first to work on endogenous proteins in PC12 cells involved in the activation of ERK (Kao et al., 2001). They showed that in PC12 cells upon both EGF and NGF stimulation, Raf-1 and B-Raf were stimulated (Fig. 1.6). They demonstrated that the activation of B-Raf contributed to about 90% of the activation of ERK showing that B-Raf is the principal MEK kinase. They demonstrated that upon both EGF and NGF stimulation, Ras was transiently activated leading to the transient activation of Raf-1. However Rap1 was transiently activated upon EGF stimulation, whereas the activation of Rap1 was prolonged upon NGF stimulation. Therefore, the sustained activation of ERK upon NGF stimulation was the result of a transient activation of Ras followed by a prolonged activation of Rap1. They

demonstrated that the longevity of the activation of ERK correlated with the stability of the complex formation at the receptor between Crk-C3G and Rap1, and therefore could cause prolonged B-Raf activation. Both EGF receptor (EGFR) and NGF receptor (TrkA) recruit a variety of signalling molecules to their receptor complexes consisting of adaptor proteins and GEFs upon stimulation. Ras activation is mediated through the association of the adaptor protein Grb2 and the GEF SOS, while the activation of Rap1 is mediated through the formation of a complex between Crk and the GEF C3G. Some of the proteins forming the receptor complexes are shared between both receptors such as phosphatidylinositol 3-kinase or adaptor proteins such as Shc and Grb2. Other proteins are specific for each receptor (Segal and Greenberg, 1996). The main difference between EGFR and TrkA is that TrkA can recruit the adaptor protein FRS2 (for fibroblast growth factor receptor substrate 2), which is a lipid-anchored docking protein. FRS2 can bind directly to TrkA through its phosphotyrosine binding domain (PTB) and is phosphorylated upon NGF stimulation. FRS2 can then recruit Grb2 and SHP-2. Kao *et al* demonstrated that the complex formed between Crk-C3G, Rap1 and B-Raf is scaffolded and stabilised at the NGF receptor by FRS2, thus leading to the sustained activation of ERK (Kao et al., 2001).

The differential regulation of the activation of the ERK pathway is a complex event that is still not completely understood and that might results from positive and negative feedback regulation as well as crosstalk with other pathways such as the cAMP pathway.

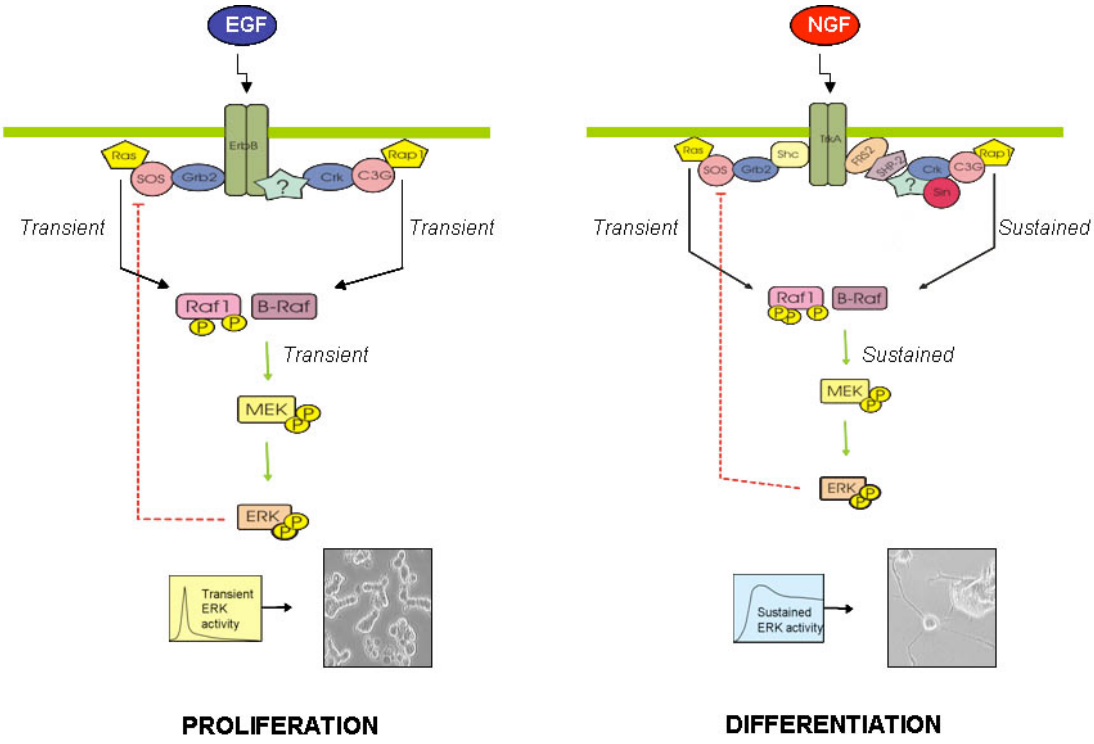


Figure 1. 6: Differential regulation of the ERK signalling pathway. In PC12 cells, EGF stimulation result in the transient activation of the ERK pathway causing the cells to proliferate, whereas NGF stimulation results in the sustained activation of the ERK pathway inducing the differentiation of the cells. According to Kao et al, the stability of the complex formation at the receptor determines the longevity of the signal and the outcome of the stimulation (Kao et al., 2001).

1.2 THE cAMP SIGNALLING PATHWAY

The activation of the cAMP signalling pathway involves the binding of an extracellular ligand to a G protein-coupled receptor (GPCR), which through G proteins regulates one of several isoforms of adenylyl cyclase (ACs) leading to the generation of cAMP. Cyclic AMP mediates the signal through its three main effectors that are protein kinase A (PKA), nucleotide exchange factors Epac (exchange protein directly activated by cAMP) and cyclic nucleotide-gated (CNG) ion channels. The level of cAMP is also regulated by the family of phosphodiesterase (PDEs) enzymes that degrade cAMP and through their different subcellular compartmentalisation shape pools of cAMP within subcellular microdomains (Houslay, 1998; Zaccolo, 2006).

1.2.1 cAMP generation and degradation

3',5'-cyclic adenosine monophosphate (cAMP) is a second messenger involved in regulation of a plethora of diverse cellular processes such as neurotransmission, muscle contraction, intracellular transport mechanisms and ion fluxes, and the regulation of metabolic processes such as glycogenolysis and lipolysis. In other contexts, cAMP can have long term effects on, for example, the regulation of the cell cycle, embryonic development, cell growth, proliferation and differentiation.

The generation of cAMP is catalysed by adenylyl cyclases (ACs) that convert ATP to cAMP and pyrophosphate (Tang and Hurley, 1998). The reaction proceeds by the inversion of configuration at the α-phosphate due to the direct displacement of pyrophosphate by attack of the 3'-OH on the α-phosphate (Eckstein et al., 1981; Tang and Hurley, 1998). Then cAMP is degraded by phosphodiesterases (PDEs) that hydrolyse cAMP into AMP (Fig. 1.7).

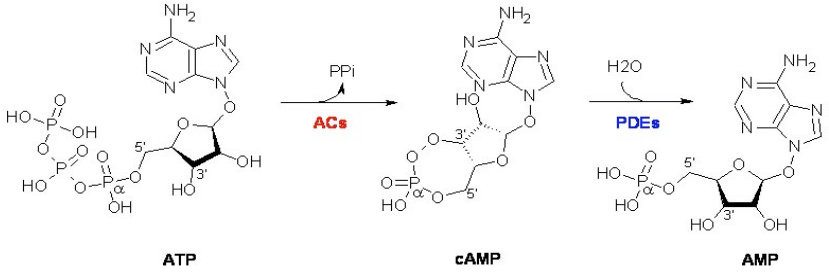


Figure 1. 7: The metabolism of cAMP. The second messenger cAMP is generated from ATP by adenylyl cyclases and hydrolysed into AMP by phosphodiesterases.

1.2.2 Adenylyl Cyclases

Nine membrane-bound isoforms (AC1 to AC9) and one soluble isoform (AC10) of mammalian ACs have been identified (Sunahara and Taussig, 2002). All membrane-bound AC isoforms are found in excitable tissues such as neurons and muscles, however AC1 and AC3 are only found in the brain. The soluble AC10 is predominantly found in the testis. The membrane-bound AC isoforms exhibit a basal activity that is enhanced upon binding of stimulatory G protein α-subunit ($G_s\alpha$) and reduced upon binding to the inhibitory G protein α-subunit ($G_i\alpha$). Their activities are differentially regulated by binding calcium/calmodulin, through phosphorylation by PKC or PKA and by the association of various G-protein subunits (Hurley, 1999). The proposed structure (Fig. 1.8) based on the amino acid sequence includes a short variable amino terminus (N), six hydrophobic transmembrane spans (M1), a large cytoplasmic domain (C1), a second set of six transmembrane spans (M2) and another large cytoplasmic domain (C2). The C1 and C2 domains are subdivided into C1a and C1b; C2a and C2b. The C1a and C2a are homologous and contain the catalytic domains. The membrane-bound ACs share about 60% of sequence similarity and the most conserved sequences are located in the cytoplasmic domains C1 and C2 (between 50-90%). Mutations of amino acids in the C1 and C2 domains have demonstrated that these domains contribute to ATP binding and catalysis (Dessauer et al., 1997).

The activation of adenylyl cyclase is initiated by the binding of hormones (Tang and Hurley, 1998), cytokines (Tachibana et al., 1998) or neurotransmitters (Spada et al., 1997) to G protein-coupled receptors at the cell surface (Robinson et al., 1968). All membrane-bound ACs can also be directly activated by forskolin (Hurley, 1999). GPCRs communicate with intracellular membrane-associated G proteins consisting of a guanosine diphosphate-bound α-subunit and a βγ heterodimer. The binding of the ligand to the GPCR induces a conformational change in the intracellular domain of the receptor that leads to the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP). The binding to GTP induces conformational changes resulting in the dissociation of the heterotrimeric G protein into α and βγ subunits. The dissociated α-subunit binds to AC, which induces a conformational change in AC that brings together the C1 and C2 domains, resulting in the activation of AC (Hurley, 1999).

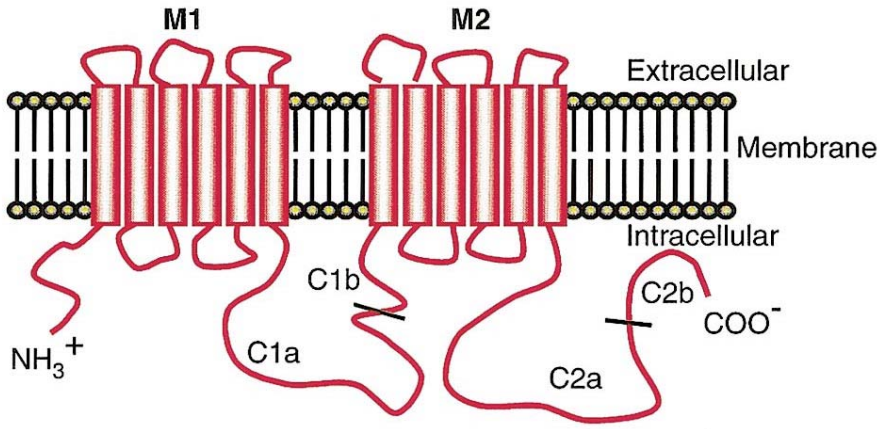


Figure 1. 8: Schematic representation of membrane-bound adenylyl cyclases. The overall structure of the membrane-bound ACs consists of a variable cytosolic N-terminus, six hydrophobic transmembrane spans (M1), a large cytoplasmic domain (C1), a second set of six transmembrane spans (M2) and another large cytoplasmic domain (C2). The C1 and C2 domains are subdivided into C1a and C1b; C2a and C2b. The C1a and C2a are homologous and contain the catalytic domains.

1.2.3 Phosphodiesterases

The termination of the cAMP signalling is controlled by phosphodiesterases (PDEs), which were first discovered shortly after the identification of cAMP (Butcher and Sutherland, 1962). PDEs hydrolyse the 3'-phosphoester bond of the 3', 5'-purine ribose cyclic monophosphate nucleotides leaving the residual 5'-monophosphate, thus terminating the cyclic nucleotide stimulated pathways in cells. This is important not only in the ablation of cellular signalling, but also in the maintenance of tight regulation of downstream effectors activity. In mammalian, there are currently 35 characterized PDEs encoded by more than 21 different genes and classified into 11 families of PDEs (see table 1.1) based on their amino acid sequences, substrate specificities, endogenous and exogenous regulators and pharmacological properties (Mehats et al., 2002). The mammalian PDEs share a common architecture consisting of a conserved catalytic domain located at the C-terminus and regulatory domains mostly at the N-terminus of the protein. The catalytic core contains common structural elements that are important for the hydrolysis of cyclic nucleotides, which consists of a PDE-specific motif (H-D-[L/I/V/M/F/Y]-X-H-X-[A/G]-X-X-N-X-[L/I/V/M/F/Y]) and two consensus divalent cation (Zn^{2+} or Mg^{2+})-binding domains (Francis et al., 2001).

PDE Family	Pharmacological classification	Cyclic nucleotide substrates	Inhibitors	No. of genes	Regulation
PDE1	Ca^{2+}/CaM stimulated PDE	cAMP and cGMP	Vinpocetine (14μM), W-7 (300μM)	3	(+)Ca^{2+}/CaM (-) PKA, CamKII
PDE2	cGMP-stimulated PDE	cAMP and cGMP	EHNA (1μM)	1	(+) cGMP, PKC (+/-) N-terminal targeting domain
PDE3	cGMP-inhibited PDE	cAMP > cGMP	Cilostamide (20nM), milrinone (150nM), zardaverine (IC50 0.5-2μM)	2	(+) PKA, PKB (-) cGMP (+/-) N-terminal targeting domain
PDE4	High affinity, rolipram-sensitive cAMP-specific PDE	cAMP	Rolipram (1μM)	4	(+) PKA, ERK, phosphatidic acid (-) ERK, caspases (+/-) N-terminal targeting domain
PDE5	cGMP-specific PDE	cGMP	Zaprinast (130nM), sildenafil (10nM)	1	(+) cGMP, PKG, PKA (-) caspases
PDE6	Phosphoreceptor cGMP-specific PDE	cGMP	Zaprinast (400nM)	3	(+) Transducin (-) cGMP
PDE7	High affinity, rolipram-insensitive cAMP-specific PDE	cAMP	IBMX (4μM)	2	(+/-) PKA
PDE8	High affinity and IBMX-insensitive cAMP-specific PDE	cAMP	Dipyridamole (9μM)	2	PAS domain
PDE9	High affinity cGMP-specific PDE	cGMP	Zaprinast (35μM)	1	
PDE10	cAMP-inhibited cGMP PDE	cAMP<cGMP	Dipyridamole (1μM)	1	(-) cAMP
PDE11	Dual specificity cGMP-binding PDE	cAMP and cGMP	Zaprinast (12μM)	1	

Table 1. 1: Properties of cyclic nucleotides PDE families

1.2.3.1 The PDE1 family of phosphodiesterases

There are three distinct isoforms of PDE1 called PDE1A, B and C. These isoforms are encoded by different genes and show differential tissue expression. The PDE1 isoforms are activated upon binding of Ca^{2+} /CaM and have different affinities for Ca^{2+} /CaM complex. The Ca^{2+} /CaM complex binds within two regions of their N-terminal regulatory domains and determines subtle changes in the regulation of activity of PDE1 isoforms (Kakkar et al., 1999). PDE1C hydrolyses both cAMP and cGMP with high affinity, while PDE1A and PDE1B selectively hydrolyse cGMP. Antagonists of CaM inhibit PDE1 activity but are not only selective for PDE1. Catalytic site inhibitors such as vinpocetine inhibit all PDE1, but PDE1C with less potency, and are not specific for any individual isoform (Kakkar et al., 1999).

1.2.3.2 The PDE2 family of phosphodiesterases

The splice variants of PDE2 (PDE2A1, PDE2A2 and PDE2A3) are encoded by a single gene. These enzymes can hydrolyse both cAMP and cGMP (Rosman et al., 1997; Yang et al., 1994). The activation of PDE2 enzyme occurs following the binding of cGMP to the GAF domains located at the N-treminus of the protein (Martinez et al., 2002), increasing the affinity of the catalytic site for cAMP. PDE2 is localised to the adrenal cortex and several areas of the brain, as well as in goblet cells, olfactory neurones and in capillary and endothelial cells (Sadhu et al., 1999). PDE2 activity has been implicated in cardiac Ca^{2+} channel control, catecholamine secretion in the central nervous system (Beavo, 1995) and in olfactory neurone signalling (Juilfs et al., 1997).

1.2.3.3 The PDE3 family of phosphodiesterases

There are two characterized PDE3 genes, *PDE3A* and *PDE3B* (Movsesian, 2002). *PDE3A* encodes for three different PDE3A isoforms PDE3A1, PDE3A2 and PDE3A3, whereas *PDE3B* encodes for a unique PDE3B enzyme (Movsesian, 2002). All PDE3 isoforms show high affinity for cAMP and cGMP. However they poorly hydrolyse cGMP. PDE3A has been reported to be expressed in blood vessels, cardiovascular system and oocytes (Movsesian, 2002; Reinhardt et al., 1995). The highest level of PDE3B has been reported in brain, renal collecting duct epithelium, hepatocytes, adipocytes and developing spermatocytes (Movsesian, 2002; Reinhardt et al., 1995). The overall structure of PDE3 enzymes consists of a C-terminal catalytic domain and two N-terminal hydrophobic membrane association regions, named NHR1 and NHR2 (Degerman et al., 1997). The catalytic domain consists of a 44 amino acid sequence that is specific for this PDE family and contributes for the catalytic activity and the inhibitor selectivity of PDE3 isoforms (Manganiello and Degerman, 1999). The two N-terminal hydrophobic domains are responsible for the membrane association of PDE3 isoforms, allowing them to have different subcellular locations (Shakur et al., 2000). They are predominantly membrane-associated but they can be either cytosolic. For example, PDE3A1 that possesses both NH1 and NH2 regions is found mainly in the particulate fraction; PDE3A2 that possesses only NH2 is found both in the cytosol and the particulate fraction; PDE3A3 which lacks both domains is cytosolic (Maurice et al., 2003). Following the N-terminal hydrophobic domains are consensus sites for phosphorylation by PKA and PKB. The phosphorylation of PDE3 isoforms by PKA or

PKB increases their PDE activity (Manganiello and Degerman, 1999; Smith et al., 1991). Taken together, it is suggested that the hydrophobic domain may be involved in the association between PDE3 with either chaperones, which assist in targeting and membrane insertion or with anchoring proteins analogous to the PKA anchoring proteins (AKAPs), which target PKA to cellular membranes such as the plasma membrane, the endoplasmic reticulum, Golgi or mitochondria, in order to target PDE3 isoforms in proximity to membrane-associated signalling molecules (Shakur et al., 2001; Shakur et al., 2000). PDE3 isoforms are also directly regulated by cGMP that competes for the cAMP binding site of the catalytic domain (Manganiello and Degerman, 1999; Tilley and Maurice, 2002). A number of PDE3 inhibitors exist, such as cilostamide, milirone and amrinone.

1.2.3.4 The PDE4 family of phosphodiesterases

PDE4 enzymes currently provide the largest known PDE family. Four genes *PDE4A-D* encode for the PDE4 isoforms, giving rise to at least 18 different splice variants. These isoforms can be grouped into long, short and super-short categories (Houslay and Adams, 2003). All PDE4 isoforms have a conserved catalytic region, located within a 360 amino acid domain. The 'long' isoforms have two of the conserved N-terminal regulatory domains, termed UCR1 (for upstream conserved region 1) and UCR2 of 59 and 79 amino acids, respectively. A linker region (LR1) of 24 amino acids separates UCR1 and UCR2, which is the site spliced to produce truncated proteins lacking UCR1 in the 'short' isoforms. The 'super-short' PDE4 species have an N-terminal truncation within the UCR2 region of the protein and unique N-terminal sequences (Houslay, 2001). The various PDE4 isoforms are expressed in almost all cell types, except blood platelets. PDE4 enzymes are specific for the hydrolysis of cAMP. Modulation of PDE4 activity is carried out by post-translational modifications such as phosphorylation, and through interaction with adapter proteins and phospholipids (Houslay, 2001). Indeed, selected PDE4 variants are localised to subcellular regions through interactions with various scaffolding or anchoring proteins including AKAPs (A kinase anchoring proteins), RACK (receptor for activated c kinase), β-arrestin (Houslay, 2001; Houslay and Adams, 2003). For example, long forms of PDE4 isoforms are activated by PKA phosphorylation, whereas long and short forms of PDE4 are differentially regulated by ERK phosphorylation (Conti et al., 2003; Houslay and Adams, 2003).

1.2.4 cAMP effectors: PKA, Epac, ion gated channels

Cyclic AMP activates several downstream signalling mechanisms through direct interaction with proteins. The best characterised cAMP effector is the protein kinase A (PKA). It was thought to be the only direct effector of cAMP (Brostrom et al., 1970; Walsh et al., 1968), with the exception of the cyclic nucleotide-gated ion channels found in specialised cells like olfactory neurons (Zufall et al., 1997), until the discovery of nucleotide exchange protein directly activated by cAMP termed Epac (de Rooij et al., 1998).

1.2.4.1 Protein Kinase A: PKA

Protein kinase A (PKA) was first characterised in 1968 (Walsh et al., 1968). It is activated through the cooperative binding of cAMP. The inactive PKA is a heterotetrameric holoenzyme consisting of two regulatory subunits (R) and two catalytic subunits (C). The cooperative binding of two cAMP molecules to each of the R subunits induces a conformational change which results in the release of the C subunits that can phosphorylate their target proteins (Johnson et al., 2001). More and more proteins that can be phosphorylated and activated by PKA are still being identified (Shabb, 2001). The consensus sites for PKA phosphorylation have been identified as R-R-X-S/T, R/K-X-X-S/T and R/K-X-S/T, where X stands for any hydrophobic amino acid (Kennelly and Krebs, 1991; Shabb, 2001). Three different genes encode for the C subunits Cα, Cβ and Cγ. Four genes express the R subunits RIα, RIβ, RIIα and RIIβ (Chrivia et al., 1988; Jahnsen et al., 1986; Scott et al., 1987). Two types of heterotetramers have been reported to exist *in vivo*: PKA holoenzymes type I ($RI\alpha_2C_2$ and $RI\beta_2C_2$) and type II ($RII\alpha_2C_2$ and $RII\beta_2C_2$). The PKAII holoenzymes are assembled preferentially over PKAI under physiological conditions (Francis and Corbin, 1994). The assembly of different catalytic and regulatory subunits gives rise to a number of PKA holoenzymes which possess different biological characteristics (Skalhegg and Tasken, 2000). The PKA type I complex is generally cytoplasmic and is activated transiently by weak cAMP signalling (Houslay and Milligan, 1997). The type II complex, however, is associated with cellular structures and organelles and responds only to high and persistent cAMP stimulation (Rubin et al., 1979; Stein et al., 1987). PKA, along with other signalling proteins, can be targeted to distinct subcellular regions through the interaction with A-kinase anchoring protein (AKAPs).

1.2.4.2 Exchange Protein Directly Activated by cAMP: Epac

More recently a new effector for cAMP, the exchange protein directly activated by cAMP or Epac, was discovered. This discovery came out when Bos' group noticed that the activation of Rap1 was insensitive to the inhibition of PKA (de Rooij et al., 1998). Epac is a cAMP-responsive guanine nucleotide exchange factor (GEF) for the small GTP-binding proteins Rap1 and Rap2 (de Rooij et al., 1998). Two isoforms have been identified, Epac1 and Epac2 (Fig. 1.9). Both isoforms have a N-terminal DEP (dishevelled, Egl-10, pleckstrin) domain involved in membrane localisation (de Rooij et al., 1998), a cAMP binding domain (CBD), a Ras exchange motif (REM) and a guanine nucleotide exchange factor homology domain (GEF) (Christensen et al., 2003). Epac 2 has another CBD and a Ras-association domain (RA) that can interact with active Ras. It is likely that the CBD folds upon the GEF domain to prevent interaction with effectors. The conformation of Epac changes when binding to cAMP, exposing the GEF domain that activates downstream targets (Qiao et al., 2002). The main effectors of Epac1 and 2 are the Ras-like small G-proteins Rap1 and Rap2 (de Rooij et al., 1998; Enserink et al., 2002; Rehmann et al., 2003).

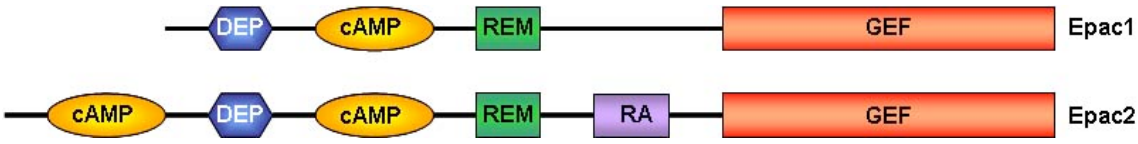


Figure 1. 9: The structure of Epac1 and Epac2. The Epac isoforms consist of a N-terminal DEP (dishevelled, Egl-10, pleckstrin) domain involved in membrane localisation, a cAMP binding domain (CBD), a Ras exchange motif (REM) and a guanine nucleotide exchange factor homology domain (GEF). Epac 2 has another CBD and a Ras-association domain (RA) that can interact with active Ras.

1.2.4.3 Cyclic Nucleotide-gated ion channels

Cyclic nucleotide-gated (CNG) ion channels on the cell membrane are biological effectors of cyclic nucleotide action. To date, over 20 genes encoding different subtypes of CNGs have been cloned from invertebrates and vertebrates (Wei et al., 1997). The CNG channels belong to the class of ligand-gated cation channels and are opened by the direct binding of cAMP or cGMP. They contribute to cellular control of the membrane potential and intracellular Ca^{2+} levels. The CNG ion channels are multi-subunit pore-forming channels. The best-studied cAMP gated channel is the olfactory cyclic

nucleotide channel, which is activated equally by both cAMP and cGMP (Shabb, 2001). This nonselective ion channel is activated in response to odorant binding to G protein-coupled olfactory receptors which, in turn, results in the activation of adenylyl cyclase (Shabb, 2001).

1.2.5 Compartmentalization of the cAMP-depedent PKA activity: AKAP

The intracellular targeting and compartmentalization of the cAMP-dependent PKA signalling is controlled through the association with A-kinase anchoring proteins or AKAPs. The AKAP family comprises about 50 anchoring proteins that have diverse structures. They bind to PKA and to co-precipitate with PKA catalytic activity (Tasken and Aandahl, 2004; Wong et al., 2004). All AKAPs contain a PKA-binding domain located at the C-terminus and a unique targeting domain in the N-terminus that participates in the association of AKAPs with membranes or organelles targeting the PKA-AKAP complex to specific subcellular compartments. In general, AKAPs bind the PKA type II (RII_2C_2) with high affinity and poorly bind to PKA type I (RI_2C_2) (Carr et al., 1992; Herberg et al., 2000). The PKA-binding domain consists of 14-18 hydrophobic amino acids forming an amphipathic α-helix that binds to the N-terminal dimerisation domain of the R subunits of PKA (Dell'Acqua and Scott, 1997; Newlon et al., 1997; Wong et al., 2004). More recently it was reported that some AKAPs bind specifically to the RI subunits of PKA (McConnachie et al., 2006; Wang et al., 2006a) and others have dual affinity for both RI and RII PKA subunits (Huang et al., 1999). AKAP79, AKAP82 and AKAP220 were characterised as dual specific AKAPs and can bind to both RI and RII PKA subunits (JarnÃ¦ss and TaskÃ©n, 2007). In addition to these domains certain AKAPs are able to form multivalent complexes by interaction with phosphatases, kinases and other proteins involved in signal transduction (Wong et al., 2004). AKAPs participate in the spatial and temporal regulation of the PKA signalling by exposing PKA to isolated cAMP gradients and allowing efficient catalytic activation and accurate substrate selection.

1.3 EVIDENCE OF CROSSTALK BETWEEN THE cAMP & THE ERK SIGNALLING PATHWAYS

1.3.1 Inhibition of Raf-1 by cAMP through PKA

In the early 1990s, it was discovered that elevation of cAMP induced a profound inhibition in the activation of ERK by growth factors in Rat1 and NIH3T3 fibroblasts and in vascular smooth muscle cells (Cook and McCormick, 1993; Hafner et al., 1994; Sevetson et al., 1993; Wu et al., 1993). Raf-1 was identified to be the target of cAMP and that PKA was required for the inhibition of Raf-1 by cAMP (Cook and McCormick, 1993; Hafner et al., 1994; Sevetson et al., 1993; Wu et al., 1993). However there are still debates about the exact mechanisms.

One possible mechanism that has been suggested involves the small G-protein Rap1 (Schmitt and Stork, 2001). Indeed Rap1 was first identified as an inhibitor of ERK in Rat-1 fibroblasts (Cook and McCormick, 1993). This model suggests that Rap1, activated in a PKA dependent manner, antagonises Ras activation of Raf-1 by binding and sequestering Raf-1 away from Ras (Okada et al., 1999; Schmitt and Stork, 2001). However, studies using Epac selective activator have demonstrated that the activation of Rap1 did not inhibit ERK activation (Enserink et al., 2002). It is unlikely that Rap1 is responsible for blocking Raf-1 activation upon elevated levels of cAMP.

Another possible mechanism involves direct phosphorylation of Raf-1 by PKA resulting in the inhibition of Raf-1. It was demonstrated that PKA can phosphorylate Raf-1 on serines 43, 233, 259 and 621 both *in vitro* and *in vivo* (Dhillon et al., 2002b; Dumaz et al., 2002; Hafner et al., 1994; Sidovar et al., 2000). These sites appear to be phosphorylated in resting cells but are hyperinduced by PKA (Dhillon et al., 2002a). Moreover, these sites work independently to inhibit Raf-1 and they have to be all mutated to render Raf-1 completely resistant to PKA inhibition (Dumaz et al., 2002; Sidovar et al., 2000). Wu *et al* demonstrated that the phosphorylation on S43, which is located just upstream to the RBD, lowers the affinity of Raf-1 to Ras-GTP that is the active form of Ras (Wu et al., 1993). Therefore the phosphorylation of S43 by PKA prevents Raf-1 to bind and to be activated by Ras. However, the phosphorylation of S43 is not exclusive for the inhibition of Raf-1 by PKA as it was demonstrated that mutation of S43 to alanine does not overcome the effect of cAMP and that Raf-1 is still

susceptible to inhibition by PKA (Dhillon et al., 2002b; Dumaz et al., 2002). Phosphorylated serines 233, 259 and 621 are docking sites for 14-3-3 proteins, which are adapter proteins that bind to short phosphorylated peptide motifs (Yaffe, 2002). It was demonstrated that S259 is the major phosphorylation site for the inhibition by PKA (Dhillon and Kolch, 2002). When 14-3-3 binds to these sites, it antagonises Ras binding. The mechanisms through which this occurs remained unclear but it is thought that 14-3-3 binding to Raf-1 might directly compete with Ras for the CRD of Raf-1 or might modify Raf-1 conformation and mask the RBD. Dumaz and Marais from their research have suggested a model for the regulation of Raf-1 by cAMP (Dumaz and Marais, 2005). They proposed that in resting cells, S259 and S621 are phosphorylated allowing 14-3-3 to bind and trap Raf-1 in an inactive form. In normal conditions, Ras activation recruits Raf-1 to the plasma membrane removing 14-3-3 from the phosphorylated S259, which can be dephosphorylated by PP2A. Once S259 is dephosphorylated then Raf-1 can be activated through a series of further phosphorylations. Upon elevated cAMP levels, activated PKA can also phosphorylate Raf-1 on S43 and S233. The phosphorylation on S233 creates a new binding site for 14-3-3. It seems that 14-3-3 has a higher affinity for phosphorylated S233 than S621, and therefore 14-3-3 would bind to phosphorylated S233 rather than to phosphorylated S621. Their hypothesis is that in this particular conformation due to high levels of cAMP, Ras cannot displace 14-3-3 from phosphorylated S259 and Raf-1 cannot be activated.

1.3.2 Activation of B-Raf by cAMP via Rap1

While it has been well documented that cAMP induces the inhibition of cell proliferation, cAMP can also stimulate cell proliferation and differentiation by promoting ERK activity in certain cell types such as ovarian granulosa cells, melanoma or neuronal cells (Houslay and Kolch, 2000; Vossler et al., 1997; Young et al., 1994). It was demonstrated that the activation of ERK by cAMP requires the activation of Rap1 and B-Raf (Dugan et al., 1999; Schmitt and Stork, 2000; Zanassi et al., 2001). As described previously in the section 1.1.4, Rap1 can bind to and activate B-Raf (Vossler et al., 1997; York et al., 1998). Furthermore, agonist screening assays showed that cAMP elevating agents such as forskolin increased the level of phosphorylation of Rap1 (Bos et al., 2001; de Rooij et al., 1998). However the way that cAMP activates Rap1 is

still subject to debate regarding the involvement of PKA. Both PKA-dependent and independent activation of Rap1 by cAMP have been proposed. In the PKA-dependent mechanism, it was suggested that PKA activates Rap1 through the phosphorylation of Src (Obara et al., 2004). In the PKA independent mechanism, the activation of Rap1 seems to be mediated by a Rap1 specific GEF, also called exchange protein directly activated by cAMP (Epac) described in the section 1.2.4.2. (de Rooij et al., 1998; Enserink et al., 2002).

The activation of B-Raf by cAMP remains controversial and the models described above have been challenged by several studies. For example, the activation of Rap1 by cAMP or Epac agonist (8-CPT(MeO)cAMP) did not always result in the activation of B-Raf (Zwartkruis et al., 1998) or the activation of ERK (Enserink et al., 2002).

1.3.3 The Crosstalk between the cAMP and the ERK signalling pathways in PC12 cells

In PC12 cells, cAMP induces neuronal differentiation (Greene and Tischler, 1976; Gunning et al., 1981; Vossler et al., 1997). As described in the sections 1.1.7 and 1.1.8, the neuronal differentiation of PC12 cells is a result of the longevity of the activation of ERK. York *et al* demonstrated that sustained activation of ERK upon NGF stimulation is the result of the activation of two distinct pathways where the small G-protein Ras initiates the activation of ERK, which is sustained by the activation of Rap1 (York et al., 1998). They identified the formation of a stable complex at the receptor between the adaptor protein Crk and the GEF C3G that selectively activates Rap1. This is supported by the work of Kao *et al* that show that the complex formation between Crk, C3G and Rap1 is scaffolded and stabilised by FRS2 that binds to TrkA (NGF receptor) but not to EGF receptor (Kao et al., 2001). They showed that the longevity of this complex correlates with the sustained activation of ERK upon NGF stimulation. They also demonstrated that in PC12 cells, endogenous B-Raf contributes to 90% of the activation of ERK, showing that PC12 cell differentiation is mainly mediated through B-Raf compare to Raf-1. Previous work from Erhardt and colleagues in PC12 cells showed that Raf-1 is inhibited by cAMP, whereas B-Raf is resistant to the inhibition by cAMP in serum-containing medium (Erhardt et al., 1995). While the mechanisms are still not totally understood, there is much evidence of the inhibition of Raf-1 by cAMP through PKA as described in the section 1.3.1. Finally, Vossler *et al* demonstrated that the

activation of ERK by cAMP requires both Rap1 and B-Raf (Vossler et al., 1997). As described in the previous section 1.3.2, the mechanisms of the activation of Rap1 by cAMP might involve PKA or Epac (the exchange factor protein directly activated by cAMP).

From these observations we hypothesised that the signal initiated by growth factors would utilise distinct pathways: Ras/Raf-1/MEK/ERK and Rap1/B-Raf/MEK/ERK. The transient activation of ERK upon EGF would be mainly mediated through Ras/Raf-1/MEK, whereas the sustained activation of ERK would be mediated through both Ras/Raf-1/MEK and Rap1/B-Raf/MEK. The cAMP pathway would interact with the ERK pathway by inhibiting Raf-1 through PKA and by activating B-Raf though the activation of Rap1 by either PKA or Epac to strengthen the mitogen signal (Fig. 1.10).

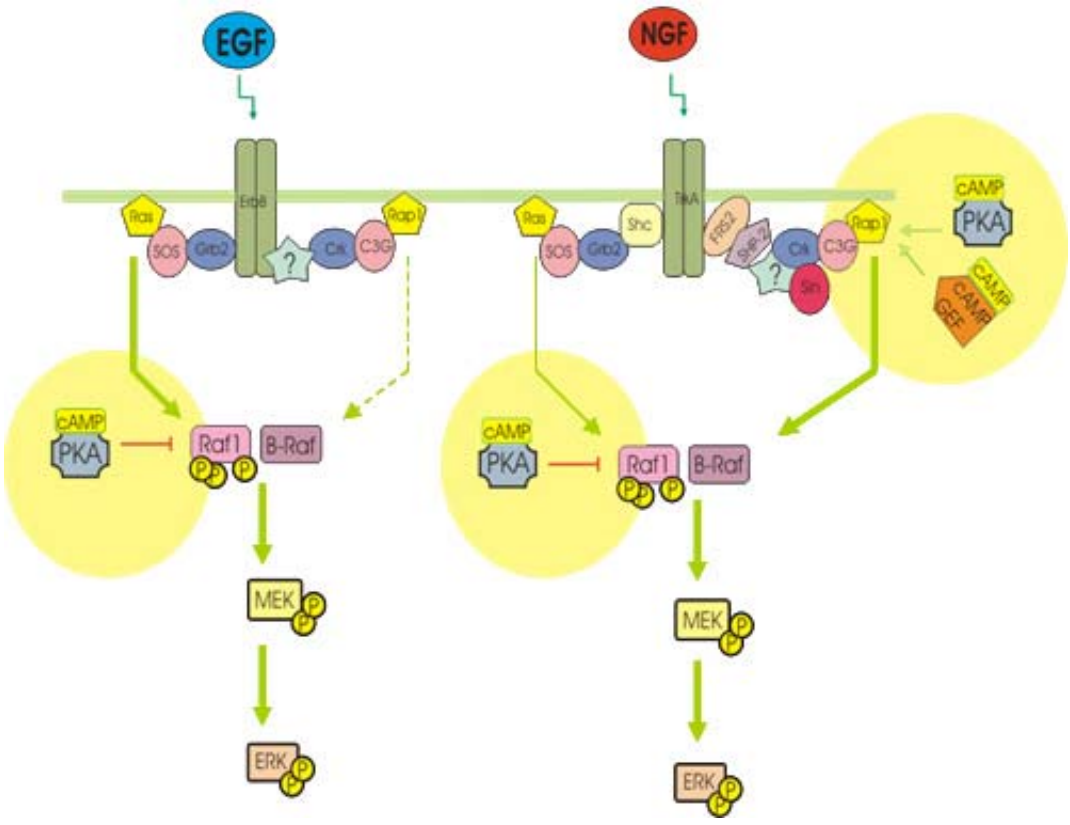


Figure 1. 10: Schematic representation of the crosstalk between the ERK and the cAMP signalling pathways. We hypothesised that the signal initiated by growth factors would utilise distinct pathways: Ras/Raf-1/MEK/ERK and Rap1/B-Raf/MEK/ERK. The transient activation of ERK upon EGF would be mainly mediated through Ras/Raf-1/MEK and transiently through Rap1/B-Raf/MEK, whereas the sustained activation of ERK would be mediated through both Ras/Raf-1/MEK and Rap1/B-Raf/MEK. The cAMP would serve to finely tune the regulation of the ERK pathway by inhibiting Raf-1 through PKA and activating B-Raf though the activation of Rap1 by either PKA or Epac.

The aim of this research is to determine the mechanisms through which cAMP crosstalk with the ERK signalling pathway occurs and to verify if PKA and Epac are involved in these mechanisms. This research also aimed to elucidate the mechanisms through which NGF mediates sustained activation of ERK and PC12 cell differentiation, while EGF mediates transient ERK activation of ERK and cell proliferation.

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 Antiserum

Primary Abs	Type	Host	WB Dilution	Supplier
Phospho-p44/42 MAPK (Thr202/Tyr204)	Monoclonal	Mouse	1:2000	Cell Signaling Technology®
Phospho-p44/42 MAPK (Thr202/Tyr204)	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
ERK	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
ERK 1 (C-16)	Polyclonal	Rabbit	1:1000	Santa Cruz Biotechnology
ERK 2 (C-14)	Polyclonal	Rabbit	1:1000	Santa Cruz Biotechnology
Anti-ERK1/2 pan Ab	Polyclonal	Rabbit	1:1000	Biosource
Phospho-MEK1/2 (Ser217/221)	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
MEK 1	Monoclonal	Mouse	1:1000	BD Biosciences
MEK 2	Monoclonal	Mouse	1:2500	BD Biosciences
MEK1/2	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
Raf-1	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
c-Raf	Monoclonal	Mouse	1:1000	BD Biosciences
Phospho-c-Raf (Ser259)	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
Phospho-c-Raf (Ser338)	Monoclonal	Rabbit	1:1000	Cell Signaling Technology®
B-Raf	Polyclonal	Rabbit	1:1000	Upstate®
Ras	Monoclonal	Mouse	1:1000	Upstate®
Rap1	Polyclonal	Rabbit	1:1000	Upstate®
Ras-GRF2 (C3G)	Monoclonal	Mouse	1:250	BD Biosciences
PDE3A (G-20)	Polyclonal	Goat	1:1000	Santa Cruz Biotechnology
PDE3B (H-300)	Polyclonal	Rabbit	1:1000	Santa Cruz Biotechnology

Primary Abs	Type	Host	WB Dilution	Supplier
Phospho-(Ser/Thr) PKA Substrate	Polyclonal	Rabbit	1:1000	Cell Signaling Technology®
AKAP 79	Polyclonal	Rabbit	1:1000	Santa Cruz Biotechnology
AKAP 82	Monoclonal	Mouse	1:5000	BD Transduction Laboratories
AKAP 220	Monoclonal	Mouse	1:250	BD Transduction Laboratories
AKAP 250	Polyclonal	Goat	1:1000	Santa Cruz Biotechnology
FLAG	Monoclonal	Mouse	1:10000	Sigma
Phospho-SRC(Tyr418)	Polyclonal	Rabbit	1:1000	Biosource

Table 2. 1: List of primary antibodies

Secondary Ab	Type	Host	Dilution	Supplier
Anti-Rabbit IgG	Peroxidase conjugate	Goat	1/5000	Sigma
Anti-Mouse IgG	Horseradish peroxidase conjugate	Sheep	1/5000	Amersham Biosciences
Anti-Goat IgG	Peroxidase conjugate	Rabbit	1/5000	Sigma
AlexaFluor® 680 Anti-Rabbit IgG	Fluorescent dye conjugate	Goat	1/2000	Molecular Probes
AlexaFluor® 680 Anti-Mouse IgG	Fluorescent dye conjugate	Goat	1/2000	Molecular Probes
IRDye™ 800 Anti-Mouse IgG	Fluorochrome conjugate	Rabbit, Goat	1/5000	Rockland Inc.
IRDye™ 800 Anti-Rabbit IgG	Fluorochrome conjugate	Donkey	1/5000	Rockland Inc.
IRDye™ 800 Anti-Goat IgG	Fluorochrome conjugate	Donkey	1/5000	Rockland Inc.

Table 2. 2: List of secondary antibodies

2.1.2 Pharmacological inhibitors

Calbiochem

Cilostamide, N-Cyclohexyl-N-methyl-4-(1,2-dihydro-2-oxo-6-quinolyloxy) butyramide

Rolipram, 4-[3-(Cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone

Sigma-Aldrich

EHNA, *erythro*-9-(2-Hydroxy-3-nonyl) adenine hydrochloride

IBMX, 3-Isobutyl-1-methylxanthine

Vinpocetine, (3α, 16α)-Eburnamenine-14-carboxylic acid ethyl ester

KT5720, (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester

2.1.3 cAMP analogues

BioLog Life Science Institute

PKA agonist, N^6- Benzoyladenosine- 3', 5'- cyclic monophosphate (6-Bnz-cAMP)

EPAC agonist, 8- (4- Chlorophenylthio)- 2'- O- methyladenosine- 3', 5'- cyclic monophosphate (8-pCPT-2'-O-Me-cAMP / 8-CPT-2'-O-Me-cAMP)

2.1.4 General Reagents

All reagents were purchased from Sigma unless listed here.

Amersham Biosciences: ECL™ Western Blotting Detection Reagent

BDH Biochemical: NaCl, Hepes

Bio-Rad Laboratories: Precision Plus Protein Prestained Standard, Bio-Rad Protein Assay

Costar: 6-well tissue culture plates

Dharmacon: Smart pools of ON-TARGET plus siRNAs for rat B-Raf and Raf-1

Fisher BioReagents: Tris/Base

ICN Biomedicals: PBS (tablets)

Invitrogen: LipofectAMINE 2000 transfection reagent, Novex™ mini-cell gel tank, Novex™ XCell II™ Blot module, NuPage® 4-12% Bis-Tris gels, NuPAGE® MES buffer, NuPAGE® MOPS buffer, NuPAGE® transfer buffer

Life Technologies/Gibco: DMEM, L-glutamine, MBP, trypsin

Nunc: tissue culture flasks

Promega: Nerve Growth Factor, mNGF (2.5S, Murine), Pure Yield™ Plasmid Maxiprep System

Qiagen: plasmid purification kits (maxi)

Roche Diagnostics/Boehringer Mannheim: EDTA, EGTA, leupeptin, Complete EDTA-Free protease inhibitor cocktail tablets

Sterilin: dishes for agar plates

Upstate: cAMP HTS Immunoassay kit, Raf-1 and B-Raf Kinase Cascade Assay Kit

Whatman: Protran BA85 nitrocellulose membrane, 3MM paper

2.2 CELL CULTURE

2.2.1 PC12 cells

The PC12 cells, a cell line derived from a pheochromocytoma of the rat adrenal medulla, were maintained in complete, antibiotic-free media made of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Horse Serum, 5% (v/v) foetal bovine serum (FBS) and 3.2 mM L-Glutamine. They were grown at 37˚C in 5% CO_2. When reaching about 80% of confluency, cells were split at a ratio of 1:4 into fresh media.

2.2.2 HEK 293 cells

The human embryonic kidney cell line, HEK 293 cells, was maintained in complete media made of DMEM complemented with 10% (v/v) FBS, 2 mM L-Glutamine, 1% (v/v) MEM and penicillin/streptomycin (100 units/ml). They were grown at 37˚C in 5% CO_2. When the cells were about 80% confluent they were splited 1:10. This was done by rinsing once the cells with 5 ml of pre-warmed and steril phosphate buffered saline (PBS) solution then by applying 1ml of trypsin for 2 min at room temperature to detach the cells from the bottom of the culture flask. The cells were diluted into 9 ml of fresh medium of which 1ml was dispensed into a new culture flask.

2.2.3 HEKβ2 cells

HEKβ2 cells were maintained in complete media made of DMEM complemented with 10% (v/v) newborn calf serum (NCS), 2 mM L-Glutamine, 1% (v/v) MEM, 2% (v/v) G-418 and penicillin/streptomycin (100 units/ml). They were grown at 37˚C in 5% CO_2. When the cells were about 80% confluent, they were trypsinised and splited 1:5 in new culture flask as described above.

2.2.4 Transfection

2.2.4.1 PolyFect® DNA transfection of HEK 293 cells

DNA transfection of HEK 293 cells was performed using PolyFect® reagent from Qiagen. The cells were plated onto 100 mm dishes and grown overnight to 50%-80% confluency. 8 µg of DNA was diluted with basal medium (DMEM) to a total volume of 300 µl. 80 µl of PolyFect® reagent was added to the DNA solution, mixed by pipetting and incubated at room temperature for 10-15 min. During that time, the medium from the culture dishes was removed and replaced with 7 ml of fresh growth medium. 1ml of normal growth medium was added to the DNA solution and mixed by gentle pipetting. The total DNA mixture was added to the cells and mixed by gentle swirling. The cells were treated and/or harvested 24-48 h after transfection.

2.2.4.2 siRNA transfection of PC12 cells with Lipofectamine™ 2000

One day before transfection, PC12 cells were plated onto poly-L-lysine pre-coated 6-well plates in normal growth medium and in an appropriate dilution that they will be 30-50% confluent at the time of transfection. The day of the transfection, the siRNA oligomer was diluted in the appropriate volume of Opti-MEM® I Reduced Serum Medium to have a final concentration of 100 nM (or 200 pmol) per well. Lipofectamine™ 2000 (4 µl/well) was diluted in the appropriate amount of Opti-MEM® I Reduced Serum Medium. Both mixtures were mixed gently and incubated for 10 min at room temperature. After the 10 min the diluted oligomer solution was combined with the diluted Lipofectamine™ 2000 solution to allow complex formation to occur. The final mixtures were mixed gently and incubated for 20 min at room temperature. During that time, the medium was removed from the well and replaced by 800 µl of pre-warmed Opti-MEM® I Reduced Serum Medium. After the 20 min, the oligomer-Lipofectamine™ 2000 complexes were added to each well and mixed by swirling the

plates. After 6 hours of incubation, the Opti-MEM® I Reduced Serum Medium was removed and replaced by fresh growth medium. The cells were incubated for 72-96 hours at 37ºC in 5% CO_2 before being treated and assessed for gene knockdown.

2.2.5 Cell Treatment

2.2.5.1 Time courses

6 well plates were coated with 10% (v/v) poly-L-lysine in PBS for 1 hour at room temperature. The poly-L-lysine was then removed and the plates were let to dry at room temperature. PC12 cells were plated onto the pre-coated plates in normal growth medium and left to adhere over night at 37˚C in 5% CO_2. 3 hours before starting an experiment, the medium was removed and replaced by a serum-free medium (DMEM supplemented with 3.2 mM L-Glutamine). The cells were then treated with indicated drugs and stimulated with either NGF or EGF for different periods of time.

2.2.5.2 Differentiation of PC12 cells

PC12 cells were plated onto poly-L-lysine pre-coated 6-well plates in normal growth medium and left to adhere over night at 37˚C in 5% CO_2. The cells were treated with the appropriate drug then stimulated with NGF or EGF for 72 hours. The cells were observed under a microscope (AxioVert B5) and for each treatment 10 phase contrast images were taken randomly with a Carl Zeiss monochrome digital camera (AxioCam MRm) and analyzed with the Axiovision software.

2.2.6 Preparation of cell lysates

Time points were sampled by discarding the medium and washing cells once with ice-cold PBS. Then, the cells were lysed by adding ice-cold 3T3 Lysis buffer or RIPA buffer supplemented with cocktails of protease and phosphatase inhibitors. Cells were removed from the bottom of the plates using a cell scraper and transferred into 1.5 ml eppendorf tubes. The tubes were rotated on a wheel for 20 min at 4˚C. To remove the unwanted cell debris, the lysates were centrifuged for 5 min at 13000 g and at 4˚C. The supernatants were transferred into fresh eppendorf tubes and used directly or stored at -20˚C. The protein concentration was measured as described in the section 2.2.2 and was normalised for each samples.

2.3 BIOCHEMISTRY

2.3.1 Buffers

- 3T3 Lysis Buffer: 25 mM Hepes, pH 7.4, 50 mM NaCl, 10% (v/v) glycerol and 1% (v/v) Triton X-100. Before use, this buffer was supplemented with Complete EDTA-Free protease inhibitor cocktail and with phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride and 5 mM sodium pyrophosphate).
- RIPA Buffer: 50 mM Tris/HCl, pH 8, 132 mM NaCl, 0.1% (v/v) SDS, 1% (v/v) triton X-100, 1.1 mM EDTA, and 5.5 mM EGTA. Before use, the RIPA buffer was supplemented with protease, phosphatase and proteasome inhibitors.
- TBS: 20 mM Tris/HCl, pH 7.6, 137 mM NaCl
- TBST: 20 mM Tris/HCl, pH 7.6, 137 mM NaCl and 1% (v/v) TWEEN 20
- Stripping Buffer: 0.75% (w/v) glycine, 0.87% (w/v) NaCl
- PDE Assay diluting buffer: 20 mM Tris/HCl, pH 7.4 and 10 mM $MgCl_2$
- 5× Sample Buffer: 10% (w/v) SDS, 300 mM Tris/HCl, pH 6.8, 0.05% (w/v) bromophenol blue, 50% (v/v) glycerol, 10% (v/v) mercaptoethanol
- Dialysis Buffer: 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5% (v/v) glycerol

2.3.2 Protein Assay

Total protein concentration of each samples were measured using the BioRad's protein assay based on the Bradford assay and carried out in 96 well microtitre plates. A spectrophotometric standard curve of protein concentration was produced using a serial dilution from 0 to 5 μg bovine serum albumin (BSA). 10 μl of diluted lysate sample was analysed in triplicate. The Bio-Rad Bradford Assay reagent was diluted 1:5 with distilled water and 200 μl was added to each well. The 96-well plate was then analysed using the Revelation package on a computer, connected to an MRX microtitre plate reader, which read absorbance at a wavelength of 590 nm. Protein concentrations were determined by plotting the standard curve and using a least squared regression analysis to obtain the line of best fit. The equation of the line was used to determine the protein concentration of each sample.

2.3.3 Western Blot

2.3.3.1 Electrophoresis

Proteins were separated according to their molecular weight by electrophoresis using the NuPAGE® Novex® system from Invitrogen. This system is based upon a Bis-Tris-HCl buffered polyacrylamide gel that works in neutral pH (pH 7.0), which minimizes protein modifications. Polyacrylamide gradient gels (4-12% Bis-Tris polyacrylamide gels) were used to optimize the separation of small to medium-sized proteins. Proteins to be analysed were denaturated using sample buffer that contains reducing agents (mercaptoethanol and SDS). SDS also confers a net negative charge on the proteins, which allow them to migrate across the gel when the electic current is applied. Thus, the denaturated and negatively charged proteins migrate through the polyacrylamide gels toward the anode depending on their size, with the smaller proteins migrating further through the gel than the larger proteins. Proteins were separated along a protein standard marker (Precision Plus Protein Prestained Standard from BioRad) to estimate the size of the proteins of interest. NuPAGE® MES buffer was used to separate small proteins (such as ERK), while NuPAGE® MOPS was used to separate larger proteins (such as Raf). Electrophoresis were run for about 45 min at 160 V.

2.3.3.2 Protein Transfer

Proteins separated in the polyacrylamide gels (as described above) were transferred onto nitrocellulose membranes by electroblotting. Basically, a polyacrylamide gel was placed on a sheet of Whatman 3MM paper. Then a nitrocellulose membrane was placed on top of the gel and the membrane was covered with another sheet of Whatman paper. The assembly was covered on both side with sponges (provided by Invitrogen) and finally the "swandich" was placed in a XCell II™ Blot Module and immersed in NuPAGE® Transfer Buffer from Invitrogen as followed: the gel facing the negative electrode and the membrane facing the positive electrode. Thus, when the electric current was applied the negatively charged proteins in the gel migrated to the nitrocellulose membrane toward to positive electrode. Transfers were carried out for 1.5 h at 30 V.

2.3.3.3 Blocking

The membranes were blocked for at least 1h with gentle shaking in 5% (w/v) milk in 1× TBS (for ODYSSEY analysis) or in 5% (w/v) milk in 1× TBST (for ECL analysis).

2.3.3.4 Immunoblotting and Detection

ODYSSEY Near-infrared Detection

After blocking, the membranes were incubated with one or two specific primaryantibodies (see table 1) in 1× TBST for 1h at room temperature, or over night at 4ºC with shaking. The membranes were then washed 3 times for 5 min each with 1× TBST. The membranes were incubated with one or two appropriate fluorescently-labelled secondary antibodies (see table 2) in 1× TBST for 1h at room temperature. Before scanning, the membranes were washed 3 times for 5 min each with 1× TBST.

ECL Detection

After blocking, the membranes were incubated with a specific primary antibody (see table 1) in 1% (w/v) milk or BSA in 1× TBST according to the manufacturer for 1h at room temperature, or over night at 4ºC with shaking. The membranes were then washed 3 times for 5 min each with 1× TBST. The membranes were incubated with the appropriate peroxidase-conjugated secondary antibody (see table 2) in 1% (w/v) milk in 1× TBST for 1h at room temperature. The membranes were washed 3 times for 5 min each in 1× TBST. Then, they were placed into ECL™ Western Blotting Detection Reagent for 1min to be revealed on a photographic film.

2.3.4 Immunoprecipitation

Cell lysates were adjusted to the concentration of 1 µg/µl of protein in a total volume of 500 µl in 3T3 lysis buffer. An aliquot of 25 µl of each cell lysates was kept to be resolved on a gel. Adequate volumes of protein G sepharose beads were washed 3 times in 3T3 lysis buffer to remove ethanol from the slurry. The beads were resuspended in 3T3 lysis buffer. The cell lysates were pre-cleared with 50 µl of washed protein G sepharose beads for 30 min at 4ºC. The beads were removed by centrifugation and the supernatants were placed into new eppendorfs. 5 µl of appropriate antibody was added to the cell lysates and the tubes were set to rotate for an hour at 4ºC. Then, 50 µl of protein G sepharose beads was added to the tubes, which were set to rotate for 2 hours or overnight at 4ºC. The protein G sepharose beads were pelleted by centrifugation at 13000 g for 1 min and at 4ºC. The supernatants were removed carefully by aspiration to avoid removing any beads. The beads were washed at least 3 times with lysis buffer. They were either resuspended in assay buffer (e.g. for Raf kinases assays) or

resuspended in 50 µl of 5×sample buffer and boiled for 5 min to be resolved by Western Blotting.

2.3.5 Phosphodiesterase Activity Assay

This assay measures the hydrolysis of cAMP by phosphodiesterases.

2.3.5.1 Activation of Dowex 1X8-400 anion exchange resin

Dowex resin was prepared in large batches. To activate the Dowex 1X8-400, 4L of 1M NaOH solution was added to 400 g of the resin and incubated at room temperature for 15 min with gentle mixing. The resin was allowed to settle by gravity. The supernatant was removed and the resin was washed at least 30× with 4L of distilled water until the pH reached 7. Then, the resin was resuspended into 4L of 1M HCl and incubated at room temperature for 15 min with gentle mixing before being allowed to settle by gravity. The resin was finally washed at least 3× with 4L of distilled water until the pH reached 3. The activated resin was stored in distilled water in a 1:1 ratio and at 4°C. Before used, the appropriate amount of beads was centrifuged to remove the water, and resuspended in absolute ethanol in a 2:1 ratio (EtOH:beads).

2.3.5.2 Protocol

Samples were diluted to the concentration of 1.5 µg/µl of protein in a total volume of 40 µl in PDE Assay diluting buffer (20 mM Tris/HCl, 10 mM $MgCl_2$, pH7.4). All tubes were set up on ice. 10 µl of PDE inhibitor at the indicated concentration was added to the protein solution. 50 µl of substrate solution (2 µM cAMP containing 3 µCi [^{3}H]cAMP in 20 mM Tris/HCL, 10 mM $MgCl_2$, pH7.4) was added to the mixture. The tubes were quickly vortexed to collect all the components at the bottom of the tubes. The tubes were incubated for 10 min at 30˚C and then for 1 min at 100˚C to inactivate any PDE present in the samples. The tubes were placed on ice to cool down. 25 µl of Snake Venom (*Crotalus atrox* venom from Sigma) at 1 mg/ml was added to the samples and the samples were incubated for 10 min at 30˚C. The Snake venom, which has 5'-nucleotidase activity, prevents hydrolysed cAMP from recircularising. The non-hydrolysed cAMP nucleotides were separated by batch binding of the mixture of Dowex-1-chloride. The Dowex removes the charged nucleotides but not the uncharged nucleosides. Dowex mixture was centrifuged to discar the dH2O and the Dowex resin was resuspended in absolute ethanol (in a 2:1 ratio). 400 µl of Dowex was added to the

tubes, mixed and incubated on ice for 20 min. The tubes were centrifuged at 13000 g for 2 min to pellet the Dowex beads. 150 µl of the clear supernatant was added to new eppendorf tubes containing 1 ml of Opti-scint scintillation fluid. The hydrolysis of cAMP was measured by using a Wallac 1409 liquid scintillation counter.

Tubes	Buffer	Inhibitor	Substrate	Snake venom&Dowex
Control	+		+	+
Samples	+	+	+	+
Blank	+		+	+
Tot Counts			+	

2.3.5.3 PDE Activity calculation

The PDE activity was calculated using the following equation:

$$\text{PDE Activity} = 2.61 \times [(\text{Control-A})/\text{Average Tot Count}] \times 10 \times (1000/\mu\text{g protein})$$

Where A represents the value obtained for each samples – blank.

2.61 stands for the Dowex practical concentration.

10 stands for the concentration of cAMP (in pM) hydrolysed in 100 µl in 10 min.

2.3.6 Raf Kinase Assays

2.3.6.1 Endogenous Raf-1 or B-Raf in PC12 cells

The activity of the Raf kinases (Raf-1 or B-Raf) was studied using the kinase cascade assay kit from Upstate. This assay kit is designed to measure the Raf dependent phosphotransferase activity in a kinase cascade reaction using inactive recombinant MEK1 as substrate for Raf kinases and inactive recombinant ERK2 as substrate for MEK1. To avoid interferences from active MEK and ERK in cell lysates, Raf-1 or B-Raf were immunoprecipitated as described in section 2.5.5. 10 µl of immunoprecipitated Raf were assayed for each condition in conjunction with 0.4 µg of inactive MEK1 and 1 µg of inactive ERK2 in Assay Dilution Buffer I (ADBI: 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) to bring the total volume of the reaction to 45 µl. The reaction was initiated by adding 10 µl of $MgCl_2$ ATP cocktail (500 µM cold ATP and 75 mM $MgCl_2$ in ADBI). The

tubes were centrifuged pulse to collect all the components at the bottom of the tubes and gently vortexed. The tubes were incubated for 30 min at 30°C. The reactions were stopped by adding 20 µl of 5× sample buffer and boiling for 5 min. The samples were analysed by Western Blot as described in section 2.5.3. The membranes were immunoblotted for phosphorylated ERK to measure Raf activity. Then the membranes were probed for total ERK and Raf-1 or B-Raf to ensure equal loading.

2.3.6.2 Transfected B-Raf in HEK 293 cells

A one-step kinase assay was used to assess the activity of transfected B-Raf in HEK 293 cells. In this assay, only inactive recombinant MEK1 was used as B-Raf substrate. FLAG-tagged transfected B-Raf was immunoprecipitated using an anti-FLAG antibody as described in section 2.5.5. 10 µl of immunoprecipitated B-Raf was assayed in combination with 1 µg of inactive recombinant MEK1 and 20 µl of $MgCl_2$ ATP cocktail (500 µM cold ATP and 75 mM $MgCl_2$ in ADBI). The total volume of the assay was adjusted to 40 µl with ADBI (Assay Dilution Buffer I). The tubes were centrifuged pulse to collect all the components at the bottom of the tubes and gently vortexed. The tubes were incubated for 30 min at 30°C. The reactions were stopped by adding 20 µl of 5× sample buffer and boiling for 5 min. The samples were analysed by Western Blot as described in section 2.5.3. The membranes were immunoblotted for phosphorylated MEK to measure Raf activity. Then the membranes were probed for total MEK and B-Raf to ensure equal loading.

2.3.7 cAMP Assay

2.3.7.1 Principle

The level of cAMP in PC12 cells was measured using the cAMP HTS Immunoassay kit from Upstate®. This assay is a competitive enzyme-linked immunosorbent assay (ELISA) for sensitive and rapid chemiluminescent quantitation of cAMP from cell extracts. The principle of a competitive ELISAs is based on the competition between labelled and unlabeled antigen for available antibody binding sites. The standard and the unknown samples are mixed with an alkaline phosphatase (AP)-labelled cAMP conjugate and a specific anti-cAMP antibody, and incubated on well of 96-well microtiter plates coated with a capture antibody. The AP-labelled cAMP conjugate will bind to the anti-cAMP antibody where its binding sites are not occupied by unlabeled cAMP. Thus, a sample containing a high concentration of cAMP will have a low

concentration of labelled cAMP. Therefore, the chemiluminescence intensity is inversely proportional to the amount of cAMP in a sample or standard.

2.3.7.2 Protocol

The cAMP standard provided was diluted in lysis buffer to generate a range of cAMP standard dilutions with the highest concentration starting at 100 pmol/µl. Then, a serial 1:10 dilution was performed. The cell lysates were prepared as described in section 2.4. The protein concentration of each cell lysate was adjusted to 1 µg/µl in assay diluent buffer. 50 µl of cAMP standards and prepared samples were added in triplicate to each well of 96-well anti-rabbit coated plate provided by the kit. 25 µl of cAMP alkaline phosphatase conjugate tracer (diluted 1:3000 with 1× assay diluent) was added to all wells. Then, 50 µl of the rabbit anti-cAMP antibody (diluted 1:2000 with 1× assay diluent) was added to all wells. The plate was covered with cling film and incubated for 30 min at room temperature with shaking. Following the incubation time, the reagents were removed by inverting the plate over the sink and blotting the plate on a clean paper towel to remove excess fluid. The wells were washed 5 times with 1× wash buffer. 100 µl of the alkaline phosphatase substrate was added to each well. The plate was covered with cling film and incubated for 30 min at room temperature with shaking. Finally the plate was read for 1.0 second with a luminometer.

2.3.7.3 Calculation

The results obtained were analysed using curve fitting software called PRISM. The data was fitted to a one-site competitive binding curve.

2.3.8 Ras and Rap1 Activation Assays

The activity of Ras and Rap1 were measured by affinity pull-down assay. This assay is based on the differential affinity of Ras-GTP and Ras-GDP for the Ras Binding Domain (RBD) of Raf-1, and of Rap1-GTP and Rap1-GDP for the Rap Binding Domain (RBD) of RalGDS. Indeed, Raf-1 RBD binds specifically to GTP loaded Ras (the active form of Ras) and that RalGDS RBD binds specifically to GTP loaded Rap1 (the active form of Rap1). The GST-tagged RalGDS-RBD beads were obtained from Upstate®. The GST-tagged RBD of Raf-1 was expressed in *E. coli* and the fusion protein was purified on glutathione-Sepharose beads as described in section 2.3.2.

Cell lysates were adjusted to the concentration of 1 µg/µl of protein in a total volume of 500 µl. An aliquot of 25 µl of each cell lysate was kept to be resolved on a gel. The cell lysates were pre-cleared with 50 µl of washed protein G sepharose beads for 30 min at 4ºC. The beads were removed by centrifugation and the supernatants were placed into new eppendorfs. 5 µl of appropriate RBD was added to the cell lysates and the tubes were set to rotate for 2 h at 4ºC. The RBD beads were pelleted by centrifugation at 13000 g for 1 min and at 4ºC. The supernatants were removed by aspiration carefully to avoid removing any beads. The beads were washed at least 3 times with lysis buffer. They were resuspended in 30 µl of 5×sample buffer and boiled for 5 min before being resolved by Western Blotting.

2.3.9 Statistical analysis

Unless stated, each data was first analysed using the non-parametric Kruskal-Wallis one-way analysis of variance method to determine if significant differences existed within the data. If $p < 0.05$, then the test was followed by a Mann-Whitney analysis to assess whether two treatments were significantly different from each other. The p value was set to 0.05.

2.4 MOLECULAR BIOLOGY

2.4.1 Large scale production of Plasmid DNA

Plasmid DNAs were prepared and purified with the PureYield^{TM} Plasmid Maxiprep System from Promega.

2.4.1.1 Preparation and lysis of bacterial cell cultures

Transformed competent *E. Coli* cells were grown overnight in 250 ml of L-broth (170 mM NaCl, 0.5 % (w/v) Bacto-Yeast extract, 1 % (w/v) Bacto-Tryptone pH 7.5) supplemented with the appropriate antibiotic at 37ºC with shaking. The cells were pelleted by centrifugation at 5000 g for 10 min. The supernatants were discarded and the tubes were drained on a paper towel to remove excess media. The cells were resuspended in 12 ml of cell resuspension solution (50 mM Tris/HCL, 10 mM EDTA

and 100 μg/ml RNase A). 12 ml of cell lysis solution (0.2 M NaOH and 1% SDS) was added to each tube and mixed by inverting the tubes 3-5 times. The tubes were incubated for 3 min at room temperature. 12 ml of neutralisation solution (4.09 M guanidine hydrochloride, 759 mM potassium acetate and 2.12 M acetic acid) was added to the lysed cells. The mixtures were mixed by inverting the tubes 10-15 times to precipitate the cellular debris. The lysates were then centrifuged at 14000 g for 20 min at room temperature to pellet the cellular debris. The lysates were filtered through a cloth and transferred to fresh tubes.

2.4.1.2 DNA purification

The lysates were poured into the blue PureYield™ clearing column assembled on top of a white PureYield™ maxi binding column fixed onto a vacuum manifold port. Maximum vacuum was applied allowing the DNA to bind the binding membrane in the PureYield™ maxi binding column. After the vacuum was completed, the blue PureYield™ clearing columns were removed and discarded. 5 ml of endotoxin removal wash (complemented with the appropriate volume of isopropanol as described by the manufacturer) was added to the PureYield™ maxi binding column and vacuumed. 20 ml of column wash (60% ethanol, 60 mM potassium acetate, 8.3 mM Tris/HCl and 0.04 mM EDTA) was loaded onto the binding column and then vacuumed to draw the wash through. The membranes were totally dried by applying a vacuum for 5-10 min. The binding columns were placed into 50 ml centrifuge tubes. The DNA was eluted by adding 1.5 ml of nuclease-free water into the binding membranes. The tubes were centrifuged at 2000 g for 5 min to collect the DNA in the 50 ml tubes. The eluates were then transferred into bijou bottles.

2.4.1.3 Quantification of DNA

DNA was quantified by spectrophotometry. 1 μl of DNA was diluted in 1 ml of deionised water and absorbance measurement was taken at 260 nm.

2.4.2 GST fusion protein synthesis

2.4.2.1 Transformation of competent *E. Coli*

Competent *E. Coli* cells were transformed with 1μl of recombinant protein-containing construct plasmid DNA of interest and incubated on ice for at least 20 min. The cells were heat shocked at 42°C for 1 min and then placed on ice. They were transferred to

1ml of L-Broth and incubated for 1 h at 37°C with shaking. 100 μl of the culture was plated onto LB agar plate supplemented with 100 μg/ml of ampicillin. The plate was incubated at 37°C overnight. Colonies were picked from the plates, transferred into 50 ml of L-broth supplemented with 100 μg/ml of ampicillin and incubated overnight at 37°C with shaking.

2.4.2.2 Protein expression

The cells from the overnight culture were used to inoculate 450 ml of L-broth supplemented with 100 μg/ml of ampicillin and incubated at 37°C with shaking for 1 to 2 hours until the absorbance at 600 nm (OD_{600}) of the culture reached 0.6 to1.0. Then, the expression of fusion protein was induced by adding 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were grown for a further 2 to 5 hours at 30°C with shaking. Samples of cells before and after induction of protein expression were taken, separated by gel electrophoresis and visualised by Coomassie blue stain to ensure protein expression. The induced cells were pelleted by centrifugation at 8000 g for 10 min at 4°C and frozen at –80°C until required.

2.4.2.3 GST fusion protein elution and purification

Frozen cells were thawed on ice and resuspended in 10 ml of ice-cold PBS (1 tablet of PBS diluted in 100 ml of dionised H_2O) supplemented with 1mg/ml of lysozyme and transferred to “oak ridge” centrifuge tubes. The cells were sonicated on ice at 40 V for 3× 30 seconds periods with 30 seconds intervals. 1/500 volume of 10% Triton X-100 was added to the mixture to lyse the cells. The cell lysate was centrifuged at 11000 g for 15 min at 4°C. The supernatant containing the protein was transferred to 15 ml centrifuge tubes. 1 ml of glutathione beads was added to the supernatant and incubated for 1 hour at 4°C with rotation to enable binding to the protein. The beads were then collected by centrifugation at 3000 g for 2 min and the supernatant discarded. The beads were washed in 5 ml of PBS. After removing the supernatant, the beads were resuspended in 1 ml PBS and transferred to eppendorf tube. The beads were washed a further 3× in 1 ml of PBS. The fusion proteins were eluted from the beads with 600 μl of elution buffer (10 mM glutathione in 50 mM Tris/HCl, pH 8) as follows: the beads were resuspended in 600 μl of elution buffer, incubated for 20 min at 4°C with rotation, and pelleted by brief centrifugation. The supernatant was carefully removed to a fresh tube. The elution step was repeated 3 times and the eluates were pooled together.

Finally, the fusion protein sample was dialysed to remove glutathione in 650 ml of dialyse buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5% (v/v) glycerol) with 3 buffer changes.

CHAPTER 3

EFFECTS OF PDE INHIBITORS ON ERK PHOSPHORYLATION AND ON PC12 CELL DIFFERENTIATION

3.1 INTRODUCTION

The second messenger cAMP regulates a large variety of biological processes from metabolic to cellular signalling pathways. It is now commonly accepted that cAMP induces cell growth inhibition (Cook and McCormick, 1993; Sevetson et al., 1993; Stork and Schmitt, 2002; Wu et al., 1993). However, in certain cell types, cAMP is associated with cell proliferation and cell differentiation (Stork and Schmitt, 2002). In PC12 cells, it has been demonstrated that cAMP induces cell differentiation into sympathetic-like neurones (Dumaz et al., 2002; Houslay and Kolch, 2000; Piiper et al., 2002; Stork and Schmitt, 2002). The mechanisms through which this occurs are not totally understood and are highly controversial.

3.1.1 Cyclic AMP induces PC12 differentiation

As described in the general introduction of this thesis (see sections 1.1.7 and 1.1.8), the differentiation and the proliferation of PC12 cells are mediated through the activation of the ERK signalling pathway (Marshall, 1995). The fate of the cells has been demonstrated to be dependent on the longevity of the signal initiated by stimuli induced by different growth factors (Kao et al., 2001; Marais et al., 1995; Traverse et al., 1992). Upon EGF stimulation, the activation of ERK is transient and the cells proliferate, whereas upon NGF stimulation, the activation of ERK is sustained and the cells differentiate (Dichter et al., 1977; Greene and Tischler, 1976). It is well known that cAMP is associated with the differentiation of the PC12 cells into sympathetic-like neurones (Houslay and Kolch, 2000; Piiper et al., 2002). As described in the section 1.3 growing lines of evidence show that the second messenger cAMP interacts with the ERK signalling pathway, identifying PKA and Epac, the main effectors of cAMP, as the link molecules between these two pathways. It is widely accepted that PKA can inhibit Raf-1, whereas Epac has been shown to activate Rap1, which according to Stork and colleagues activates B-Raf (Stork and Schmitt, 2002). Raf-1 and B-Raf are the main protein kinases transducing the signal through the ERK pathway. Therefore, cAMP might play an important role in the mechanisms of the regulation of the ERK signalling pathway.

3.1.2 Selective Phosphodiesterase Inhibitors

The level of cAMP has to be elevated in the cells in order to study the crosstalk between the cAMP and the ERK signalling pathways. In theory, this can be done by either activating the production of cAMP using adenylyl cyclase activators such as forskolin (Hansen et al., 2003; Vossler et al., 1997) or pituitary adenylate cyclase-activating polypeptide PACAP (Deutsch and Sun, 1992), or by blocking the hydrolysis of cAMP by inhibiting the phosphodiesterases. Selective phosphodiesterase inhibitors have been synthesised to specifically inhibit the different PDE isoforms. The research and development on PDE inhibitors is growing rapidly and there are already three drugs targeting PDE5 (sildenafil, tadalafil and vardenafil) and three drugs targeting PDE3 (anagrelide, pimobendan, milirone) available on the market. There are two drug candidates of PDE4 inhibitors that are waiting for approval and about twenty PDE4 inhibitors are undergoing clinical studies. Vinpocetine, cilostamide and rolipram are specific inhibitors for PDE1, PDE3 and PDE4 respectively (Fig. 3.1). These compounds are competitive inhibitors for the cAMP binding site within the catalytic domain of their respective PDE. All PDEs contain a conserved catalytic domain of about 270 amino acids at the carboxyl terminus that consists of three helical subdomains: an N-terminal cyclin-fold region, a linker region and a C-terminal helical bundle. These three subdomains form a deep hydrophobic pocket. This pocket is composed of four sites (Fig. 3.2): a metal-binding site (M site), a core poket (Q pocket), a hydrophobic pocket (H pocket) and a lid region (L region) (Houslay and Adams, 2003; Jeon et al., 2005). The M site contains metal atoms likely to be zinc and magnesium, which are though to be involved in the stabilization of the structure and in the activation of hydroxide to mediate the catalysis. The Q pocket provides the specific hydrogen bonding between a conserved glutamine residue to all PDEs with the substrate or with the inhibitor. Vinpocetine is a potent vasodilatator and has been designed by different pharmacological companies as a drug for the potential treatment of vascular diseases including cerebrovascular and ophtalmological diseases, but due to poor efficacy and side effects (dizziness) the drugs were withdrawn. PDE1 inhibitors are also been investigated in the treatment of neurodegenerative diseases such as Parkinson's disease (Kakkar et al., 1996). Rolipram is the most studied PDE4 inhibitor and has been investigated for its anti-inflammatory effect in asthma (Giembycz, 2000; Houslay and Adams, 2003). However rolipram induces central nervous system and cardiovascular side effects. PDE3 inhibitors have inotropic and vasodilatory actions and were

developed as therapeutic drugs for the treatment of ischemic and idiopathic dilated cardiomyopathy, which is a syndrome characterised by impaired myocardial contractibility and inappropriate systemic and pulmonary vasoconstriction. PDE3 inhibitors have also been investigated in the treatment of type 2 diabetes mellitus (Reinhardt et al., 1995). It has also been suggesting that phoshpodiesterase inhibitors might serve as anti-cancer drugs (Hirsh et al., 2004). PDE inhibitors, by elevating the intracellular levels of cAMP, could suppress the activity of the oncogene Ras and therefore blocking the constitutive activation of ERK and induce apoptosis in certain type of cancer cells. PDE inhibitors might improve the chemotherapeutic of cancer treatments by reducing side effects.

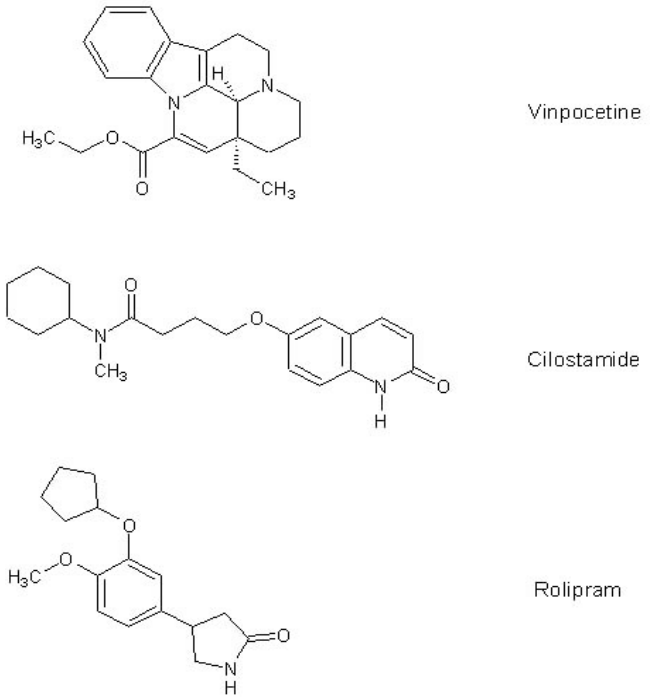


Figure 3. 1: Chemical structures of Vinpocetine, cilostamide and rolipram that are selective inhibitors for PDE1, PDE3 and PDE4 respectively.

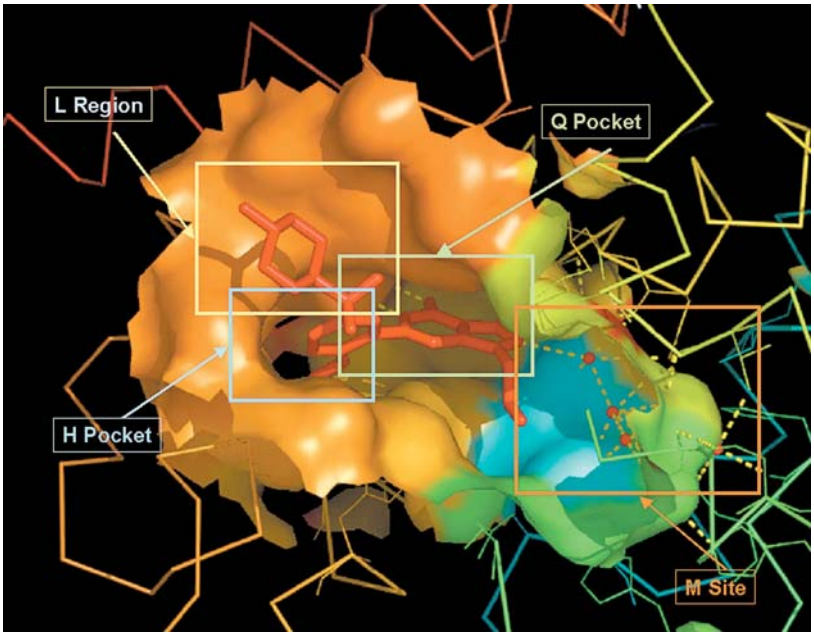


Figure 3. 2: Representation of the surface of the active site of PDE5A occupied by sildenafil (a PDE5 inhibitor). The active site is composed of four subsites: a metal-binding site (M site), a core pocket (Q pocket), an hydrophobic pocket (H pocket) and a lid region (L region). This picture was taken from (Jeon et al., 2005).

In this chapter, the effects of cAMP on the activation of the ERK signalling pathway and the differentiation of PC12 cells were investigated by elevating the levels of cAMP using phosphodiesterase inhibitors to block the degradation of cAMP. This chapter also aims to investigate if the signal initiated by NGF or EGF is mediated through distinct pathways or not.

3.2 RESULTS

3.2.1 ODYSSEY Near-Infrared Imaging System

3.2.1.1 Near-infrared fluorescence vs. chemiluminescence: stable signal and accuracy

Most of the Western blots performed throughout this work were analyzed and quantified using the ODYSSEY Near-infrared imaging system. This method was used preferably to conventional chemiluminescent detection methods that are less accurate for quantification. Chemiluminescent detection methods rely on enzymatic reactions producing light, which is detected on photographic films. However enzymatic reactions are not linear, and the horseradish peroxidase used is inhibited by substrate. Therefore, the light emission is not constant and is changing over time. Moreover, the dynamic range of X-ray film is limited, and especially when highly diverse signals are to be compared the detection by film also becomes non-linear. Consequently the exposure time to generate the data has to be optimized. This time-dependence and non-linearity of signal detection compromises quantification and accuracy of these methods.

In comparison, fluorescent detection is static. The amount of light produced from the excitation of a fluorescent dye is constant when the fluorescent dye is excited. This property makes fluorescent detection a more precise and accurate measurement of the differences in signal produced by fluorescent-labelled antibodies bound to proteins on a Western blot.

Fluorescent detection enables accurate quantification of proteins because the signal generated by the different amounts of proteins on a blot is linear and the dynamic range of fluorescence detection spans several orders of magnitude.

3.2.1.2 Sensitivity and low background

The ODYSSEY Near-infrared system is highly sensitive as near-infrared fluorophores available to label secondary antibodies provide excellent sensitivity. Moreover, in the near-infrared nitrocellulose membranes and biomolecules exhibit reduced autofluorescence, resulting in lower background and less signal interference.

3.2.1.3 Multiplexed detection and quantification

Multiplexed detection is another advantage of the ODYSSEY system. This scanner uses two separate near infrared (NIR) lasers and detectors to image labelled antibodies at 700 and 800nm simultaneously. Therefore, using appropriate primary and secondary antibodies that avoid cross-reactivity and using spectrally distinct fluorophores, two proteins or two different activity states of a protein (e.g. phosphorylation state) can be analysed at the same time. For example, one detection channel can be used to detect a phosphorylated protein and the second channel to detect the total amount of the protein of interest to accurately normalize the signal intensities and to correct for loading and sampling errors.

The ODYSSEY software provides tools to visualise, find and quantify the bands detected on the nitrocellulose membranes. The fluorescence detected in the 700nm channel image is displayed in red, while the fluorescence detected in the 800nm channel is displayed in green. Where the fluorescence from the two channels overlap, the image displayed is yellow (Fig. 3.3). Each band is automatically or manually found and a feature is drawn around the bands of interest. The image data within the feature are quantified immediately. When a feature is quantified, integrated intensity is calculated. The integrated intensity has also been referred to as pixel volume in other software. The integrated intensity is the sum of the intensity values for all pixels enclosed by a feature, multiplied by the area of the feature (counts×mm^2). Since background pixels should not be part of this calculation, the background is calculated and subtracted.

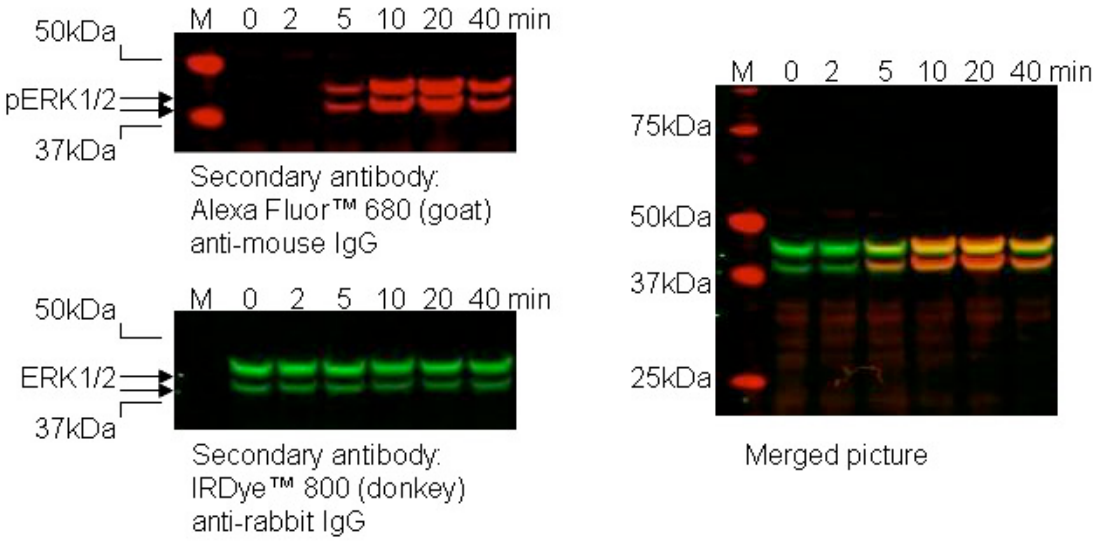


Figure 3. 3: Analysis of Western Blot using the ODYSSEY Near-Infrared imaging system. This technique allows accurate and reproducible quantification of Western Blots. Serum starved PC12 cells were stimulated with NGF (100ng/ml) for 0, 2, 5, 10, 20 and 40 min. The phosphorylation of ERK was evaluated by probing Western blots of cell lysates with mouse anti-phospho-ERK1/2 antibody detected with an Alexa fluor™ 680nm anti-mouse IgG secondary antibody. The total amount of ERK was evaluated by probing Western blots with rabbit anti ERK1/2 antibody detected with an IRDye™ 800nm anti-rabbit IgG secondary antibody. M stands for protein marker.

3.2.2 Kinetics of the phosphorylation of ERK upon NGF and EGF stimulation in PC12 cells

PC12 cells are a commonly used model cell system to study the ERK signalling pathway. These cells have the property to either proliferate or differentiate depending on the type of mitogen stimulation. Upon epidermal growth factor (EGF) stimulation cells proliferate. However, upon nerve growth factor (NGF) stimulation the cells differentiate into sympathetic-like neurons (Dichter et al., 1977; Greene and Tischler, 1976). These two easily distinguishable and mutually exclusive biological responses make the PC12 cell line an ideal model to study the diverse biological functions of ERK signalling pathway. The fate of the cell has been shown to depend on the longevity of the ERK signal (Kao et al., 2001; Marshall, 1995; Traverse et al., 1992). It is commonly accepted that EGF causes a transient activation of the ERK pathway (Kao et al., 2001) allowing the cells to continue to divide. However, NGF causes a sustained activation of the pathway causing the cells to withdraw from the cell cycle and differentiate (Traverse et al., 1992; Yao et al., 1998).

To verify that the PC12 cells used for this research behaved as described above, the cells were serum starved for 3 to 4 hours and then stimulated with either NGF or EGF for the indicated periods of time. Cell lysates were probed using antibodies against phosphorylated ERK to measure the activation of ERK, and against ERK to measure the total amount of protein loaded in the gel. The anti phospho-ERK antibody used was a monoclonal antibody raised in mouse, whereas the total ERK antibody was a polyclonal antibody raised in rabbit thereby avoiding cross-reactivity between both primary antibodies. The anti phospho-ERK antibody was detected using a secondary antibody directed against mouse IgG and labelled with a fluorophore that fluoresces at 700nm. The total ERK antibody was detected using a secondary antibody directed against rabbit IgG and labelled with a fluorophore that fluoresces at 800nm. Again, the two secondary antibodies were raised in different species (donkey and goat) to avoid any cross-reactivity. After scanning the nitrocellulose membranes with the ODYSSEY imager (Fig. 3.4.a), the bands detected were quantified using the ODYSSEY software. A ratiometric analysis was used to normalise the amount of phosphorylated ERK protein over the total amount of protein to correct any loading errors (Fig. 3.4.b). The results obtained were expressed as percentage of the highest ratio.

Upon NGF stimulation the peak of activation of the ERK pathway was around 10 minutes and ERK remained phosphorylated after 40 minutes. Whereas, upon EGF stimulation, the activation of ERK was quicker, reached the maximum activation around 5 minutes and was back to basal levels at 40 minutes. As expected, the activation of the ERK pathway in PC12 cells was sustained upon NGF stimulation, whereas the signal was transient upon EGF stimulation.

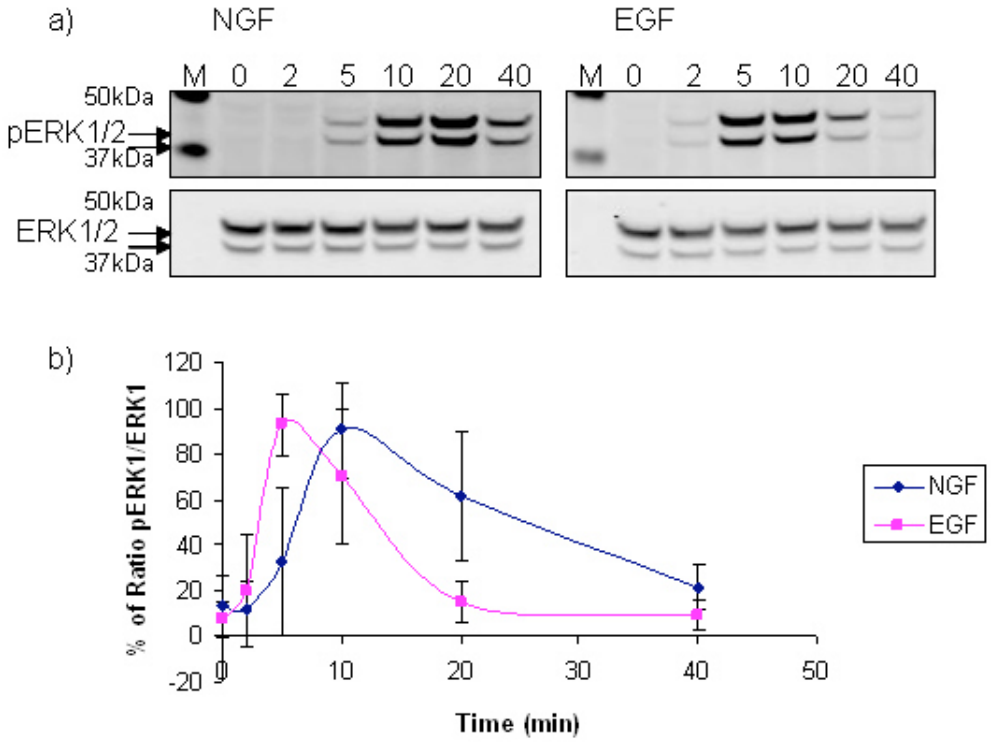


Figure 3. 4: Phosphorylation of ERK under NGF or EGF stimulation. Serum starved PC12 cells were stimulated with NGF (100ng/ml) or EGF (100ng/ml) for 0, 2, 5, 10, 20 and 40 min. a) The activation of ERK was evaluated by probing Western blots of cell lysates with mouse anti-phospho-ERK1/2 antibody detected with an Alexa fluor™ 680nm anti-mouse IgG secondary antibody. The total amount of ERK was evaluated by probing Western blots with rabbit anti ERK1/2 antibody detected with an IRDye™ 800nm anti-rabbit IgG secondary antibody. B) The values are expressed as the percentage of the highest ratio of the integrated intensities of pERK over totalERK. The values shown are the means ± S.D. of 7 independent experiments. A repeated measures ANOVA test was performed: $p<0.001$, which confirmed that these curves are significantly different. M stands for protein marker.

3.2.3 Effect of PDE inhibitors on the phosphorylation of ERK

To assess the existence of a possible crosstalk between the ERK and the cAMP signalling pathways, the first step was to increase the level of cAMP in PC12 cells. Instead of stimulating the production of cAMP using adenylyl cyclase activators, such as forskolin (Hansen et al., 2003; Vossler et al., 1997) or pituitary adenylate cyclase-activating polypeptide PACAP (Deutsch and Sun, 1992), a different approach was taken. Phosphodiesterase (PDE) inhibitors were used to inhibit specific isoforms of PDEs in order to block the degradation of cAMP (Manallack et al., 2005). It is generally accepted that PDEs control discrete pools of cAMP in specific localisations within the cells (Fischmeister et al., 2006; Houslay and Adams, 2003; Mongillo et al., 2004; Shakur et al., 2000). Therefore, by using specific PDE inhibitors to block the degradation of cAMP, the pool of cAMP that might regulate the ERK pathway can be identified. Different PDE inhibitors available were screened and the phosphorylation of ERK was measured by quantifying Western blots using the ODYSSEY imager. It was also expected from this experiment to obtain some clues about which of York's or Kao's models (described in section 1.1.8) to support.

The PC12 cells were serum starved for 3 to 4 hours in a serum-free medium. The cells were treated for 10 minutes with the different PDE inhibitors before being stimulated with either NGF or EGF for the indicated periods of time. The cell lysates were analysed by Western blot using the ODYSSEY imager. The nitrocellulose membranes were probed with antibodies against phosphorylated ERK to measure the activation of ERK, and against ERK to measure the total amount of protein loaded in the gel. These primary antibodies were detected with different fluorescent secondary antibodies as described in the section 3.1.2. Then, the ratio between pERK and total ERK (integrated intensity values) were calculated and normalised as percentage of the highest ratio, which was the peak of activation upon NGF or EGF stimulations without the PDE inhibitor treatment. The results obtained were plotted onto a graph (Fig. 3.5).

First of all, upon any PDE inhibition and without mitogen stimulation, ERK was not phosphorylated (results not shown). This meant that the accumulation of cAMP in the basal state was not enough to activate the ERK pathway.

However, upon NGF stimulation, the phosphorylation of ERK was increased when the cells were treated with the PDE inhibitors. The signal was the strongest when PDE3 was

inhibited by cilostamide, where the phosphorylation of ERK was significantly increased 2.6 fold after 10 min of stimulation. Unexpectedly, the inhibition of PDE4 (a cytosolic PDE) by rolipram did not significantly increase the phosphorylation of ERK.

Upon EGF stimulation, every PDE inhibitor increased the phosphorylation of ERK. More remarkably the maximum of phosphorylation was located between 5 to 10 minutes instead of 5 minutes in normal conditions. Again the activation of ERK was the strongest when PDE3 was inhibited by cilostamide, which was significantly different from the control after 10 of stimulation. But ERK phosphorylation was only increased by 1.6 fold.

The results obtained upon EGF stimulation were quite surprising because it was expected that upon high levels of cAMP the ERK signalling pathway would be switched off resulting in no ERK phosphorylation. According to the York *et al*'s models (York et al., 1998), it was expected that the signal initiated by EGF would utilise only the Ras/Raf-1/MEK/ERK pathway. Research had shown that cAMP activates PKA that inhibits Raf-1 (Dumaz and Marais, 2003; Sidovar et al., 2000). Therefore, the pathway should be switched off at Raf-1 level and the signal should no longer be transduced toward ERK. The results obtained suggested that high level of cAMP upon mitogen stimulation activate or switch to other pathways such as Rap1/B-Raf/MEK/ERK (Dumaz and Marais, 2005; Houslay and Kolch, 2000; Stork and Schmitt, 2002), which support the Kao *et al*'s model (Kao et al., 2001). Another explanation is that Ras activates B-Raf and therefore would overcome the inhibition of Raf-1. This will be investigated further in the next chapters.

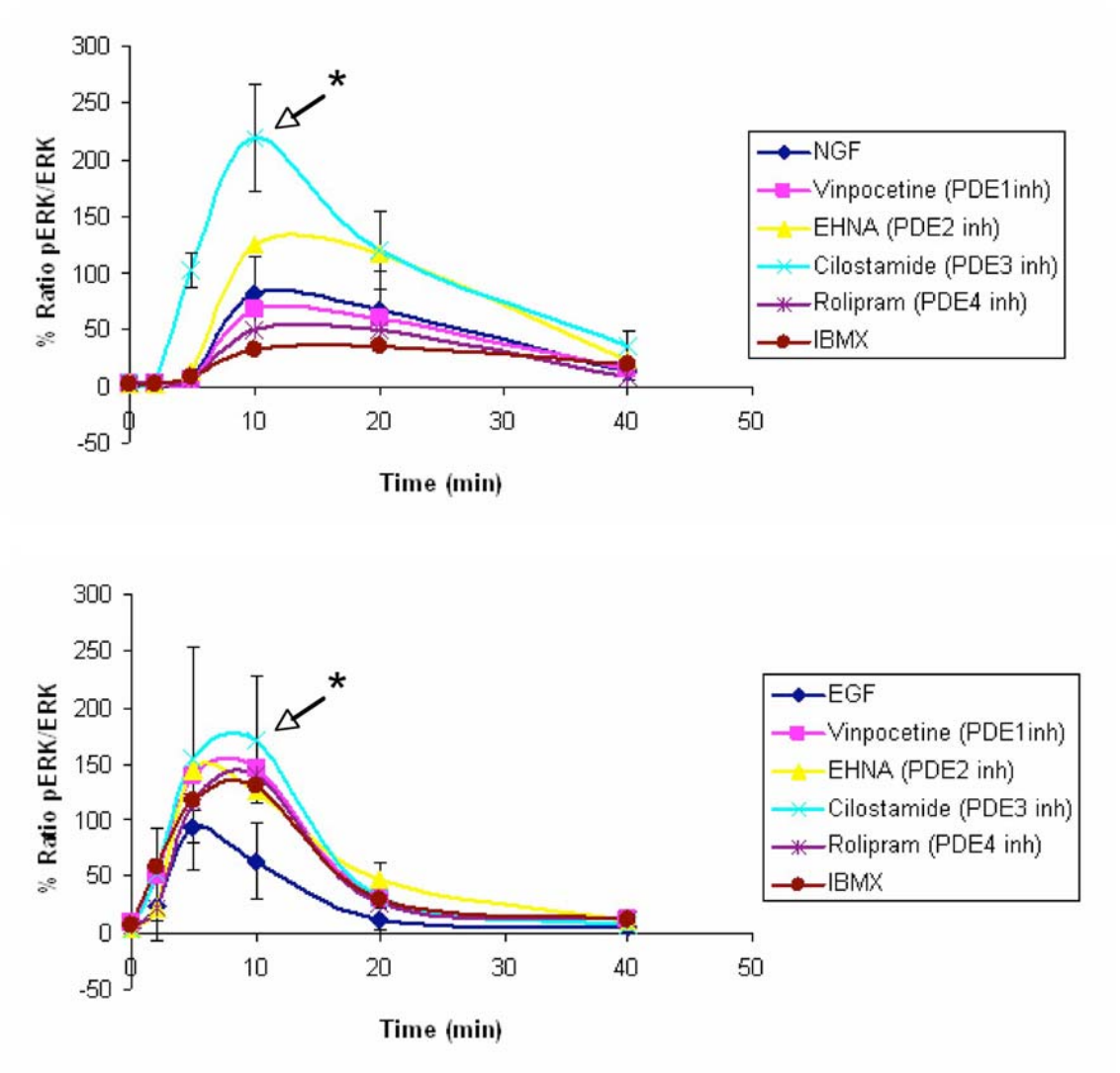


Figure 3. 5: Effect of PDE inhibitors on the phosphorylation of ERK. Serum starved PC12 cells were treated with Vinpocetine (100µM), EHNA (10µM), Rolipram (10µM), Cilostamide (10µM) and IBMX (100µM) for 10 min before being stimulated with either a) NGF (100ng/ml) or b) EGF (100ng/ml) and incubated at 37ºC for 0, 2, 5, 10, 20 and 40 min. The activation of ERK was evaluated by probing Western blots of cell lysates with anti-phospho-ERK1/2 antibody. The total amount of ERK was evaluated by probing Western blots with anti ERK1/2 antibody. The values shown are the mean of 3 independent experiments ± S.D. Effects of each PDE inhibitors were compared to respective basal stimulation values at time 10 min, *$p < 0.05$. Only the error bars for NGF and EGF stimulations without treatment and upon cilostamide treatments are shown for graph clarity.

3.2.4 Assaying changes in cAMP levels caused by PDE inhibitors

In order to study the effects of cAMP on the ERK signalling pathway, the approach chosen was to raise the concentration of cAMP by blocking its degradation using PDE inhibitors. From the results described above (section 3.1.3), it has been demonstrated that the treatment with specific PDE inhibitors modulated the phosphorylation of ERK by enhancing or decreasing it. It was then crucial to assess if the level of cAMP actually rose within the cells.

PC12 cells were plated onto 10 cm dishes pre-coated with poly-L-Lysine and left to set overnight. They were treated (or not in the control) for 30 min, the time necessary to achieve total inhibition, with the different PDE inhibitors at the concentrations indicated below. Then the cells were stimulated for 10 min with forskolin to activate the production of cAMP by the adenylyl cyclases. The concentration of cAMP was analysed from cell lysates using a cAMP HTS Immunoassay kit from Upstate®, which is a competitive ELISA. The samples were added in triplicates along with cAMP alkaline phosphatase conjugate tracer onto a 96 well-plate pre-coated with anti-rabbit antibody. Then, a rabbit anti-cAMP antibody was added to all wells. Therefore, the cAMP contained in each sample and the cAMP alkaline phosphatase conjugate tracer competed to bind to the anti-cAMP antibody, which bind to the anti rabbit antibody coated to the bottom of the wells. After incubation time followed by vigorous washings, alkaline phosphatase substrate was added to all samples. The samples were read using a luminometer. Because this assay is a non-competitive ELISA, high concentration of cAMP gives low intensity. The results obtained were analysed using curve fitting software called PRISM. The data was fitted to a one-site competitive binding curve (Fig. 3.6.b) and the results were plotted on the graph below (Fig. 3.6.c).

As expected, upon forskolin stimulation, cAMP accumulated when the cells were treated with specific cAMP-phosphodiesterase inhibitors compare to the control where the cells were only stimulated with forskolin. However, it was expected that the increase in the level of cAMP would be much greater upon the treatment with cilostamide following the results described in section 3.1.3. Surprisingly, the increase in cAMP level was approximately the same in response to the different PDE inhibitor treatments. Then, how was it possible that each PDE inhibitor treatment had such different effects on the phosphorylation of ERK? This result might be evidence of the compartmentalisation of cAMP controlled by PDEs, because only the pool of cAMP

regulated by PDE3 can affect the phosphorylation of ERK. This question will also be discussed further in the discussion part of this chapter.

It has to be notified that the basal concentration of cAMP was not actually measured in this experiment. It was the changes in the accumulation of cAMP induced by forskolin and the inhibition of specific PDEs that was measured in this assay. Indeed, this assay was not sensitive enough to detect any cAMP concentration when the production of cAMP was not stimulated. Therefore the production of cAMP had to be induced by an elevating cAMP agent such as forskolin, which is the most commonly used adenylyl cyclase activator. However this experiment reflected the changes in the concentration of cAMP and was suitable to demonstrate that in the presence of PDE inhibitors, then cAMP accumulated within the cells. To consolidate these results, it would have been interesting to the same experiment in a different cell line, such as HEK293 cells that are an ideal model to study the cAMP signalling. It would have been also judicious to use other techniques to measure the level of cAMP in the cells. There are other tools available such as fluorescence resonance energy transfer (FRET) for example. This technique is based on the energy transfer mechanism that occurs between two chromophores that are in molecular proximity to each other. This technique allows monitoring the changes in the distance of chromophores attached to either two interacting proteins or to a single protein with a changing conformation. PKA can be used as cAMP sensors. This technique is based on the dissociation of the PKA subunits. In the absence of cAMP, PKA is a tretrameric holoenzyme consisting of two regulatory (R) subunits and two catalytic (C) subunits. In the presence of cAMP, the two C subunits dissociate from the R subunits. The R and the C subunits can be genetically labelled with cyan (CFP) and with yellow (YFP) fluorescent proteins respectively. Therefore, in the presence of cAMP, PKA is in a tetrameric configuration in which the C and R subunits are bound, which result in a high FRET signal. In the presence of cAMP, the R and the C subunits dissociate resulting in the loss of the FRET signal. This technique is used to monitor the changes in cAMP concentration and compartmentation in living cells using confocal microscopy. However this technique is difficult to carry out and has some disadvantages, such as transfection or the fact that active PKA phosphorylates and activates PDE4 to hydrolyse cAMP, which interferes with the accuracy of the measurement of cAMP (Nikolaev and Lohse, 2006).

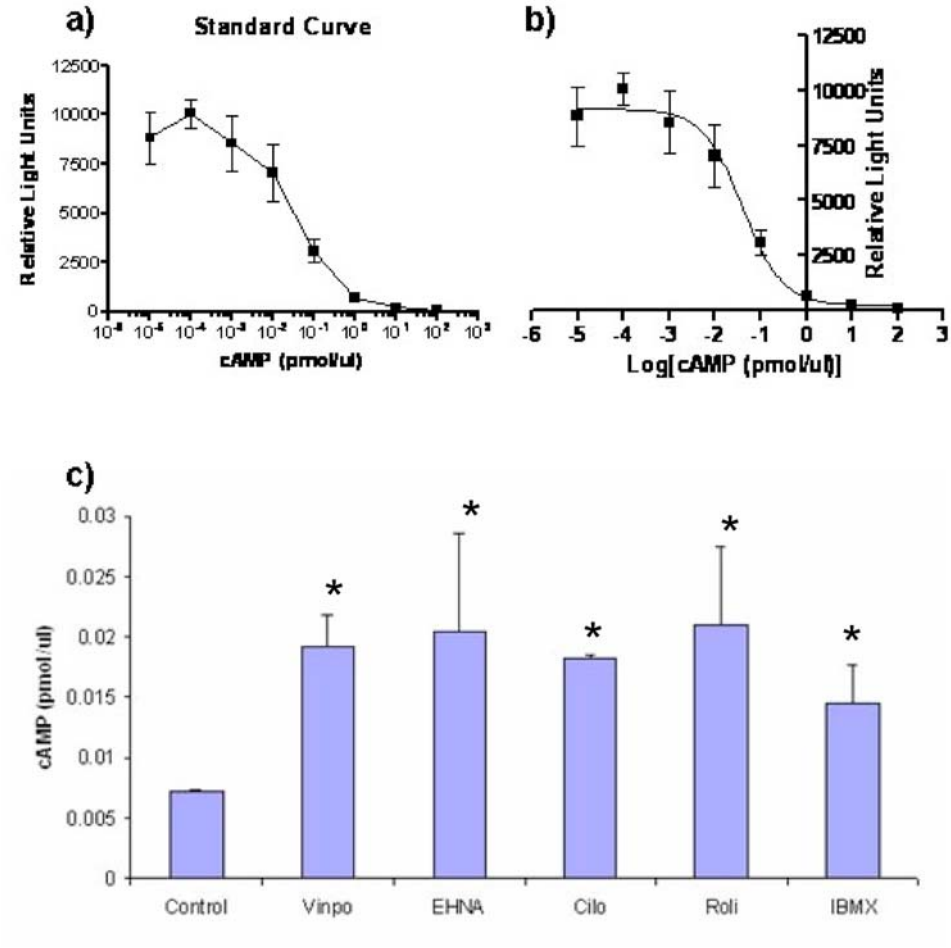


Figure 3. 6: Measurements of the level of cAMP when PDEs are inhibited with specific inhibitors. PC12 cells were treated with Vinpocetine (100µM), EHNA (10µM), Rolipram (10µM), Cilostamide (10µM) and IBMX (100µM) for 10 min before being stimulated with Forskolin (100µM) to generate the production of cAMP. The amount of cAMP was measured using the cAMP HTS Immunoassay kit from Upstate®, which is a competitive enzyme-linked immunosorbent assay (ELISA). The results were analyzed using PRISM software. The values shown are the mean of 3 different experiments ± SEM. *$p < 0.05$ compared to the control.

3.2.5 Analysis of PDE3 and PDE4 activities in PC12 cells

Since it was demonstrated that the inhibition of PDEs enabled a raise in the level of cAMP within the cell and that resulted in enhancing the phosphorylation of ERK upon cilostamide and rolipram treatments, the activity of both PDE3 and PDE4 had to be measured in order to understand why cilostamide had a greater effect on ERK phosphorylation than rolipram upon mitogen stimulation.

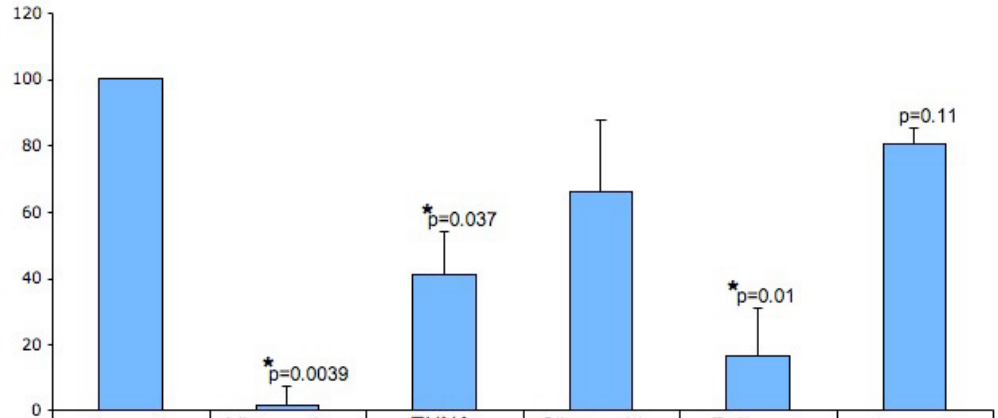
PC12 cells were seeded onto 6-well plates pre-coated with poly-L-Lysine and left to set overnight. They were harvested and lysed as described in the section 2.1.6 of the previous chapter. The principle of the assay is to measure the hydrolysis of cAMP by phosphodiesterases. To measure the activity of one specific PDE, this PDE is specifically inhibited and the activity of this PDE is then defined as the fraction of the total PDE activity that has been inhibited:

Specific PDE activity = Total PDE activity – PDE activity treated with inhibitor

Each lysate was assayed for the total PDE activity (control and IBMX, which is a non-specific PDE inhibitior), PDE1, PDE2, PDE3 and PDE4 activities. The activity of PDE1, PDE3 and PDE4 were measured in the presence of vinpocetine, EHNA, cilostamide and rolipram, respectively, at the indicated concentrations.

The results show that the contribution of PDE3 to the total PDE activity is almost 4 times higher than PDE4 (Fig. 3.7). This would explain why the inhibition of PDE3 by cilostamide has a greater effect on the phosphorylation of ERK compared to the inhibition of PDE4 by rolipram. However, this does not eliminate the hypothesis stipulating that PDEs are localised within discrete subcellular microdomains delimiting specific pools of cAMP, hence PDE3 would control a pool of cAMP surrounding the ERK signalling pathway in PC12 cells.

From these results it seems that the contribution of PDE2 to the total PDE activity is also high. However, this might not reflect the reality due to the fact that EHNA is not a very potent and selective PDE2 inhibitor (Chambers et al., 2006). Therefore, EHNA might also inhibit other phosphodiesterases compromising the real contribution of PDE2 to the total PDE activity.



	Control	Vinpocetine PDE1	EHNA PDE2	Cilostamide PDE3	Rolipram PDE4	IBMX
PDE activity %	100	1	41	66	17	80
in pmol/mg/min	64.2-135.5	0-6.7	16.5-50.3	23.8-87.9	0-23.9	52.3-109.6

Figure 3. 7: Activity of PDE3 and PDE4 in PC12 cells. This graph shows the total cAMP PDE activity in PC12 cells assayed with 1μM cAMP as substrate. PDE3 activity is defined as the fraction inhibited by 10 μM of Cilostamide, PDE4 activity is defined as the fraction inhibited by 10 μM of Rolipram, PDE1 is defined as the fraction inhibited by 100 μM of Vinpocetine and PDE2 is defined as the fraction inhibited by 10 μM of EHNA. The values shown are means ± S.D. of 6 different experiments. The activity of PDE1, PDE2 and PDE4 were compared to the activity of PDE3, * $p < 0.05$

3.2.6 Analysis of the total PDE activity upon mitogen stimulation

So far, it has been demonstrated that a high level of cAMP regulated by PDE3 enhances the phosphorylation of ERK upon mitogen stimulation. Little is known about the mechanisms through which this occurred. This is likely due to the activation of PKA and Epac by cAMP (Stork and Schmitt, 2002), which will be discussed in the next chapter. It was then important to investigate how the level of cAMP might change within the cells. Two different hypotheses had to be investigated in order to understand the cross-talk between the ERK and the cAMP signalling pathways. The first hypothesis is that mitogens activate adenylyl cyclase that would increase the production of cAMP. Unfortunately, the results obtained, using the Upstate kit to measure cAMP (results not shown), were not significant enough to allow a definitive conclusion. However, it has been demonstrated by different research groups that NGF increases the level of cAMP by activating soluble adenylyl cyclase (Golubeva et al., 1989; Knipper et al., 1993; Stessin et al., 2006) in PC12 cells. The second hypothesis is that mitogens inhibit PDEs to block the degradation of cAMP. In non-neuronal cells, it has been demonstrated that activated ERK upon mitogen stimulation, can phosphorylate and inhibit PDE4 (Baillie et al., 2000; Hoffmann et al., 1999).

To test this hypothesis, PC12 cells were seeded onto 6-well plates pre-coated with poly-L-lysine and left over night to set. After they had been serum starved for 3 to 4 hours, the cells were stimulated with either NGF or EGF at the concentrations indicated below for 0, 2, 5, 10, 20 and 40 minutes. The lysates were assayed with 1µM of cAMP as described in the section 2.2.6 to measure the total PDE activity.

The results (Fig. 3.8) demonstrated that there was no significant effect on the total PDE activity upon mitogen stimulation over time. Thus, within the sensitivity of the assay mitogens do not seem to regulate the activity of PDEs in PC12 cells. Therefore, it was assumed that the production of cAMP in response to mitogen stimulation was due to stimulation of adenylate cyclases rather than inhibition of PDEs.

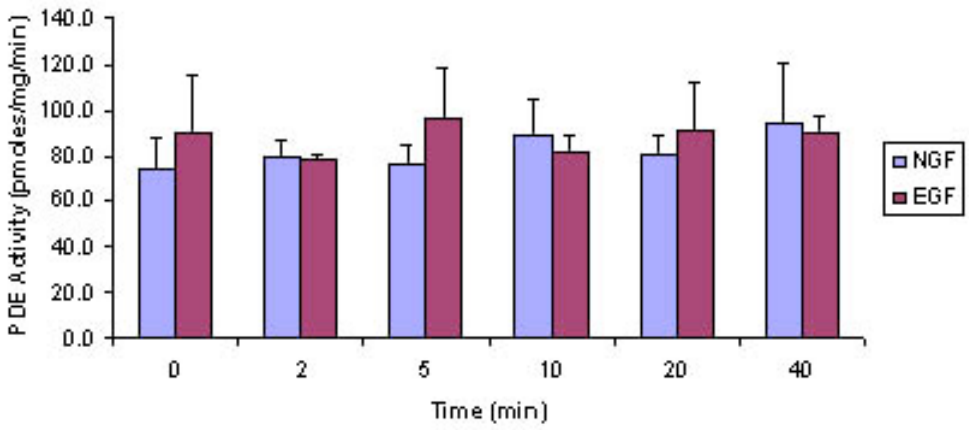


Figure 3. 8: Mitogens have no effect on the total activity of phosphodiesterases in PC12 cells. PC12 cells were stimulated with either NGF (100ng/ml) or with EGF (100ng/ml) for 0, 2, 5, 10, 20 and 40 minutes. The lysates were assayed with 1μM of cAMP as substrate to measure the total activity of PDEs. The results shown are expressed in pmoles/mg/min and are the mean ± S.D. of 3 independent experiments. The PDE activity for each time was compared to time 0 of the respective mitogen stimulation, * $p < 0.05$.

3.2.7 Effect of PDE inhibitors on PC12 cell differentiation

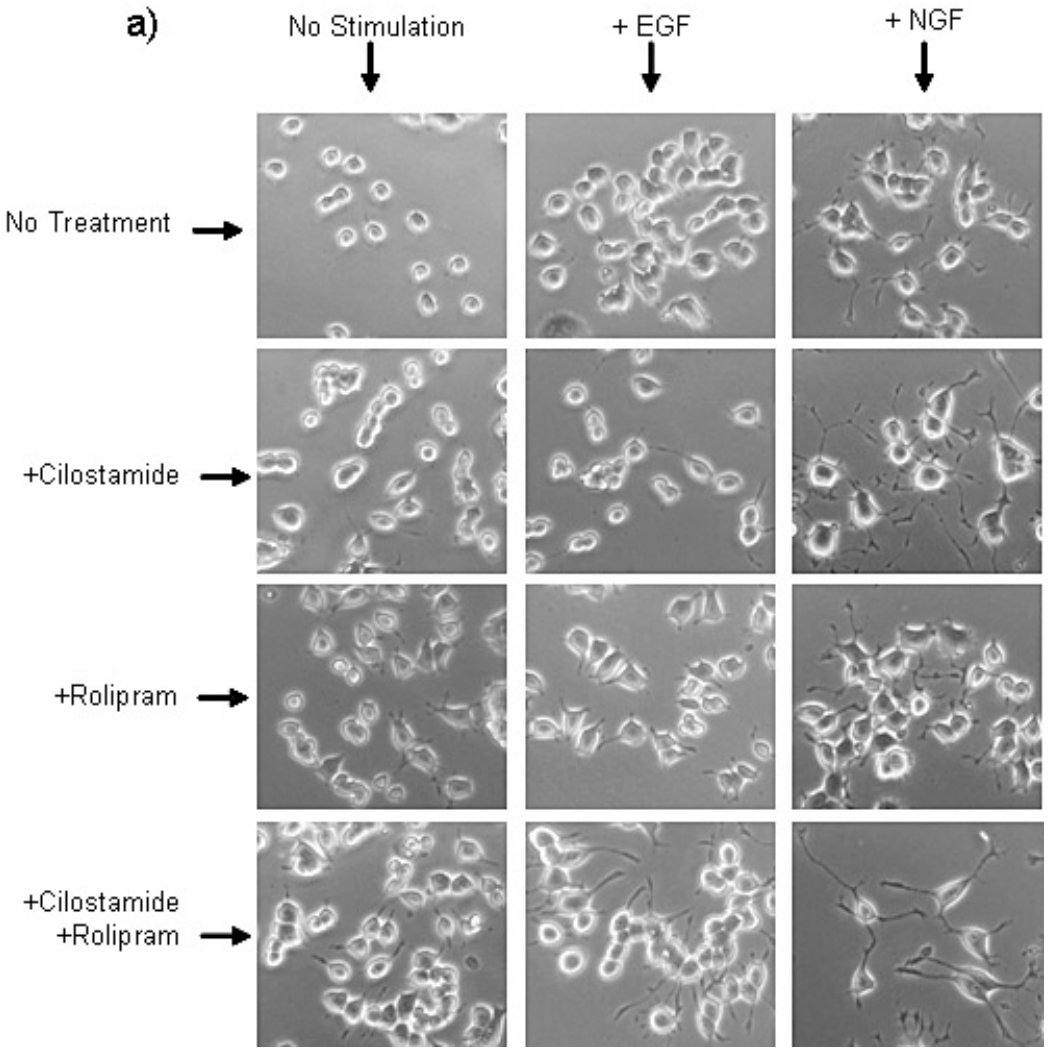
It is well established that NGF mediates PC12 cells differentiation (Deutsch and Sun, 1992; Greene and Tischler, 1976; Severin and Kondratyev, 1988; Sun et al., 2006; Traverse et al., 1992) by activating and sustaining the ERK signalling pathway. The exact mechanisms through which this occurs remain unclear at present and are at the centre of this research. The second messenger cAMP might be a key component in cell differentiation. Several research demonstrated that cAMP analogues (Schubert et al., 1977; Vossler et al., 1997), pituitary adenylate cyclase-activating polypeptide PACAP (Deutsch and Sun, 1992) or forskolin (Hansen et al., 2003; Vossler et al., 1997), which all increase the level of cAMP, promote the differentiation of PC12 cells.

Since the inhibition of PDEs has been demonstrated to increase the level of cAMP and to enhance the phosphorylation of ERK, it was crucial to examine whether it can also affect the differentiation of PC12 cells. To assess the possible involvement of cAMP in cell differentiation, PC12 cells were seeded onto 6-well plates pre-coated with poly-L-lysine and incubated in normal medium at 37ºC and allowed to set over-night. At day 0, the cells were studied under the microscope to ensure that no cells were differentiated. The cells were then treated with cilostamide, rolipram or both PDE inhibitors at the indicated concentrations for 10 minutes before being stimulated with either NGF or EGF. The cells were then incubated for 72 hours at 37ºC. About 10 phase-contrast images of the cells were taken randomly for each treatment to have a significant number of cells. A cell was only considered fully differentiated if the neurite extension was twice the length of the cell body. The percentage of differentiated cells was expressed as the number of neurite-extending cells compared to the total number of cells for each treatment (Fig. 3.9).

As expected, without stimulation or upon EGF stimulation and after 72h of incubation, PC12 cells remained round in morphology, meaning they did not differentiate. Upon NGF stimulation PC12 cells presented long neurites and 42% of the cells had differentiated.

When the cells were treated with either cilostamide or rolipram and without mitogen stimulation, cells remained undifferentiated. Upon NGF stimulation, the treatment with cilostamide dramatically increased the differentiation of PC12 cells and 69% of the cells

differentiated. The treatment with rolipram did not increase the differentiation of the cells upon NGF stimulation and the cells differentiated normally. PDE4 being an ubiquitously expressed phosphodiesterase, it was expected that rolipram would have the same effect than cilostamide on the cell differentiation. However, these results are in line with the previous results (see section 3.2.3) showing that rolipram did not increase the phosphorylation of ERK upon NGF stimulation. Interestingly, when both PDE inhibitors were combined 75% of the cells differentiated, which was not significantly different from the treatment with cilostamide only, but these cells presented longer and more robust neurites. The neurites were twice longer than the neurites seen on the cells that had differentiated upon NGF stimulation alone or with cilostamide treatment. The most interesting effect was the combination of both PDE inhibitors upon EGF stimulation. Under these conditions, 34% of the cells differentiated significantly compared to the treatment with EGF, which was not expected as EGF promotes only proliferation but not differentiation of the cells. Thus, the "proliferative" effect of EGF was turned into a "differentiative" effect. Moreover, 17% of the cells had differentiated without mitogen stimulation when both PDE inhibitors were combined, suggesting that the accumulation of cAMP resulting from the combination of different PDE inhibitions can activate pathways involved in cell differentiation.



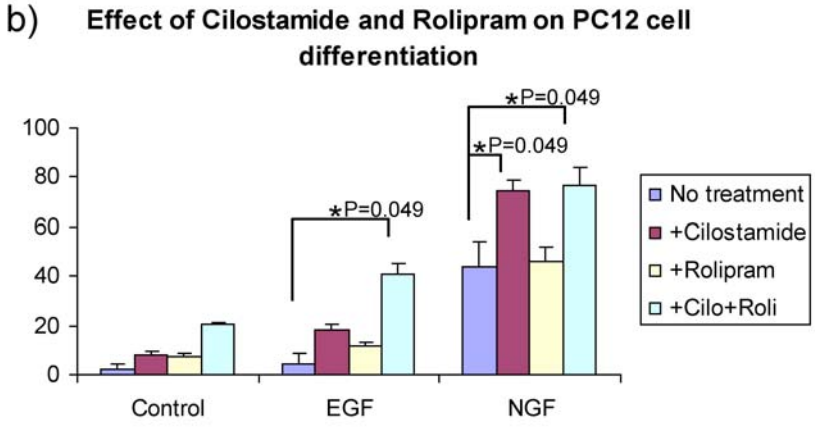


Figure 3. 9: Effect of Cilostamide and Rolipram on PC12 cell differentiation. A) PC12 cells were treated with Cilostamide (10μM) or Rolipram (10μM) for 10 min before being stimulated with either NGF (100ng/ml) or EGF (100ng/ml) and incubated at 37ºC. After 72h of incubation, phase-contrast images of representative cells were taken. B) The percentage of differentiated cells was determined by comparing the number of neurons over the total number of cells for each treatment. The values shown are means ± S.D. of the mean of 3 independent experiments. * $p < 0.05$.

3.3 DISCUSSION

Within the past decade, several lines of evidence for a crosstalk between the ERK and the cAMP pathways have been demonstrated (Houslay and Kolch, 2000; Qiu et al., 2000; Stork and Schmitt, 2002). It seems that the second messenger cAMP might be involved in the regulation of the ERK signalling pathway and might play an important role in the duration and the strength of the signal transduced. The choice of the cell line to study this crosstalk was really important. The PC12 cell line is an ideal model and is widely used to study the ERK signalling pathway. Upon different mitogen stimuli these cells have the property to either proliferate or differentiate, which are two biological processes easily distinguishable under a microscope. Therefore, PC12 cells were a perfect cell line to study cell differentiation. Both proliferation and differentiation are the results of the activation of the ERK pathway (Greene and Tischler, 1976; Houslay and Kolch, 2000; Kao et al., 2001). It was demonstrated that the fate of the cell depends on the longevity of the activation of the pathway (Traverse et al., 1992). Upon EGF stimulation, the signal transduced through the ERK pathway is transient and PC12 cells proliferate, whereas upon NGF the signal is sustained and the cells differentiate into sympathetic-like neurones.

Western blotting was the technique used to measure the phosphorylation of ERK throughout this research. This choice can be contested and some would argue that protein kinase assay was a more suitable technique to use because it is a more sensitive and it is a better quantitative assay than Western Blot. However, due to the number of samples to be analysed at once, protein kinase assays were too inconvenient for different reasons. First of all, a protein kinase assay is based on the phosphorylation of a substrate using the transfer of the γ-phosphate of adenosine-5'-[^{32}P] triphosphate ([γ-^{32}P] ATP) by the protein of interest. Therefore, this assay is highly radioactive and is very difficult to handle with a large number of samples. The second disadvantage is that kinase assays require to immunoprecipitate the total amount of the active kinase from the whole cell extract, which contains detergents and biochemicals in the lysis buffer that may affect the kinase activity. Moreover, immunoprecipitation is time consuming and part of the protein of interest can be lost during manipulation, which may alter the accuracy of this technique. Therefore Western blotting with antibodies indicative of activation was preferred to protein kinase assay to measure the activity of ERK. It had

been demonstrated and it is generally accepted that for ERK in particular, the results obtained by protein kinase assays correlate with the results obtained by Western blotting with activation specific (i.e. phosphorylation specific) antibodies. Western Blot is an easier and less time consuming technique. However, for the reasons described above it is not a good technique for quantification when the commonly used ECL technique is employed. Fortunately, we had an ODYSSEY scanner available. As described in the section 3.1.1 of this chapter, the ODYSSEY scanner allows rapid and accurate quantifications of blots probed with fluorescent-labelled secondary antibodies. It enables the comparison of different blots because pERK and the total amount of ERK were measured at the same time and on 2 different channels, which allows the normalisation of the results as a ratio pERK/ERK for each samples and therefore to correct any loading sample errors. Western blots analysed with the ODYSSEY scanner are well suited for quantification and for handling a large number of samples.

To investigate the crosstalk between the ERK and the cAMP pathways, the level of cAMP had to be increased. Two different approaches were considered. The first approach was to activate the production of cAMP by activating adenylyl cyclases using activators such as forskolin or PACAP. Several studies already had demonstrated that forskolin and PACAP increased the level of cAMP and enhanced the differentiation of PC12 cells (Deutsch and Sun, 1992; Hansen et al., 2003; Vossler et al., 1997). The second approach was to inhibit the degradation of cAMP using PDE inhibitors. It is generally accepted that PDEs control discrete pools of cAMP in specific localisations within the cells (Fischmeister et al., 2006; Houslay and Adams, 2003; Mongillo et al., 2004; Shakur et al., 2000). This approach was chosen not only because it has never been investigated previously, but also because it was a more subtle way to study the crosstalk between the ERK and cAMP pathways. The second messenger cAMP is involved in a plethora of other pathways than the MAPK pathway (Tasken and Aandahl, 2004). Therefore, increasing the general content of cAMP in the cell by activating adenylyl cyclases might affect these other pathways. However, inhibiting specific isoforms of PDEs using specific inhibitors can affect the level of cAMP within discrete cellular compartments, which might avoid crosstalk with other pathways. The results obtained show that not only the inhibition of specific PDEs increased the level of cAMP when the cells were stimulated with forskolin (Fig. 3.6) but the inhibition of PDE3 greatly increased the phosphorylation of ERK (Fig. 3.5) and the differentiation of PC12 cells (Fig. 3.9). These observations suggest the existence of the compartmentalisation of

cAMP, where PDE3 is likely to control a pool of cAMP controlling the ERK signalling pathway in PC12 cells. This is also supported by the experiment where both PDE3 and PDE4 inhibitors were combined. The results show that cilostamide treatment (PDE3 inhibitor) increased the differentiation of PC12 cells, whereas rolipram treatment had a minor effect on PC12 cell differentiation (Fig. 3.9). However, when both inhibitors were combined, the differentiation of PC12 cells was dramatically enhanced upon NGF stimulation and the proliferative effect of EGF was turned into a differentiation effect. The neurites produced were much longer and more "robust" upon NGF stimulation, and 40% of cells had differentiated upon EGF, mimicking the normal effect of NGF stimulation.

To consolidate these findings, I attempted to characterise PDE3 in PC12 cells by either probing Western Blots of cell lysates or PDE3 immunoprecipitations with different anti-PDE3 antibodies. Unfortunately, the antibodies used (anti-PDE3A and anti-PDE3B from Santa Cruz) could not detect any PDE3 isoforms probably due to poor affinity.

Several studies on cardiac myocytes demonstrated that PDEs are organised functionally and spatially to control the flux of cAMP (Fischmeister et al., 2006; Mongillo and Zaccolo, 2006). The second messenger cAMP is produced from adenylyl cyclases at the membrane that are activated by distinct transmembrane G protein-coupled receptors (GsPCRs). Different isoforms of PDEs regulate subcellular compartments of cAMP to control distinct downstream effects such as coordination and cell contractility. For example, PDE3 and PDE4 were shown to modulate the cAMP response to β_1 adrenergic receptors (β_1-AR) stimulation, whereas PDE4 exclusively regulates the cAMP response generated by stimulation of the glucagon receptors (Glu-R) (Fischmeister et al., 2006; Rochais et al., 2006; Vandecasteele et al., 2006). Moreover Vandecasteele *et al* (Vandecasteele et al., 2006) suggested that the different PDE isoforms are organised in different subsets to delimitate compartments of cAMP within subcellular microdomains and to control the flux of cAMP from the membrane to the cytoplasm (Fig. 3.10). We can therefore imagine a similar organisation of PDE isoforms to regulate the level of cAMP surrounding the ERK signalling pathway in PC12 cells. This might explain why upon cilostamide (PDE3 inhibitor), but not upon other PDE inhibitor treatments, the phosphorylation of ERK is stronger and that the combination of cilostamide and rolipram dramatically enhanced the differentiation of PC12 cells. It would be interesting to assess the phosphorylation of ERK upon cilostamide and rolipram compare to the

phosphorylation of ERK upon cilostamide or rolipram alone over a time course to verify if there is any difference in the signal strength and then to confirm this theory.

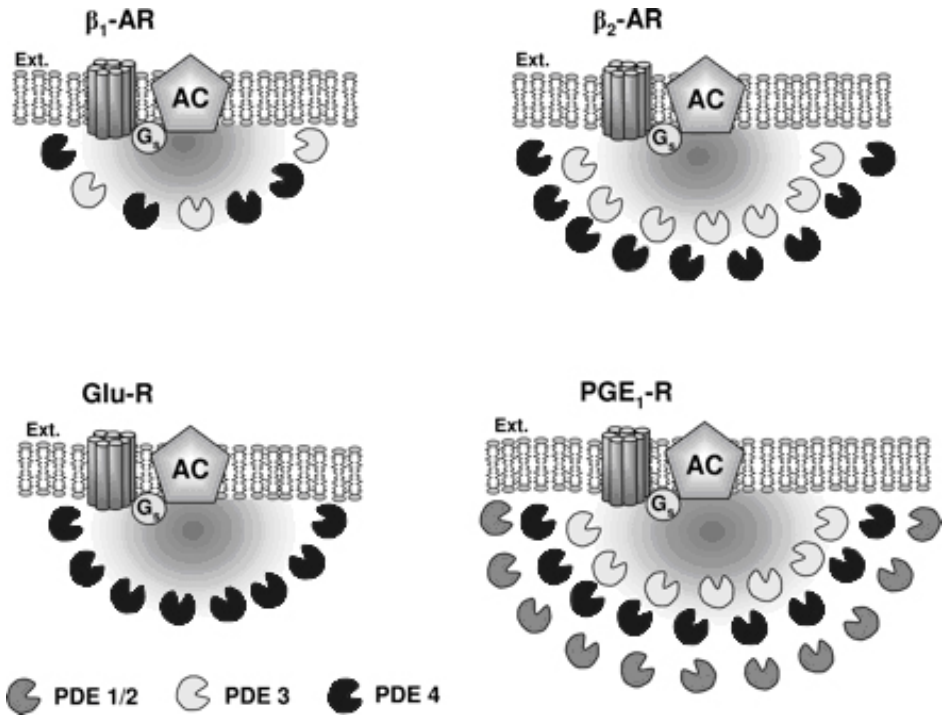


Figure 3. 10: Schematic representation of PDE-dependent cAMP compartmentalisation. This scheme represents the putative organisation of the different PDE isoforms in cardiac myocytes. Depending on the G-coupled receptor, PDEs are organised in different subsets to delimitate pools of cAMP and to control the flux of cAMP through the cytoplasm. This scheme was taken from a review by Fischmeister *et al* (Fischmeister et al., 2006).

Another important finding is that the results gave some clues to resolve the "architecture" of the ERK pathway. It is not clear how the signal is coupled from the receptor to the ERK cascade and which small G-proteins and Raf kinases (Ras/Rap1 and Raf-1/B-Raf) are involved. As described in the introduction, different models have been proposed to solve the sustained/transient activation of ERK. Even though it is commonly agreed that the signal triggered by both NGF and EGF utilises the Ras/Raf-1/MEK/ERK pathway that results in the transient activation of ERK, it is not clear how the signal is sustained upon NGF stimulation. On one hand, York *et al* suggest that the sustained activation of ERK is due to the activation of Rap1/B-Raf upon NGF stimulation (York et al., 1998). Their model suggests that upon EGF stimulation, the signal is only transiently mediated through Ras/Raf-1/MEK/ERK and upon NGF the signal is mediated through two distinct pathways, Ras/Raf-1/MEK/ERK and Rap1/B-Raf/MEK/ERK that is responsible for the activation of ERK. On the other hand, Kao *et*

al demonstrated that NGF and EGF stimulations can activate both Ras and Rap1 (Kao et al., 2001). They demonstrated that EGF can transiently stimulate Rap1 and that upon NGF the activation of Rap1 correlates with the sustained activation of ERK. They suggested that the difference in the longevity of the signal is due to Rap1, which is strongly coupled to the NGFR by the Crk-C3G complex scaffolding by FRS2. Therefore they proposed a model in which upon EGF stimulation the signal is transiently mediated through both Ras/Raf-1/MEK/ERK and through Rap1/B-Raf/MEK/ERK, whereas upon NGF stimulation the signal is transiently mediated through Ras/Raf-1/MEK/ERK followed by the sustained activation of Rap1/B-Raf/MEK/ERK. The study of a crosstalk with cAMP may help to clarify this point. Indeed, upon high level of cAMP, PKA is active and can phosphorylate and inhibit Raf-1 (Dumaz and Marais, 2003; Sidovar et al., 2000). If the signal triggered by EGF was mediated only through Ras/Raf-1/MEK/ERK, therefore the pathway would be blocked at Raf-1 level upon high levels of cAMP. However, the results obtained during this research, show that upon high levels of cAMP, the signal is still switched on and is even longer upon EGF stimulation. This suggested that a second pathway other than Ras/Raf-1/MEK/ERK had transduced the signal. This finding supports the Kao *et al*'s model and more experiments to confirm this hypothesis, particularly on Ras/Rap1 activity and on the knockdown of Raf-1 and C3G, have been investigated and will be discussed in the next chapters.

Finally, the neuronal differentiation of PC12 cells is characterised by cell cycle arrest, activation of specific genes and neuritogenesis. Upon NGF stimulation, the phosphorylation of ERK is sustained allowing the translocation and the accumulation of phosphorylated ERK in the nucleus. Therefore, in the nucleus phosphorylated ERK can activate specific transcription factors and can induce the expression of specific genes involved in cell differentiation.

I tried an experiment to investigate at which point in time the commitment to differentiation is made. To do so, I used the MEK inhibitor U0126 to block the ERK pathway. PC12 cells were plated onto poly-L-lysine pre-coated 6-well plates and left overnight to set. The cells were starved for at least 3h and then they were treated or untreated with U0126 (20μM) and stimulated with NGF (100ng/ml) for 72h as followed. The positive control consisted of cells untreated with U0126 and stimulated with NGF to estimate the basal differentiation of the cells. To evaluate when the

differentiation of the cell occurs, the cells were first stimulated with NGF before being treated with U0126 after 1h and 24h following the NGF stimulation. The negative control consisted of cells treated with U0126 for 10 min before NGF stimulation. As expected, PC12 cells differentiated when they were stimulated with NGF. They did not differentiate when they were pre-treated with U0126, which confirmed that U0126 can block the differentiation of PC12 cells. The results obtained showed that when the cells were first stimulated with NGF then treated with U0126 after 1h or 24h after stimulation, they had differentiated. The results confirmed that the determination of the fate of the cell occurs within the first hour of NGF stimulation. I did not present these results in the result section of this chapter because I only repeated these results twice. This experiment was only a test experiment. I wanted to deepen this experiment investigating what would happen when the cells were treated with U0126 after 5 min, 10 min (that is the peak of the phosphorylation of ERK after NGF stimulation), 30 min and 1h after NGF stimulation. However I encountered some problems, such as cell contaminations, and I could not repeat the results. I think it was important to mention this experiment and it would be interesting to repeat the same experiment trying different MEK inhibitor such as PD98059.

To conclude, these results demonstrate that deregulating the cAMP pathway by inhibiting its degradation deregulates the ERK pathway, increases the differentiation of the cells and more dramatically can modify the mitogen signal. Indeed, upon high levels of cAMP, EGF can promote differentiation, which is the wrong signal! Moreover, the use of specific PDE inhibitors enabled to determine that PDE3 controls a pool of cAMP surrounding the ERK pathway. PDEs are essential to control the appropriate level of cAMP across the cells and then to maintain the function of the ERK signalling pathway and therefore the fate of the cell.

CHAPTER 4

ROUTE FOR cAMP REGULATION OF ERK ACTIVATION

4.1 INTRODUCTION

The second messenger cAMP was discovered in the sixties and was widely studied for its role in the regulation of metabolic pathways in response to hormones in mammalian cells. Over the years growing evidence has shown the involvement of cAMP in the proliferation and differentiation of cell (Gottesman et al., 1986). It was particularly demonstrated that cAMP can activate or inhibit the ERK signalling pathway depending on the cell type (Houslay and Kolch, 2000; Qiu et al., 2000). In the previous chapter it was demonstrated that cAMP increases the phosphorylation of ERK and the differentiation of PC12 cells. Surprisingly, high levels of cAMP could even turn the proliferation effect of EGF into a differentiation effect. These results proved that the level of the cAMP is crucial to maintain the correct function of the ERK signalling pathway and the fate of the cell (Dumaz et al., 2002; Stork and Schmitt, 2002). Disturbing the level of cAMP leads to altering the mitogen signal. The mechanisms through which cAMP regulates the ERK pathway have in part been elucidated (Stork and Schmitt, 2002) but many details remain unclear. Protein kinase A (PKA) was thought to be the only direct effector of cAMP (Brostrom et al., 1970; Walsh et al., 1968), with the exception of the cyclic nucleotide-gated ion channels found in specialised cells like olfactory neurons (Zufall et al., 1997). However, recently guanine nucleotide exchange factors directly activated by cAMP (Epacs) were discovered (Bos, 1998; de Rooij et al., 1998). This discovery raised the possibility that part of the effects attributed to the activation of PKA is in fact mediated by the activation of Epac.

4.1.1 Protein Kinase A: the most studied effector of cAMP mediates the activation of ERK by cAMP through Rap1 but inhibits Raf-1

The major effector of cAMP discovered in the seventies is the cAMP-dependent protein kinase A (PKA), which is a serine/threonine specific protein kinase (see chapter 1-general introduction for further details). PKA is a heterotetramer consisting of two regulatory subunits (RI or RII) and two catalytic subunits (C). When cAMP binds to the R subunits, the C subunits are released and can phosphorylate a wide range of cytosolic and nuclear proteins. Four regulatory subunits have been identified (RIα, RIβ, RIIα and RIIβ). The R subunits exhibit different affinities for cAMP and are localised in different subcellular compartments. While the RI holoenzyme is found predominantly in the

cytoplasm, the RII holoenzyme is associated with cell structures, membranes and organelles. This localisation is due to anchoring by a family of specialised scaffold proteins called the A-kinase anchoring proteins or AKAPs (Houslay and Kolch, 2000; Wong et al., 2004). Over the years, it has emerged that PKA is at the centre of the regulation of cell proliferation and differentiation by cAMP (Stork and Schmitt, 2002; Vossler et al., 1997; Yao et al., 1998). Interestingly, cAMP is involved in both inhibition and activation of cell proliferation. It was demonstrated that PKA can inhibit Raf-1 by phosphorylation on serines 43, 233, 259 and 621 (Dhillon et al., 2002b; Dumaz et al., 2002) and promote its binding to 14-3-3 blocking Raf-1 recruitment to the plasma membrane (Dumaz and Marais, 2003). On the other hand, cAMP is involved in cell differentiation, and PKA was demonstrated to be required for the sustained activation of ERK by NGF (Yao et al., 1998; York et al., 1998). It was demonstrated that PKA can directly activate Rap1 (Vossler et al., 1997) *in vitro* and *in vivo*. However, it is still controversial whether the activation of Rap1 by PKA is a direct activation or if it occurs through the intermediate of a guanine exchange factor such as Epac or C3G (Dumaz and Marais, 2005; Stork and Schmitt, 2002), or through the tyrosine kinase Src (Obara et al., 2004).

4.1.2 Exchange Protein directly Activated by cAMP: new perspectives

More recently a new effector for cAMP, the exchange protein directly activated by cAMP or Epac, was discovered. This discovery came out when Bos' group noticed that the activation of Rap1 was insensitive to the inhibition of PKA (de Rooij et al., 1998). Epac is a cAMP-responsive guanine nucleotide exchange factor (GEF) for the small GTP-binding proteins Rap1 and Rap2 (de Rooij et al., 1998). Two isoforms have been identified, Epac1 and Epac2. Both isoforms have an N-terminal DEP (dishevelled, Egl-10, pleckstrin) domain involved in membrane localisation (de Rooij et al., 1998), a cAMP binding domain (CBD), a Ras exchange motif (REM) and a guanine nucleotide exchange factor homology domain (GEF) (Christensen et al., 2003). Epac 2 has another CBD and a Ras-association domain (RA) that can interact with active Ras. It is likely that the CBD folds upon the GEF domain to prevent interaction with effectors. The conformation of Epac changes when binding to cAMP, exposing the GEF domain that activates downstream targets (Qiao et al., 2002). The main effectors of Epac1 and 2 are the Ras-like small G-proteins Rap1 and Rap2 (de Rooij et al., 1998; Enserink et al.,

2002; Rehmann et al., 2003). The fact that Epac activates Rap1, and that Rap1 mediates sustained activation of ERK (York et al., 1998) makes it logical to assume that Epac might be involved in the regulation of ERK by cAMP. Moreover the PACAP-stimulated neuritogenesis was demonstrated to not be blocked by PKA inhibitor (Lazarovici et al., 1998), suggesting that cAMP can activate the ERK pathway independently of PKA. This could be a model to explain the activation of ERK by cAMP in a PKA-independent manner. A recent study (Kiermayer et al., 2005) has shown that Epac activation increased the duration of PKA-dependent ERK activation and caused cell differentiation. However, the regulation of ERK by cAMP mediated by Epac in a PKA-dependent or independent manner remains controversial, and Bos' group is not convinced that cAMP-induced activation of Rap1 mediated by Epac can regulate ERK (Bos, 2003).

4.1.3 Specific Agonists for the activation of PKA and Epac

Different cAMP analogues were designed by Bos' group to specifically activate either PKA or Epac (Christensen et al., 2003; Enserink et al., 2002). They compared the amino-acid sequences of the cAMP-binding domains of Epac and PKA. The major difference is that Epac does not have a glutamate residue that forms hydrogen bonds with the 2'-hydroxyl of the cAMP ribose group (Su et al., 1995). This hydroxyl group was replaced by a 2'-O-methyl group on the ribose ring of cAMP. The deletion of the 2'-OH impaired the ability of the cAMP analogue to bind PKA but not Epac. Thus, 2'-O-Me-cAMP analogues bind selectively to Epac. However, the affinity of a 2'-O-Me-cAMP for Epac was 8-fold less than that of cAMP. The introduction of a parachlorophenylthio (pCPT) group at position 8 on the adenine moiety of 2'-O-Me-cAMP improved the affinity of the cAMP analogue to Epac. In addition, the presence of a hydrophobic pCPT increased the membrane permeability of the cAMP analogue *in vivo* (Enserink et al., 2002). *In vivo*, it was also demonstrated that the 8-pCPT-2'-O-Me-cAMP activated Rap1 without stimulating CREB (Christensen et al., 2003), which is a well-known PKA substrate, proving that this analogue can activate specifically Epac but not PKA *in vivo*. It was demonstrated that the cAMP analogues modified at the position 6 of the adenine ring, particularly 6-Bnz-cAMP, stimulate strongly the phosphorylation of CREB without activation of Rap1 *in vivo* (Christensen et al., 2003) and therefore 6-Bnz-cAMP activate specifically PKA but is not able to activate Epac.

In this chapter, the role of cAMP in the regulation of ERK was investigated by decomposing the effect of cAMP on ERK phosphorylation using cAMP analogues that specifically activate either PKA or Epac. As described above, 8-pCPT-2'-O-Me-cAMP activates specifically Epac without activating PKA, whereas 6-Bnz-cAMP activates specifically PKA. In addition the activity of Ras and Rap1, upon NGF and EGF with or without cilostamide, was assessed by affinity pull-down assay in order to find out which small G-protein is involved in the cross-talk between the ERK and the cAMP signalling pathways.

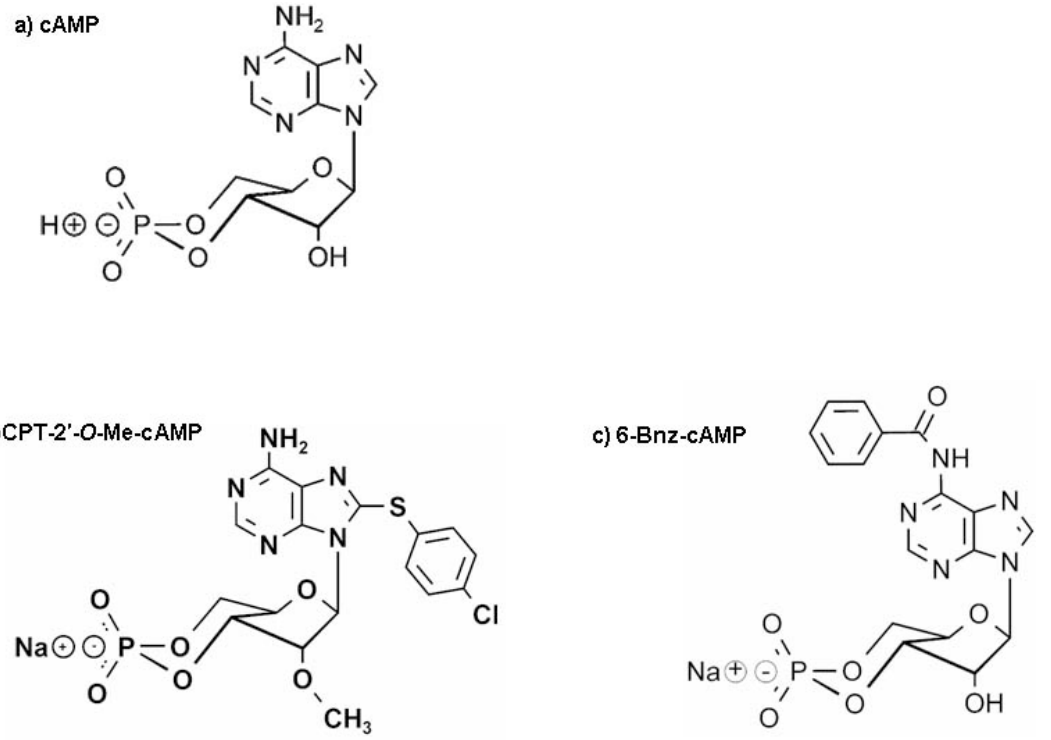


Figure 4. 1: Structures of cAMP analogues that specifically activate either PKA or Epac. a) Structure of cAMP. b) The hydroxyl group on the ribose ring of cAMP was substituted by a 2'-O-methyl group to impair the ability of the cAMP analogue to bind to PKA. The parachlorophenylthio (pCPT) group at position 8 on the adenine improved the affinity of the cAMP analogue to Epac and improved cell permeability. This analogue is a specific activator for Epac without activating PKA. c) This analogue contains a benzoyl group on the position 6 on the adenine ring that improves its affinity for its binding to PKA. This analogue can activate PKA without activating Epac.

4.2 RESULTS

4.2.1 Ras and Rap1 activation upon NGF and EGF: cAMP sensitises Rap1

In the previous chapter it was demonstrated that accumulation of cAMP induced by PDE inhibition, increased the phosphorylation of ERK upon NGF and EGF stimulation. The results obtained pertaining to EGF stimulation were surprising because it was expected that cAMP would activate PKA, which would inhibit Raf-1 and therefore would switch off the ERK pathway. From these results, it was hypothesised that cAMP in response to mitogen stimulation could activate a second pathway, Rap1/B-Raf, to activate ERK. It was then crucial to investigate through which small G-proteins, i.e. Ras and/or Rap1, the signal is mediated in response to NGF and EGF stimulation.

The activity of Ras and Rap1 were measured by affinity pull-down assay. This assay is based on the differential affinity of Ras-GTP and Ras-GDP for the Ras Binding Domain (RBD) of Raf-1, and of Rap1-GTP and Rap1-GDP for the Rap Binding Domain (RBD) of RalGDS. It was demonstrated that Raf-1 RBD binds specifically to GTP loaded Ras (the active form of Ras) and that RalGDS RBD binds specifically to GTP loaded Rap1 (the active form of Rap1) (Franke et al., 1997; Taylor and Shalloway, 1996). The GST-tagged RalGDS-RBD beads were obtained from Upstate®. The GST-tagged RBD of Raf-1 was expressed in *E. coli* and the fusion protein was purified on glutathione-Sepharose beads. Figure 4.2 shows the purity of the Raf-1-RBD beads. The lanes "RBD" prove that there was no Ras detected in the beads. The lane "Control-RBD" corresponds to the cell lysate before the affinity pull-down, which shows the presence of Ras in the lysate. The lanes "Control+RBD, NGF and EGF" in the left panel correspond to the RBD beads after being incubated in the cell lysates of unstimulated and stimulated cells. It demonstrates that a small portion of Ras corresponding to GTP-Ras was pulled-down. The lanes "Control+RBD, NGF and EGF" on the right panel, which correspond to the cell lysates after affinity pull-down, show that GDP-Ras remains in the lysate.

To measure the activity of Ras and Rap1, PC12 cells were seeded onto poly-L-lysine pre-coated 10 cm dishes and let to set overnight. The cells were serum starved in serum free medium for 3 to 4 hours. The cells were treated with cilostamide for 10 min before being stimulated with either NGF or EGF for the indicated periods of time. Cell lysates

were prepared as described in the section 2.1.6, and the protein concentration was normalised for each sample. 30µl of each lysate was set apart to be resolved by electrophoresis and probed for Ras, Rap1, pERK (to measure the activity of ERK) and for ERK. The rest of the lysates was assayed for Ras and Rap1 activity using *in vitro* affinity pull-down assays described in section 2.2.10. The Raf-1-RBD beads and the RalGDS-RBD beads were resolved by electrophoresis. After the transfer of the proteins onto nitrocellulose membranes, the membranes were probed for Ras-GTP or Rap1-GTP, the active forms of Ras and Rap1, using anti-Ras and anti-Rap1 antibodies respectively.

This experiment has only been repeated twice for each mitogen stimulation, therefore it is not possible to draw any conclusion from the results obtained.

Upon EGF stimulation (Fig. 4.3), the time courses were shorten from 0 to 5 min in order to detect Ras-GTP (or active Ras). This indicated that the activation of Ras was rapid. Ras was the most active at 1.5 min, and the activation of Ras was back to basal already after 5 min of stimulation. The treatment with cilostamide seemed to slightly increase the activation of Ras at 1.5 min, but did not modify the kinetic of the Ras activation. In contrast, no Rap1-GTP was pulled down upon EGF stimulation. This might indicate that Rap1 was not activated in response to EGF, which would support the results from York's group (York et al., 1998). Interestingly, when the cells were pre-treated with cilostamide, Rap1-GTP was detected at times 0 and 1.5 min. However, ERK was not phosphorylated at time 0. Thus, the pre-treatment with cilostamide seemed to sensitised Rap1 but was not sufficient to activate ERK. Taken together these results might demonstrate that EGF might activate Ras but not Rap1. Cilostamide possibly increased the activation of Ras. Moreover, cilostamide on its own seemed to activate Rap1, but it was not sufficient to activate ERK. Rap1 remained active after 1.5 min of stimulation with EGF, which seemed to correlate with the increase in the phosphorylation of ERK. From these results, it seems that the increase in the phosphorylation of ERK due to cilostamide and upon EGF stimulation, might be due to the activation of both Ras and Rap1.

Upon NGF stimulation (Fig. 4.4), unfortunately the results obtained for Ras activation were not convincing enough in the two experiments made, and therefore no conclusion can be made regarding to the effect of cilostamide on the activation of Ras. Although, in

the literature it is generally accepted that Ras is activated in response to NGF stimulation (Kao et al., 2001; York et al., 1998).
In contrast to the cells stimulated with EGF, Rap1 was activated in response to NGF . Its activation started around 5 min and remained activated after 20 min, which correlates with the sustained activation of ERK upon NGF stimulation. The pre-treatment with Cilostamide again seemed to activate Rap1 independently of the mitogen stimulation but was not sufficient to activate ERK. The activation of Rap1 was stronger and started after 2 min of NGF stimulation, and remained activated up to 20 min of stimulation. Again, these results correlate with the increase of the activation of ERK upon cilostamide treatment, which might confirm the role of Rap1 in the sustained activation of ERK.

These results confirm that EGF uses only Ras to mediate the signal toward the ERK pathway but was not able to activate Rap1, whereas NGF can mediate the signal through Rap1 (and certainly Ras according to the literature). The activation of Rap1 correlates with the sustained activation of ERK upon NGF stimulation. Then, the results show that cilostamide increases the activation of Ras upon EGF stimulation. The most important findings are that cilostamide seemed to sensitise Rap1 independently to mitogen stimulation; that Rap1 becomes active upon EGF stimulation; and finally that Rap1 is activated earlier and stronger upon NGF stimulation.

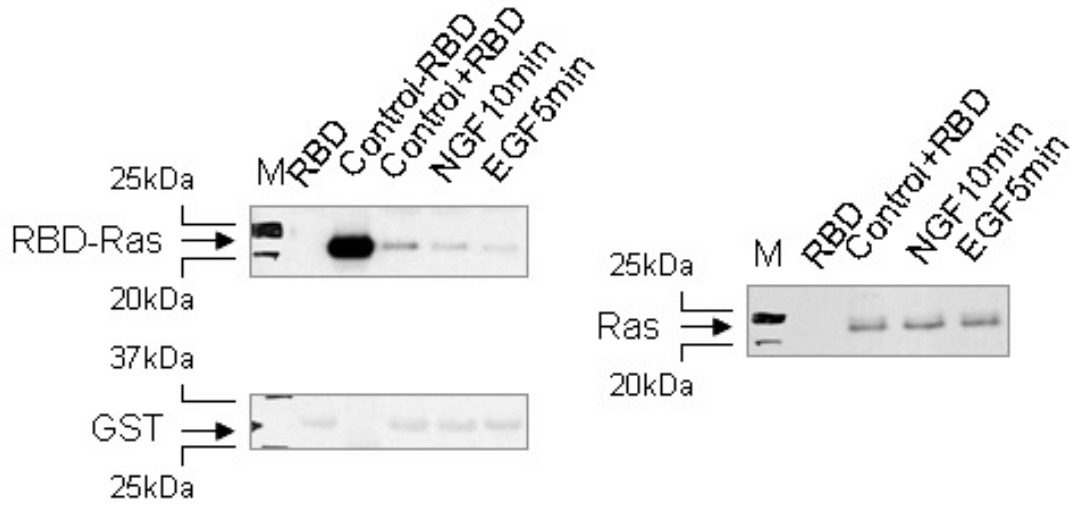


Figure 4. 2: Control of the purity of the Raf-1-RBD beads. GST-tagged fusion protein corresponding to the human Ras Binding Domain (RBD) of Raf-1 was expressed in *E. coli*. Left panel: The lysates of unstimulated cells (control+RBD) or cells stimulated with NGF or EGF were incubated with 2µL of Raf-1-RBD beads to pull-down GTP-Ras. The beads were resolved by electrophoresis along side with the cell lysate (control-RBD) to visualise Ras, and probed for Ras and GST.
Right panel: After the pull-down of GTP-Ras, the remaining cell lysates were probed for Ras. M stands for protein marker. The blots presented are representative of 3 repeated blots.

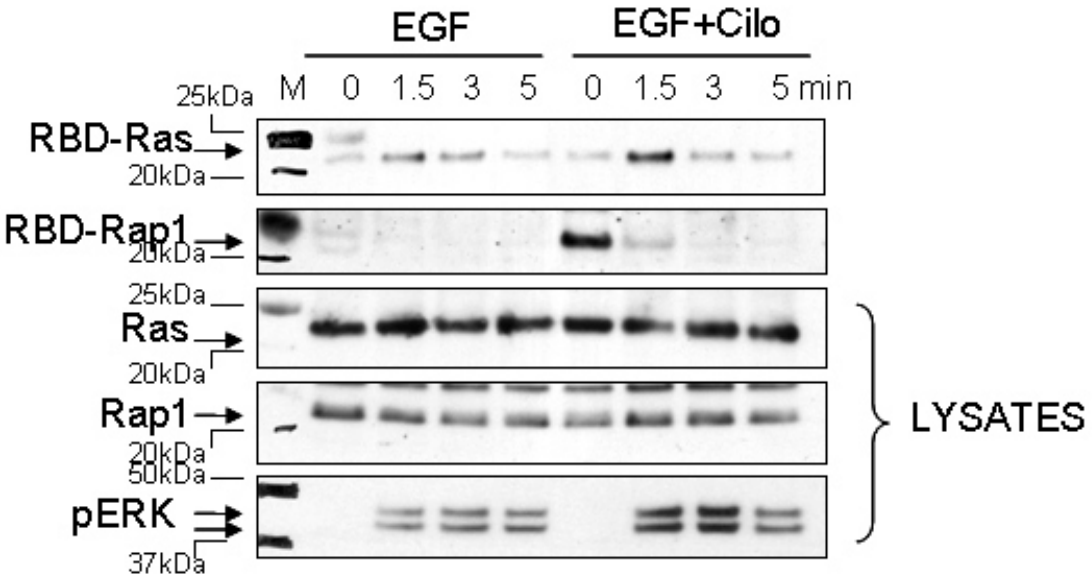


Figure 4. 3: Activity of Ras and Rap1 upon EGF stimulation in PC12 cells. Serum starved PC12 cells were treated for 10 min with Cilostamide (10 μM) before being stimulated with EGF (100 ng/ml) for the indicated periods of time. GTP-Ras and GTP-Rap1 were pulled down with Raf-1 RBD and Ral-GDS beads respectively. The beads were probed with anti Ras and anti Rap1 antibodies and the lysates were probed for Ras, Rap1 and pERK. M stands for protein marker. This blot is representative of two independent experiments.

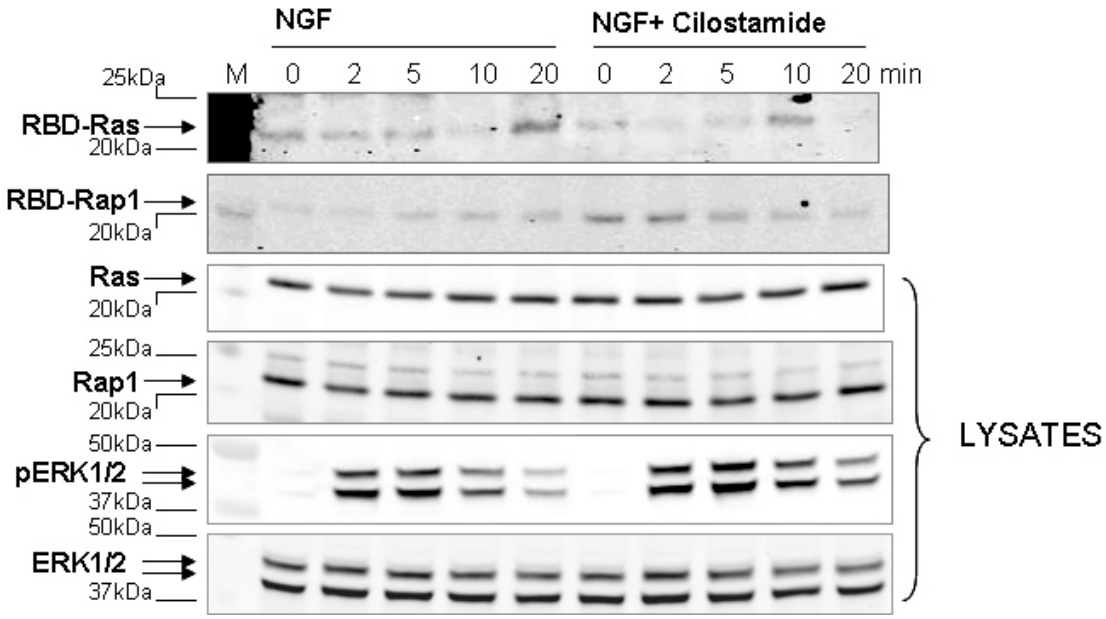


Figure 4. 4: Activity of Ras and Rap1 upon NGF stimulation in PC12 cells. Serum starved PC12 cells were treated for 10 min with Cilostamide (10 μM) before being stimulated with NGF (100 ng/ml) for the indicated periods of time. GTP-Ras and GTP-Rap1 were pulled down with Raf-1 RBD and Ral-GDS beads respectively. The beads were probed with anti Ras and anti Rap1 antibodies and the lysates were probed for Ras, Rap1 and pERK. M stands for protein marker. This blot is representative of two independent experiments.

4.2.2 Effect of PKA and Epac agonists on ERK phosphorylation

In the previous chapter it was demonstrated that the inhibition of PDE3 by cilostamide raised the level of cAMP, which increased the phosphorylation of ERK and enhanced the differentiation of PC12 cells. However, the exact molecular mechanisms through which the crosstalk between the ERK and the cAMP signalling pathway occurs are unknown. As described in the introduction, different research groups (Kiermayer et al., 2005; Vossler et al., 1997; Yao et al., 1998) have identified Ras, Rap1, PKA and Epac as the molecules mediating the crosstalk between the ERK and the cAMP signalling pathways. To investigate this hypothesis, cAMP analogues were used to activate specifically either PKA or Epac.

PC12 cells were plated onto poly-L-lysine pre-coated 6-well plates in normal medium and left overnight to set. Then, they were starved for 3 to 4 hours in a serum free medium. The cells were treated for 10 minutes with PKA agonist (6-Bnz-cAMP), a specific Epac agonist (8-pCPT-2'-O-Me-cAMP/8-CPT-2'-O-Me-cAMP) and cilostamide (PDE3 inhibitor). The cells were then stimulated with either NGF or EGF for the indicated periods of time. The cell lysates were analysed by Western Blotting using the ODSSEY imager. The nitrocellulose membranes were probed with antibodies against phosphorylated ERK to measure the activation of ERK, and against ERK to measure the total amount of protein loaded on the gel. These primary antibodies were detected with fluorescently labelled secondary antibodies as described in the section 3.1.2 (Fig. 4.5). Then, the ratio between pERK and total ERK (integrated intensity values) were calculated and normalised as percentage of the highest ratio, which was the peak of activation upon NGF or EGF stimulations. The results obtained were plotted onto a graph (Fig. 4.6).

First of all, phosphorylated ERK was not detected without mitogen stimulation, which meant that neither cilostamide nor any of the cAMP analogues at the concentrations used could activate the ERK pathway on their own (Fig. 4.5.a).

Upon NGF stimulation (Fig. 4.6.a) the phosphorylation of ERK increased 2.5 fold when the cells were treated with cilostamide (PDE3 inhibitor). Treating the cells with a cAMP analogue that specifically activates Epac had a similar effect on the ERK activation. However, the treatment of the cells with a cAMP analogue that specifically activates PKA had no effect on ERK phosphorylation, and did not block the ERK pathway.

Surprisingly, the combination of both cAMP analogues did not mimic the effect of cilostamide and the phosphorylation was only increased by 1.5 fold at the peak of activation. This may suggest that the enhancement of ERK phosphorylation by the Epac agonist might be reduced by the effect of PKA agonist.

Upon EGF stimulation (Fig. 4.6.b), again the treatment with cilostamide increased the phosphorylation of ERK 2 fold. The treatment with the Epac agonist also increased the phosphorylation of ERK. However the treatment with the PKA agonist had an inhibition effect and decreased the activation of ERK. However, ERK was still phosphorylated meaning that the ERK pathway was still switched on. Surprisingly the combination of both cAMP analogues had no effect on the phosphorylation of ERK and did not mimic the effect of cilostamide.

In order to reduce possible manipulation errors that might occur during a time course, a similar but simpler experiment (same protocol as the previous experiment) was made. Instead of stimulating the cells over a time course (Fig. 4.5.b) and c) and Fig. 4.6), cells were stimulated for 10 minutes with NGF and for 5 minutes with EGF, which are the peaks of activation of ERK by NGF and EGF respectively (Fig. 4.7). The results obtained were similar to the results described above. The activation of Epac using a cAMP analogue had a similar effect on the phosphorylation of ERK than the treatment with cilostamide (or as increasing the level of cAMP by inhibiting PDE3 with cilostamide), which was significantly different from the control in both case. This remains to be confirmed by doing an experiment where Epac would be inhibited. Unfortunately, at this time there was no tool available to inhibit Epac (siRNA or an Epac inhibitor). The results also showed that the PKA agonist used did not have a significant effect on the phosphorylation of ERK upon NGF stimulation compare to the control. However, the activity of ERK was significantly decreased due to the treatment with the PKA agonist upon EGF stimulation compare to the control, which meant that the ERK pathway was altered likely because of the activation of PKA that might inhibit Raf-1.

These results are consistent with the results described in the previous chapter and add more information. In the previous chapter it was demonstrated that high level of cAMP can activate or switch the mitogen signal from Ras/Raf-1/MEK/ERK to other pathways such as Rap1/B-Raf/MEK/ERK. The results presented here confirm the existence of at

least two independent pathways to mediate the mitogen signal to the nucleus. Based on the fact (Fig. 4.3) that upon EGF stimulation the signal is transiently mediated only through Ras/Raf-1 (Bos, 1998) and that activated PKA inhibits Raf-1 (Dumaz and Marais, 2003; Sidovar et al., 2000), therefore the signal should be switched off upon PKA agonist treatment. Here the results demonstrate that upon EGF stimulation and PKA agonist, the signal was still transduced although it was diminished, which confirms the contribution of another pathway. According to Kao et al (Kao et al., 2001), the signal upon EGF is transiently mediated through Ras/Raf-1 but might also be transduced through B-Raf via Ras or Rap1. According to the previous results, cAMP sensitises Rap1 so it is possible that the PKA agonist can activate Rap1 upon EGF stimulation, or Ras activates B-Raf.

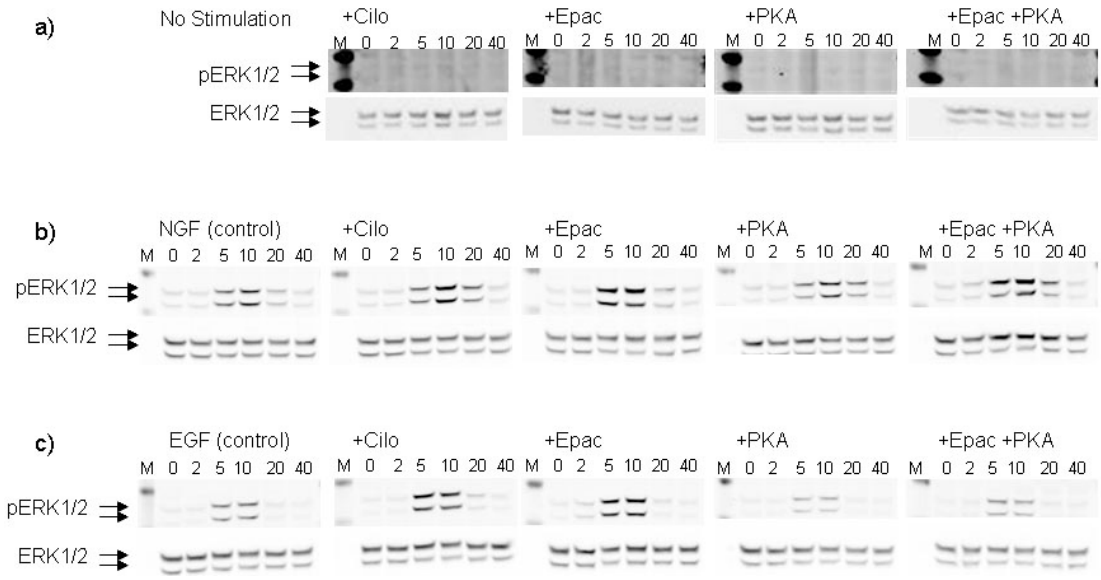


Figure 4. 5: Effect of EPAC and PKA agonists on ERK phosphorylation. Serum starved PC12 cells were treated with Cilostamide (10 μM), Epac agonist (8-pCPT-2'-O-Me-cAMP/8-CPT-2'-O-Me-cAMP at 10 μM) or with PKA agonist (6-Bnz-cAMP at 10 μM) for 10 min before being stimulated with b) NGF (100 ng/ml) or with c) EGF (100 ng/ml) and incubated at 37ºC for 0, 2, 5, 10, 20 and 40 min. The activation of ERK was evaluated by probing Western blots of cell lysates with anti-phospho-ERK1/2 antibody. The total amount of ERK was evaluated by probing Western blots with anti ERK1/2 antibody. M stands for protein marker.

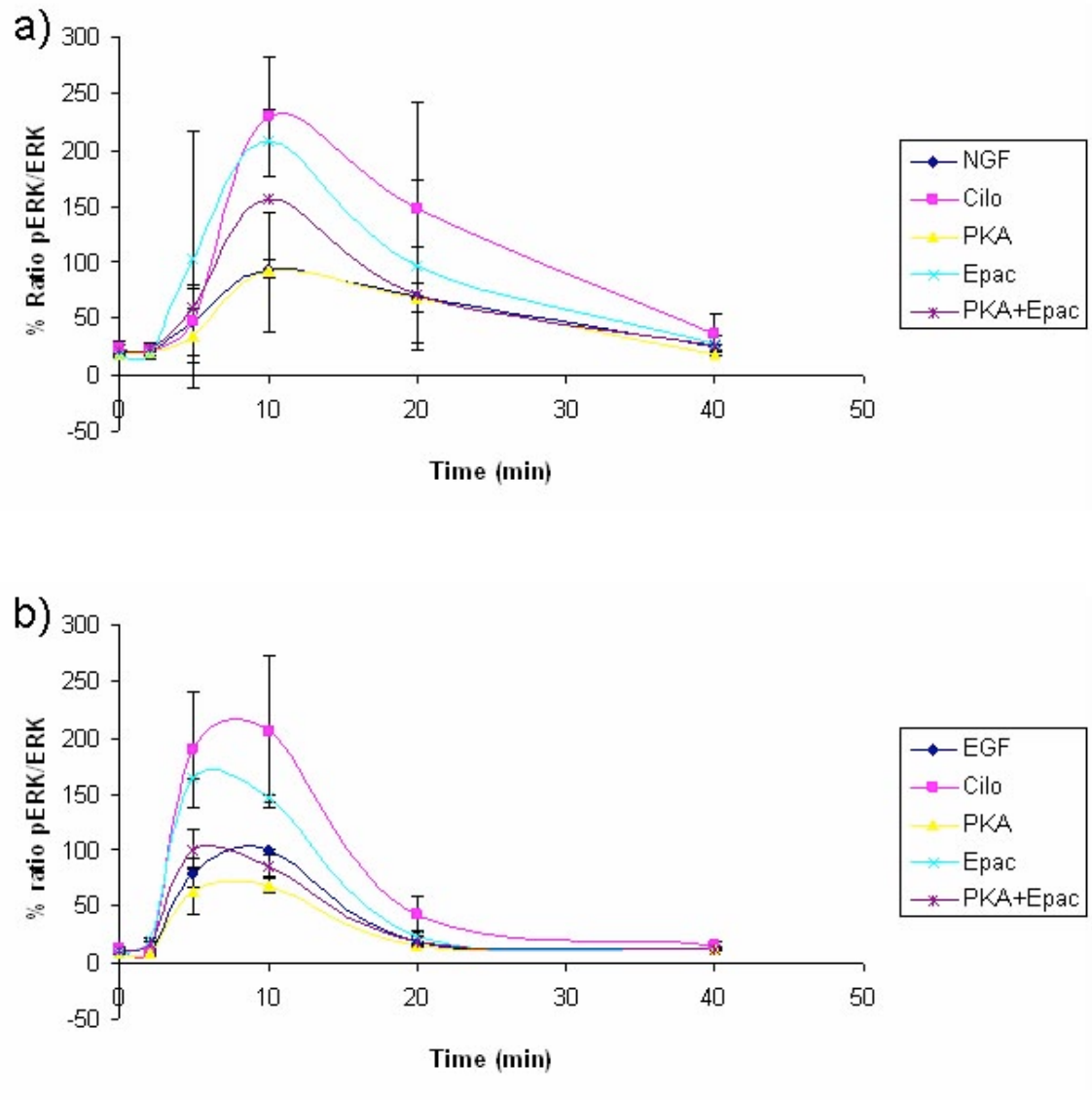


Figure 4. 6: Effect of EPAC and PKA agonists on ERK phosphorylation. Serum starved PC12 cells were treated with Cilostamide (10 μM), Epac agonist (8-pCPT-2'-O-Me-cAMP/8-CPT-2'-O-Me-cAMP at 10 μM) or with PKA agonist (6-Bnz-cAMP at 10 μM) for 10 min before being stimulated with a) NGF (100 ng/ml) or with b) EGF (100 ng/ml) and incubated at 37ºC for 0, 2, 5, 10, 20 and 40 min. The activation of ERK was evaluated by probing Western blots of cell lysates with anti-phospho-ERK1/2 antibody. The total amount of ERK was evaluated by probing Western blots with anti ERK1/2 antibody. The values represent the ratio between pERK and ERK and are expressed as the percentage of the highest value in the control. The values shown are the mean of 3 independent experiments ± S.D. The error bars for the treatment with PKA+Epac agonists upon NGF stimulation were removed for the clarity of the graph.

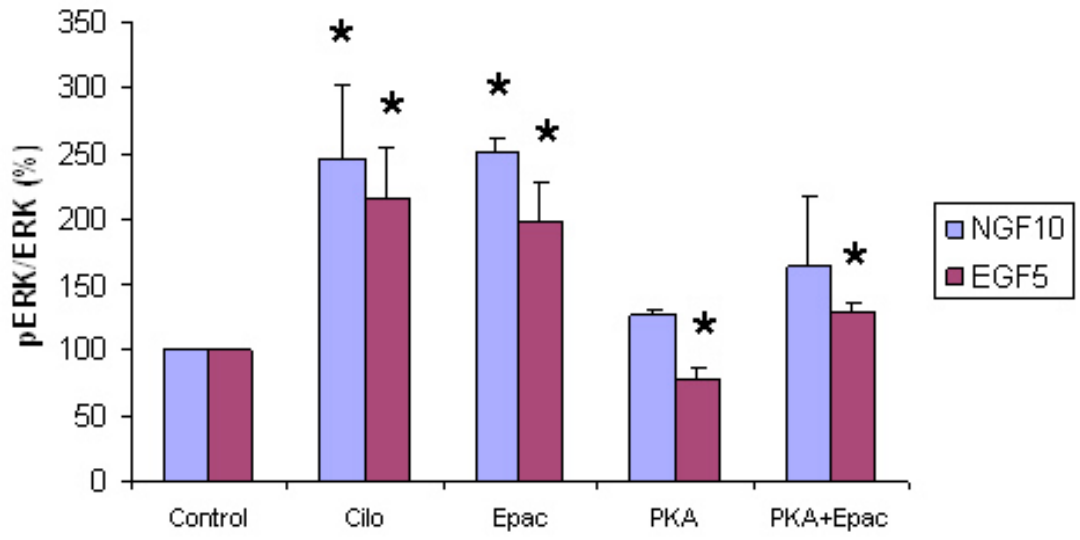


Figure 4. 7: Effect of cAMP analogues on ERK phosphorylation at the peak of activation by mitogens. Serum-starved PC12 cells were treated with Cilostamide (10 μM), Epac agonist (10 μM), PKA agonist (10 μM) or with both Epac and PKA agonists for 10 min before being stimulated with b) NGF (100 ng/ml) for 10 min or with c) EGF (100 ng/ml) for 5 min. The activation of ERK was evaluated by probing Western blots of cell lysates with anti-phospho-ERK1/2 antibody. The total amount of ERK was evaluated by probing Western blots with anti ERK1/2 antibody. The values represent the ratio between pERK and ERK and are expressed as the percentage of the highest value in the control. The values shown are the mean of 3 independent experiments ± S.D. The effects of the different treatments were compared to the respective control, * $p < 0.05$.

4.2.3 Effect of PKA and Epac agonists on PC12 cells differentiation

Since it was demonstrated that both PKA and Epac agonists can modulate the phosphorylation of ERK, it was crucial to examine whether or not they can also affect the differentiation of PC12 cells.

To assess the possible involvement of PKA and/or Epac in the differentiation of PC12 cell, the cells were seeded onto 6-well plates pre-coated with poly-L-lysine, incubated in normal medium at 37ºC and let to set over-night. At day 0 the cells were examined under the microscope to ensure that no cells were differentiated. The cells were then treated with Cilostamide, Epac agonist (8-pCPT-2'-O-Me-cAMP/8-CPT-2'-O-Me-cAMP) or PKA agonist (6-Bnz-cAMP) at the indicated concentrations for 10 minutes before being stimulated with either NGF or EGF. The cells were then incubated for 72 hours at 37ºC. About 10 phase-contrast images of the cells were taken randomly for each treatment to have a significant number of cells. A cell was only considered fully differentiated if the neurite extension was at least twice the length of the cell body. The percentage of differentiated cells was expressed as the number of cells with neurites compared to the total number of cells for each treatment (Fig. 4.8).

Without mitogen stimulation, PC12 cells did not differentiate, which meant that neither cilostamide nor both Epac and PKA agonists at the concentrations used here, could trigger cell differentiation. However, other research showed that these agonists were able to initiate neurite extension, but at least 10 times more agonist were used (Christensen et al., 2003; Kiermayer et al., 2005). Upon EGF, 15% more cells differentiated upon cilostamide treatment compare to the untreated cells. Unexpectedly, none of the cAMP analogues that specifically activate Epac or PKA significantly contributed to enhance the differentiation of the cells. Upon NGF, cilostamide increased the differentiation of PC12 cells by 36%. There was a slight (but not significantly different from the control) increase of the differentiation by 10% in the presence of Epac agonist in the medium. It seems also that PKA agonist slightly (but not significantly) blocked the differentiation of the cells and only 40% of the cells differentiated compare to 45% of differentiated cells upon NGF stimulation only. Unexpectedly, the Epac agonist did not increased considerably the differentiation of PC12 cells as expected. This will be discussed further in the discussion section of the chapter.

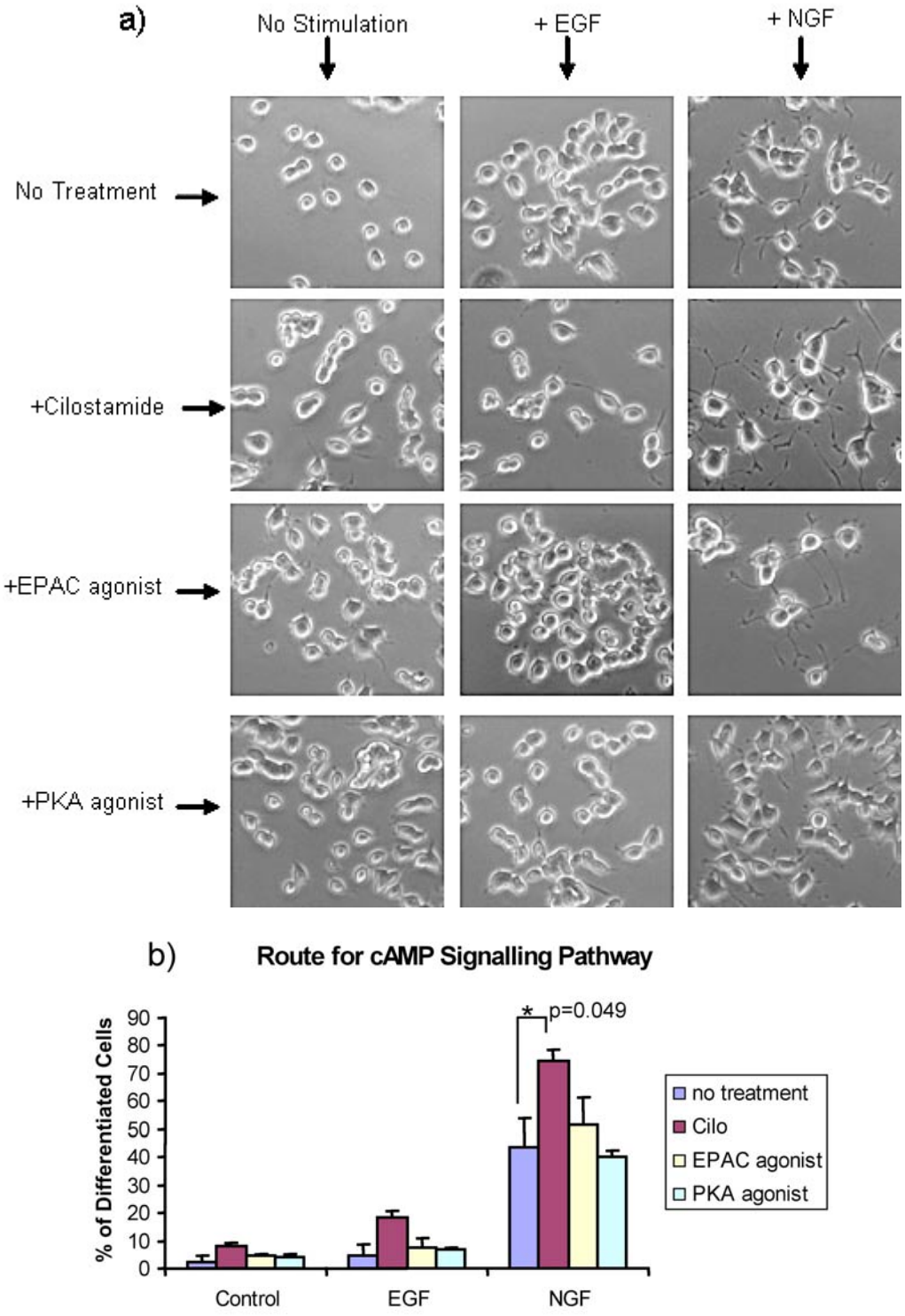


Figure 4. 8: Effect of EPAC and PKA agonists on PC12 cell differentiation. a) PC12 cells were treated with Cilostamide (10 μM), Epac agonist (10 μM) or with PKA agonist (10 μM) for 10 min before being stimulated with NGF (100 ng/ml) or with EGF (100 ng/ml) and incubated at 37ºC. After 72h of incubation, phase-contrast images of representative cells were taken. b) The percentage of differentiated cells was determined by comparing the number of neurons over the total number of cells for each treatment. The values shown are means ± S.E. of 4 independent experiments. * $p < 0.05$.
Note: the pictures for "no treatment" and for "+Cilostamide" were shown on figure 3.9.

4.3 DISCUSSION

The aim of this chapter was to investigate the route and the mechanisms through which cAMP regulates the activation of ERK and particularly to assess the role of PKA, Epac, Ras and Rap1 in these mechanisms. The chosen approach was to decompose the effect of cAMP by activating its two main effectors PKA and Epac using specific cAMP analogues.

One of the main aims of this research was also to understand how the signal by NGF is sustained whereas it is transient upon EGF stimulation and if cAMP is involved in these mechanisms. It is generally accepted that both NGF and EGF can activate the small G-protein Ras, which in turn activate the protein kinase Raf-1. However, this activation is transient and does not sustain the activation of ERK. According to recent studies NGF can also activate B-Raf through Rap1 and that the activation of B-Raf is required to sustain the phosphorylation of ERK (York et al., 1998). Therefore it was hypothesised at the start of this research that NGF and EGF would use different pathways to modulate the longevity of the activation of ERK. Thus, upon EGF the signal would be transiently mediated through the activation of Ras/Raf-1, whereas upon NGF the signal would also be transiently mediated through Ras/Raf-1 and sustained by the activation of Rap1/B-Raf. According to several lines of research (Dhillon et al., 2002b; Dumaz and Marais, 2003) PKA can directly inhibit Raf-1 by phosphorylating Raf-1 on ser259 (Dhillon and Kolch, 2002). Thus, it was expected that an elevation of cAMP that activates PKA would switch off the ERK pathway. However, in the previous chapter, it was demonstrated that upon EGF and high level of cAMP, the phosphorylation of ERK increased. This result proved that cAMP could activate ERK through a different pathway than Ras/Raf-1, which might be the Rap1/B-Raf pathway. Here, the results show that the signal triggered by NGF was mediated through Rap1 and according to the literature through Ras, whereas the signal was only mediated through Ras upon EGF stimulation. However, when the level of cAMP was high (cilostamide treatment) Rap1 was sensitised, and Rap1 became transiently active upon EGF stimulation correlating with the increase of the phosphorylation of ERK.

It was then important to investigate whether NGF and EGF can activate Raf-1 and/or B-Raf. To do so I tried to measure the activation of both kinases upon NGF and EGF

stimulation, with or without cilostamide treatment, by an *in vitro* non radioactive kinase assay (results not shown). This assay consisted of immunoprecipitation of the kinase of interest using a specific antibody immobilised on sepharose beads. Then the kinase is incubated with inactive recombinant MEK and ERK as substrates. The reaction is initiated by adding ATP and incubated at 30ºC. The reaction is stopped by adding sample buffer. The samples are resolved by electrophoresis following by Western Blotting. The membrane is then blotted for phospho-ERK using a phospho-specific antibody giving the readout of the kinase activity. Unfortunately, I did not manage to get significant results. It is probably because this assay was not sensitive enough on endogenous proteins. However, Kao *et al* showed that Raf-1 is transiently activated in response to NGF and EGF, and that B-Raf is transiently activated in response to EGF and its activation is sustained in response to NGF (Kao et al., 2001). Moreover it was demonstrated that elevation of cAMP in rat fibroblasts and vascular smooth muscle cells inhibits the ERK pathway (Cook and McCormick, 1993; Wu et al., 1993), which is the result of the inhibition of Raf-1 by PKA (Dhillon et al., 2002b; Hafner et al., 1994).

However, our results suggest that NGF mediates the signal through both Ras and Rap1 to sustain the activation of ERK, and that EGF mediates the signal only through Ras but elevated cAMP concentration sensitised the activation of Rap1. Then, the route for cAMP in the regulation of ERK was investigated by decomposing the effect of cAMP using different cAMP analogues to activate specifically either PKA or Epac. The results obtained showed that upon NGF stimulation, the effect of Epac agonist mimicked the effect of cilostamide that increased the phosphorylation of ERK. In contrast, PKA activation did not modify the phosphorylation of ERK, suggesting that the possible inhibition of Raf-1 by PKA does not affect the activation of ERK through Rap1/B-Raf. The effect of Epac agonist was reduced in presence of the PKA agonist. This was surprising because it was expected that the activation of PKA would not interfere with the activation of Epac. Christensen *et al* showed the same phenomenon using the same agonists (Christensen et al., 2003). They suggested that “the activation of Epac had a strongly enhancing effect on PKA” and they showed that the activation of Epac enhanced the activation of PKA activator. Upon EGF stimulation, again the Epac agonist mimicked the effect of cilostamide on the activation of ERK. In contrast to NGF stimulation, the PKA agonist had an inhibitory effect on the phosphorylation of ERK. This makes sense because contrary to the NGF stimulation, the signal is only mediated through Ras/Raf-1 upon EGF stimulation. Therefore, if PKA does inhibit Raf-1, then

the pathway should be switched off. However ERK was still slightly phosphorylated. This is probably because Ras can activate B-Raf. The effect of Epac agonist on ERK activation was totally abolished in the presence of the PKA agonist.

The results obtained on the differentiation of PC12 cells were disappointing. Neither Epac nor PKA agonists did improve the differentiation of the cells upon EGF stimulation similar to cilostamide. Upon NGF stimulation, only the Epac agonist slightly increased cell differentiation. It would have been interesting to investigate if the combination of both agonists would mimic the effect of cilostamide and improve cell differentiation upon both NGF and EGF stimulation. Christensen *et al* demonstrated that the Epac agonist on its own could induce neurite extension (Christensen et al., 2003) but they used 60 times more Epac agonist than I did. From their results they concluded that Epac has an important role in the cAMP-induced neurite extension in PC12 cells. Kiermayer *et al* did similar experiments using the same PKA agonist, but they used 2-Me-cAMP as Epac agonist (Kiermayer et al., 2005). They showed that on their own none of the agonists could initiate neurite outgrowth. However, when they combined the two agonists together and stimulated PC12 cells with EGF, cells elicited neurite outgrowth. They concluded that cAMP-induced neurite outgrowth requires the activation of both Epac and PKA.

To summarise, these results taken together confirm that EGF utilises the Ras/Raf-1 pathway to transduce the extracellular signal and that NGF transduces the signal through Ras/Raf-1 and Rap1/B-Raf. Elevation of cAMP can switch on the Rap1/B-Raf pathway likely though Epac that strengthens the activation of ERK upon both NGF and EGF stimulations. Furthermore, these results show that the differentiation of PC12 cells induced by cAMP is mainly mediated through Epac rather than PKA as it was suggested in previous research (Vossler et al., 1997; Yao et al., 1998; York et al., 1998). However, the activation of PKA and Epac together seems essential to initiate the differentiation of the cells. It seems that in response to cAMP level PKA and Epac synergise together to mediate the correct signal (Christensen et al., 2003; Kiermayer et al., 2005).

CHAPTER 5

THE ROLE OF Raf-1 AND B-Raf IN THE REGULATION OF ERK BY cAMP

5.1 INTRODUCTION

In the previous chapter it was demonstrated that NGF mediates its signal through the small G-proteins Ras and Rap1 whereas EGF mediates its signal through Ras only. In addition, it was demonstrated that an increase in cAMP by cilostamide sensitised Rap1 that became active upon mitogen stimulation. In these conditions the signal initiated by EGF was also mediated through Rap1. The activation of Rap1 upon both NGF and EGF stimulations correlated with the increase in the phosphorylation of ERK suggesting that Rap1 is involved in the regulation of ERK by cAMP. In contrast, when decomposing the effect of cAMP using cAMP analogues, it was demonstrated that specific PKA agonist slightly inhibited the phosphorylation of ERK upon EGF stimulation. Ras and Rap1 are the small G-proteins that link the mitogen receptors to the ERK kinase cascade by activating Raf-1 and B-Raf (Marshall, 1998; Stork and Schmitt, 2002; Zwartkruis and Bos, 1999). While Ras activates both Raf-1 and B-Raf (Kao et al., 2001; Marais et al., 1997), Rap1 was reported to be a selective activator of B-Raf in PC12 cells (Marais et al., 1997; Vossler et al., 1997; York et al., 1998). It is still unclear how signalling is coordinated between Ras/Rap1 and Raf-1/B-Raf and how cAMP is involved in these mechanisms. This chapter is dedicated to determine through which Raf kinases the signal is mediated in response to NGF or EGF, what the involvement of Rap1 is, and also to investigate a potential molecular mechanism for the inhibition of Raf-1 by PKA.

5.1.1 Activation of Raf-1 and B-Raf kinases

The Raf kinases are activated by small G-proteins of the Ras family in response to extracellular stimuli. The Ras proteins are targeted to the plasma membrane and their activation involves the recruitment of guanine nucleotide exchange factors (GEFs) to activated growth factor receptors or activated G-protein coupled receptors. The Ras GEFs stimulate the release of GDP from Ras to allow GTP to bind, causing a conformational change of Ras that enables it to interact with Raf kinases and other effectors with high affinity (Avruch et al., 2001). Activated Ras recruits Raf from the cytosol to the plasma membrane where Raf activation takes place. Ras binds to the Ras binding domain (RBD) and to the cysteine-rich domain (CRD) of Raf (Brtva et al., 1995; Hu et al., 1995). Although both Raf-1 and B-Raf are activated by binding to Ras, their respective activation mechanisms are different (Fig. 5.1). Raf-1 requires secondary

signals to be fully activated, whereas B-Raf can be activated by Ras alone. Raf-1 requires the phosphorylation of four sites located in the kinase domain (CR3) to be fully activated: S338 and Y341 within the negative-charge regulatory region (Fabian et al., 1993; King et al., 1998), and T491 and S494 within the activation loop (Chong et al., 2001). Raf-1 requires a Src family tyrosine kinase to phosphorylate Y341 (Marais et al., 1997) and S338 is probably phosphorylated by different kinases including PAK3 (King et al., 1998; Sun et al., 2000). The activation loop sites T599 and S602, equivalent to T491 and S494 respectively, are conserved in B-Raf. S446 corresponding to S338 is also conserved in B-Raf; however this serine is constitutively phosphorylated. Furthermore, Y341 is replaced by an aspartic acid (D449) in B-Raf (Mason et al., 1999) conferring a negative charge to the protein. Therefore, B-Raf does not require phosphorylation by a tyrosine kinase and other kinases, and hence requires fewer events to be fully activated (Marais et al., 1997).

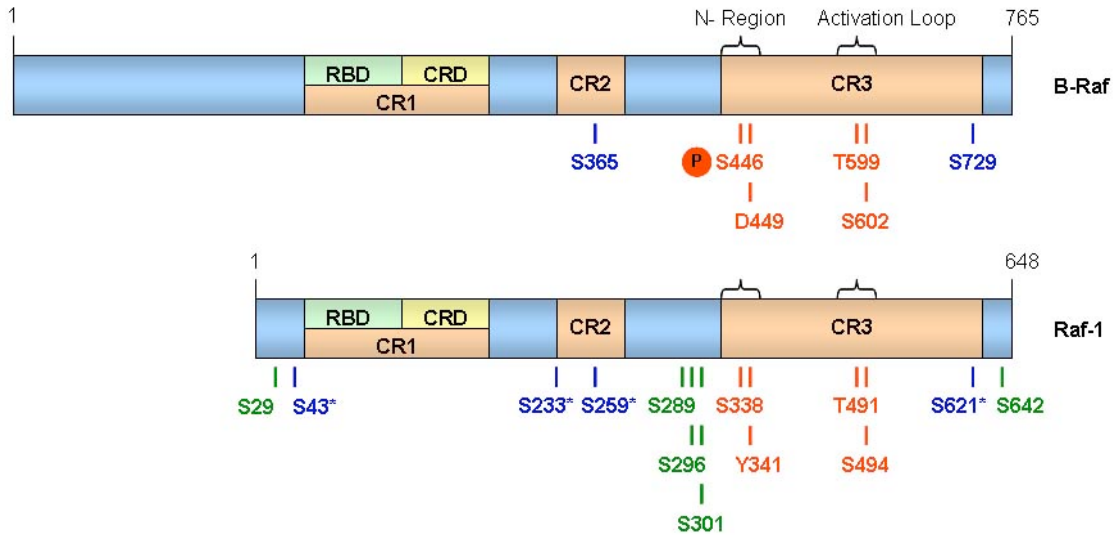


Figure 5. 1: Phosphorylation sites for the activation and inhibition of Raf-1 and B-Raf kinases. Both Raf isoforms share a common architecture with three conserved regions (CR1, CR2 and CR3). The kinase domain resides in the CR3 at the C-terminus. Raf activation occurs at the plasma membrane. Active Ras recruits Raf to the plasma membrane by direct binding to the Ras binding domain of Raf (RBD) and the cysteine-rich domain of Raf (CRD). The activation of B-Raf requires fewer events than Raf-1 mainly because S446 is constitutively phosphorylated, and because the amino acid corresponding to Y341 in Raf-1 is replaced by aspartic acid in B-Raf. Raf-1 requires secondary signals to be fully activated such as Src family tyrosine kinases, whereas Ras alone can fully activate B-Raf. Deactivation of Raf-1 involves feedback phosphorylation by ERK on serines 29, S43, S289, 296, 301 and 642 (Dougherty et al., 2005). The activating phosphorylation sites are highlighted in red. The phosphorylation sites involved in the regulation of Raf are highlighted in blue. The asterix indicates the PKA phosphorylation sites. The inhibitory ERK feedback phosphorylation sites are indicated in green.

Another major difference in the activation of Raf-1 and B-Raf is that B-Raf can be activated by Rap1, which is a Ras-related small G-protein (Kao et al., 2001). Rap1 can be activated via a large range of receptors such as the NGF receptor in response to different stimuli. Like Ras, Rap1 requires GEFs to exchange GDP to GTP to be activated. Crk SH3 domain-binding exchange guanine nucleotide factor (C3G) was the first Rap1 GEF to be cloned (Gotoh et al., 1995) and Crk can bind to various tyrosine kinase receptors targeting Rap1 to the plasma membrane. Rap1 is specific for B-Raf and does not activate Raf-1.

5.1.2 PKA inhibits Raf-1 by direct phosphorylation

It is generally accepted that cAMP inhibits Raf-1 through direct phosphorylation by PKA, but there are still debates about the exact mechanisms. It was demonstrated that PKA can phosphorylate Raf-1 on serines 43, 233, 259 and 621 both *in vitro* and *in vivo* (Dhillon et al., 2002b; Dumaz et al., 2002; Hafner et al., 1994; Sidovar et al., 2000). These sites appear to be phosphorylated in resting cells but are hyperinduced by PKA (Dhillon et al., 2002a). Moreover, these sites work independently to inhibit Raf-1 and they have to be all mutated to render Raf-1 completely resistant to PKA inhibition (Dumaz et al., 2002; Sidovar et al., 2000). Wu *et al* demonstrated that the phosphorylation on S43, which is located just upstream to the RBD, lowers the affinity of Raf-1 to Ras.GTP that is the active form of Ras (Wu et al., 1993). Therefore the phosphorylation of S43 by PKA prevents Raf-1 binding and activation by Ras. However, the phosphorylation of S43 is not exclusive for the inhibition of Raf-1 by PKA as it was demonstrated that mutation of S43 to alanine does not overcome the effect of cAMP and that Raf-1 is still susceptible to inhibition by PKA (Dhillon et al., 2002b; Dumaz et al., 2002). S43 is also one of the six feedback phosphorylation sites that are phosphorylated by activated ERK and contribute to the deactivation of Raf-1 (Dougherty et al., 2005). Phosphorylated serines 233, 259 and 621 are docking sites for 14-3-3 proteins, which are adapter proteins that bind to short phosphorylated peptide motifs (Yaffe, 2002). It was demonstrated that S259 is the major phosphorylation site for the inhibition by PKA (Dhillon and Kolch, 2002). When 14-3-3 binds to these sites, it antagonises Ras binding. The mechanisms through which this occurs remained unclear but it is thought that 14-3-3 binding to Raf-1 might directly compete with Ras for the CRD of Raf-1 or might modify Raf-1 conformation and mask the RBD. Dumaz

and Marais from their research have suggested a model for the regulation of Raf-1 by cAMP (Dumaz and Marais, 2005). They proposed (Fig. 5.2) that in resting cells, S259 and S621 are phosphorylated allowing 14-3-3 to bind and trap Raf-1 in an inactive form. In normal conditions, Ras activation recruits Raf-1 to the plasma membrane removing 14-3-3 from the phosphorylated S259, which can be dephosphorylated by PP2A. Once S259 is dephosphorylated then Raf-1 can be activated through a series of further phosphorylations (see section 5.1.1). Activated PKA can also phosphorylate Raf-1 on S43 and S233. The phosphorylation on S233 creates a new binding site for 14-3-3. It seems that 14-3-3 has a higher affinity for phosphorylated S233 than S621, and therefore 14-3-3 would bind to phosphorylated S233 rather than to phosphorylated S621. Their hypothesis is that in this particular conformation, Ras cannot displace 14-3-3 from phosphorylated S259 and Raf-1 cannot be activated.

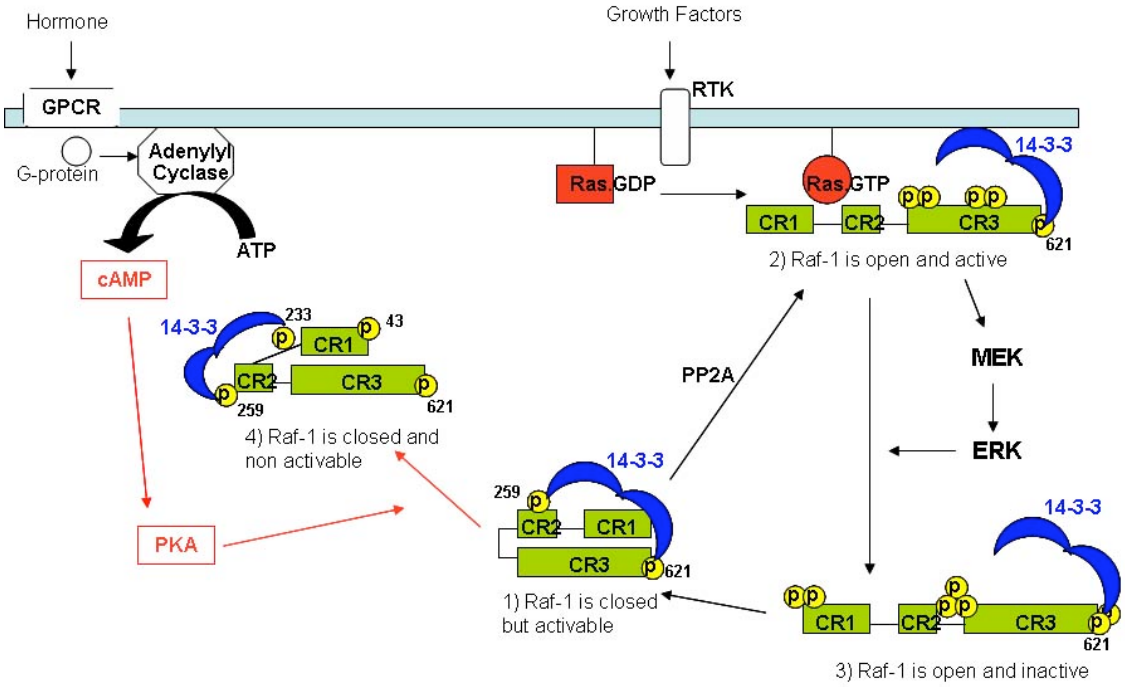


Figure 5. 2: Model for the regulation of Raf-1 by PKA. 1) In resting cells, Raf-1 is trapped in a closed but activatable conformation stabilised by 14-3-3 binding to S259 and S621. 2) Active Ras can displace 14-3-3 from S259, allowing PP2A to dephosphorylate S259 at the plasma membrane. Raf-1 can now be phosphorylated for activation. 3) Active Raf-1 stimulates MEK, which activates ERK. ERK terminates the signaling by phosphorylating Raf-1 locking it into an open but inactive conformation. Rephosphorylation of S259 allows 14-3-3 to bind and stabilises the inactive conformation. 4) When cAMP is elevated, PKA phosphorylates Raf-1 on S43 and S259 blocking the interaction with Ras and the recruitment of Raf-1 to the plasma membrane. PKA phosphorylates S233 creating a new binding site for 14-3-3, causing a rearrangement that switches 14-3-3 from S621 to S233 but maintaining contact with S259. Raf-1 is locked in a conformation that cannot be activated by Ras. Figure reproduced from (Dumaz and Marais, 2005).

5.1.3 AKAPs: A Kinase Anchoring Proteins

The AKAP family comprises about 50 anchoring proteins that have diverse structures. But they have in common that they bind to PKA and co-precipitate with PKA catalytic activity (Tasken and Aandahl, 2004; Wong et al., 2004). All AKAPs contain a PKA-binding domain located at the C-terminus and a unique targeting domain in the N-terminus that participates in the association of AKAPs to membranes confining the PKA-AKAP complex to specific subcellular structures. All AKAPs bind to the RII subunits of PKA with high affinity. The PKA-binding domain consists of 14-18 hydrophobic amino acids forming an amphipathic α-helix that binds to the N-terminal dimerisation domain of the R subunits of PKA (Dell'Acqua and Scott, 1997; Newlon et al., 1997; Wong et al., 2004). More recently it was reported that some AKAPs bind specifically to the RI subunits of PKA (McConnachie et al., 2006; Wang et al., 2006a) and others have dual affinity for both RI and RII PKA subunits (Huang et al., 1999). AKAP79, AKAP82 and AKAP220 were characterised as dual specific AKAPs and can bind to both RI and RII PKA subunits (JarnÃ¦ss and TaskÃ©n, 2007). In addition to PKA regulatory subunits, AKAPs are also able to bind to phosphatases as well as kinases and other proteins involved in signal transduction, forming multivalent signal transduction complexes (Baillie et al., 2005; Dodge-Kafka and Kapiloff, 2006; Tasken and Aandahl, 2004; Wong et al., 2004).

5.2 RESULTS

5.2.1 Depletion of Raf-1 in PC12 cells

In the previous chapter it was demonstrated that the signal initiated by NGF is mediated through Ras and Rap1, whereas the signal initiated by EGF is mediated only through Ras. It was also demonstrated that cilostamide sensitises Rap1 to become active upon mitogen stimulation correlating with the increase and the duration of the phosphorylation of ERK. A siRNA approach was used to investigate which Raf kinases are activated by small G-proteins to transduce the signal.

PC12 cells were plated onto poly-L-lysine pre-coated 6-well plates and let to set overnight. The cells were transfected with a Raf-1 siRNA from Qiagen for 6 hours in a reduced serum medium, following which the cells were incubated for 72 hours in normal medium. The transfected cells were serum starved for 3 to 4 hours and then they were treated for 10 min with cilostamide before being stimulated for 10 minutes with NGF or for 5 minutes with EGF at the indicated concentrations. The cell lysates were resolved by electrophoresis and Western Blotting. The nitrocellulose membranes were probed for C3G, phosphorylated ERK and ERK as loading control (Fig. 5.3). Each band was quantified by the ODYSSEY software, and the amount of phosphorylated ERK was normalised to the total amount of ERK. The percentage of the activation of ERK was calculated from the value “NGF” or “EGF” corresponding to the maximal activation of ERK upon mitogen stimulation (Fig. 5.3.c).

The results show that the Raf-1 siRNA used for this experiment knocked down about 80% of Raf-1 (Fig. 5.3.b). Upon NGF stimulation, the depletion of Raf-1 did not modify the activation of ERK in cells either treated or not with cilostamide (Fig. 5.3 panels a and c). These results might show that Raf-1 is not essential in the activation of ERK upon NGF stimulation, suggesting that B-Raf might be responsible for the majority of the activation of ERK by NGF. This is consistent with the works from Kao *et al* (Kao et al., 2001) who demonstrated that B-Raf is responsible for 90% of the activation of ERK in response to NGF stimulation. However, Raf-1 was not completely knockdown and it might be possible that the remaining Raf-1 was enough to activate ERK in response to NGF stimulation.

In the previous chapter it was demonstrated that EGF in normal conditions, activates Ras and was unable to activate Rap1. From these results it was hypothesised that EGF would mediate the signal preferably through Ras/Raf-1. Therefore, knocking down Raf-1 would result in the loss of ERK activation upon EGF stimulation. Unexpectedly, the depletion of Raf-1 did not reduce the activation of ERK upon EGF stimulation. Once again, the activation of ERK in response to EGF in the Raf-1-depleted cells might be due to the fact that the knockdown of Raf-1 was not complete. Another hypothesis is that the signal might have been mediated through a different route. As described in the introduction, Ras can activate B-Raf (Kao et al., 2001; Kuroda et al., 1996; Marais et al., 1997). It might be possible that upon EGF stimulation the signal is not only mediated through Raf-1 but also through B-Raf and that B-Raf compensates the depletion of Raf-1. Furthermore, in the Raf-1 knockdown, the activation of ERK was slightly increased upon EGF stimulation and cilostamide treatment compared to the cells expressing Raf-1. According to the results obtained in the previous chapter, it was demonstrated that cAMP sensitised Rap1 that became active upon EGF stimulation and high level of cAMP. This suggests that activated Rap1 is likely to activate B-Raf that strengthens the signal. However, this can only be confirmed by measuring the activity of B-Raf.

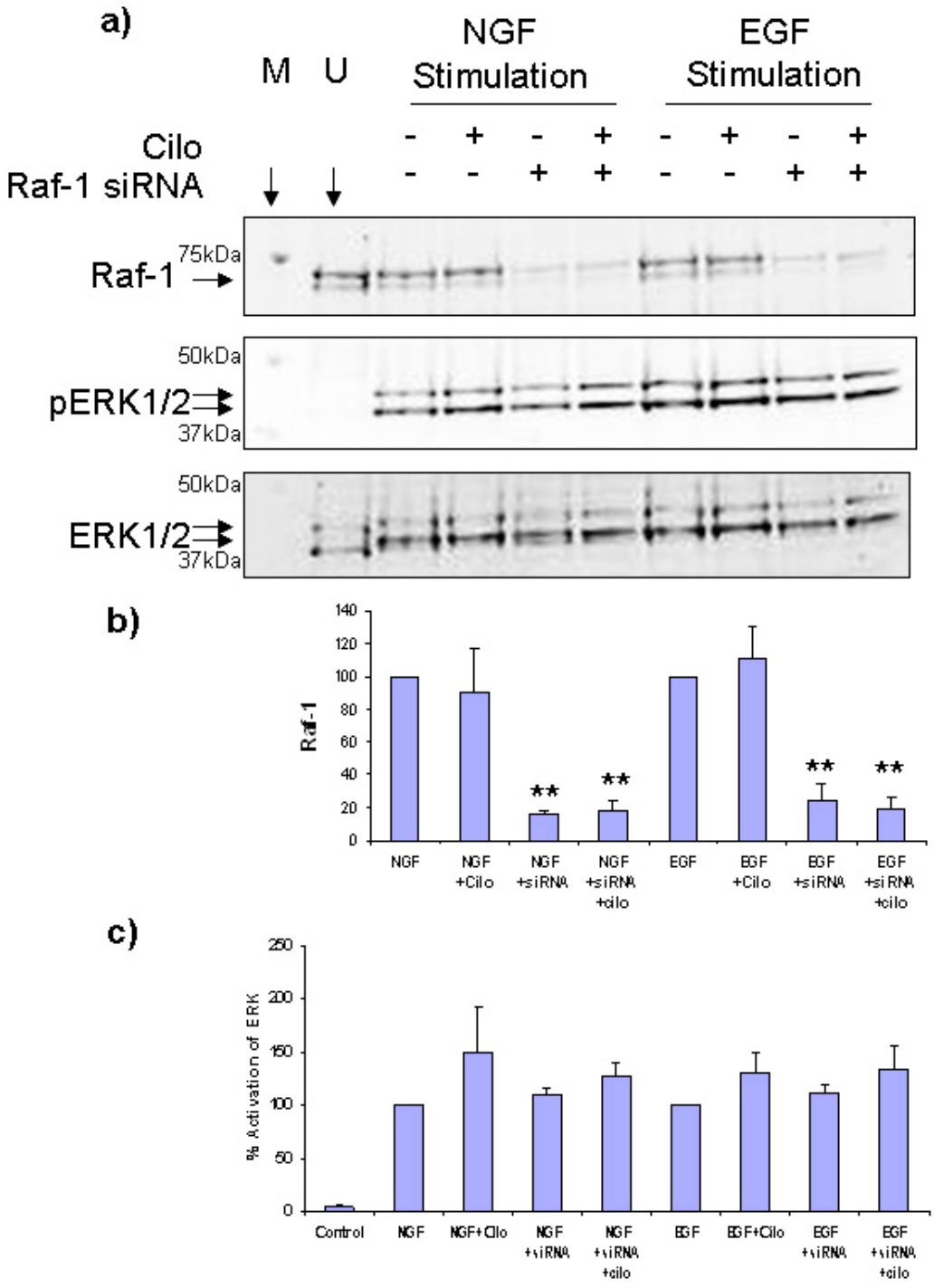


Figure 5. 3: Depletion of Raf-1 in PC12 cells. a) PC12 cells were transfected with or without siRNA against Raf-1 using Lipofectamine™ 2000 for 6 hours in Opti-MEM® I Reduced Serum Medium. The cells were incubated for 72 hours in normal medium, and serum starved for at least 3 hours. Then they were treated with Cilostamide (10 μM) for 10 min before being stimulated with NGF (100 ng/ml) for 10 min or with EGF (100 ng/ml) for 5 min, as indicated. The lysates were analysed by Western Blotting and the membranes were probed for Raf-1, phospho-ERK and ERK as loading control. b) The graph represents the percentage of the depletion of Raf-1. c) The graph represents the percentage of ratio between pERK/ERK. The values shown are the mean of 3 different experiments ± S.D. The effect of each treatment was compared to their respective control, ** $p < 0.01$. M stands for protein marker and U stands for unstimulated.

5.2.2 Effect of C3G depletion on the activation of ERK

Four different siRNAs against B-Raf from different companies were tested to deplete PC12 cells from B-Raf without success (results not shown). Therefore a protein upstream of B-Raf was targeted to be knocked down. This protein was the Rap1-GEF C3G (Crk SH3 domain guanine nucleotide exchanger). The association of C3G with the Crk adaptor molecule allows C3G to be recruited to the plasma membrane via the SH2 domain of Crk (Feller, 2001; Wang et al., 2006b). The membrane-translocated Crk-C3G complex is active and enables receptor tyrosine kinases to activate Rap1 (Kao et al., 2001; York et al., 2000; Zwartkruis and Bos, 1999).

PC12 cells were plated onto poly-L-lysine pre-coated 6-well plates and let to set overnight. The cells were transfected with C3G pool of four siRNAs from Qiagen for 6 hours in a reduced serum medium, following of which the cells were incubated for 72 hours in normal medium. The transfected cells were serum starved for 3 to 4 hours and then they were treated for 10 min with cilostamide before being stimulated for 10 minutes with NGF or for 5 minutes with EGF at the indicated concentrations. The cell lysates were resolved by electrophoresis and analysed by Western Blotting. The nitrocellulose membranes were probed for C3G, phospho-ERK and ERK as loading control (Fig. 5.4). Each band was quantified by the ODYSSEY software, and the amount of phosphorylated ERK was normalised to the total amount of ERK. The percentage of the activation of ERK was calculated from the value “NGF” or “EGF” corresponding to the maximal activation of ERK upon mitogen stimulation (Fig. 5.4.c).

The results show that the C3G pool of siRNAs knocked down significantly about 60 to 80% of C3G (Fig. 5.4.b). Upon NGF stimulation (Fig. 5.4 panels a and c), the depletion of C3G did not impair the activation of ERK. However, when the cells were treated with cilostamide to increase the level of cAMP, the phosphorylation of ERK tended to be reduce when C3G was knocked down compared to the cells expressing C3G and treated with cilostamide. Upon EGF stimulation, the depletion of C3G had no effect on the phosphorylation of ERK and did not impair the activation of ERK by cAMP (cilostamide treatment).

It was expected that the depletion of C3G would reduce the activation of ERK upon NGF stimulation, abrogate the enhancing effects of cilostamide, and even cause further reduction of ERK phosphorylation in the presence of cilostamide. According to Kao *et*

al the activation of ERK in response to NGF is mainly due to the action of B-Raf (Kao et al., 2001). They estimated that Raf-1 contributes to only 10% of the activation of ERK. However, this remaining Raf-1 activity conceivably could take over when B-Raf activation is blocked, and may explain why upon NGF stimulation and depletion of C3G ERK can still be phosphorylated. However, it was expected that when the level of cAMP is high as under cilostamide treatment, then Raf-1 would be inhibited by cAMP-dependent PKA resulting in the total switch off of the ERK pathway. However, the C3G knock-down was not complete and there is the possibility that the remaining C3G is sufficient for maintaining ERK activation. Another explanation would be that Ras might activate B-Raf and compensate the loss of C3G. Alternatively, it is possible that C3G is not the only route of Rap1 activation. C3G, which links Rap1 to the mitogen receptors, resides upstream B-Raf and Rap1. Therefore depleting C3G does not exclude the possibility that Rap1 can be activated independently from C3G, suggesting that cAMP might activate Rap1 via another route, likely through Epac.

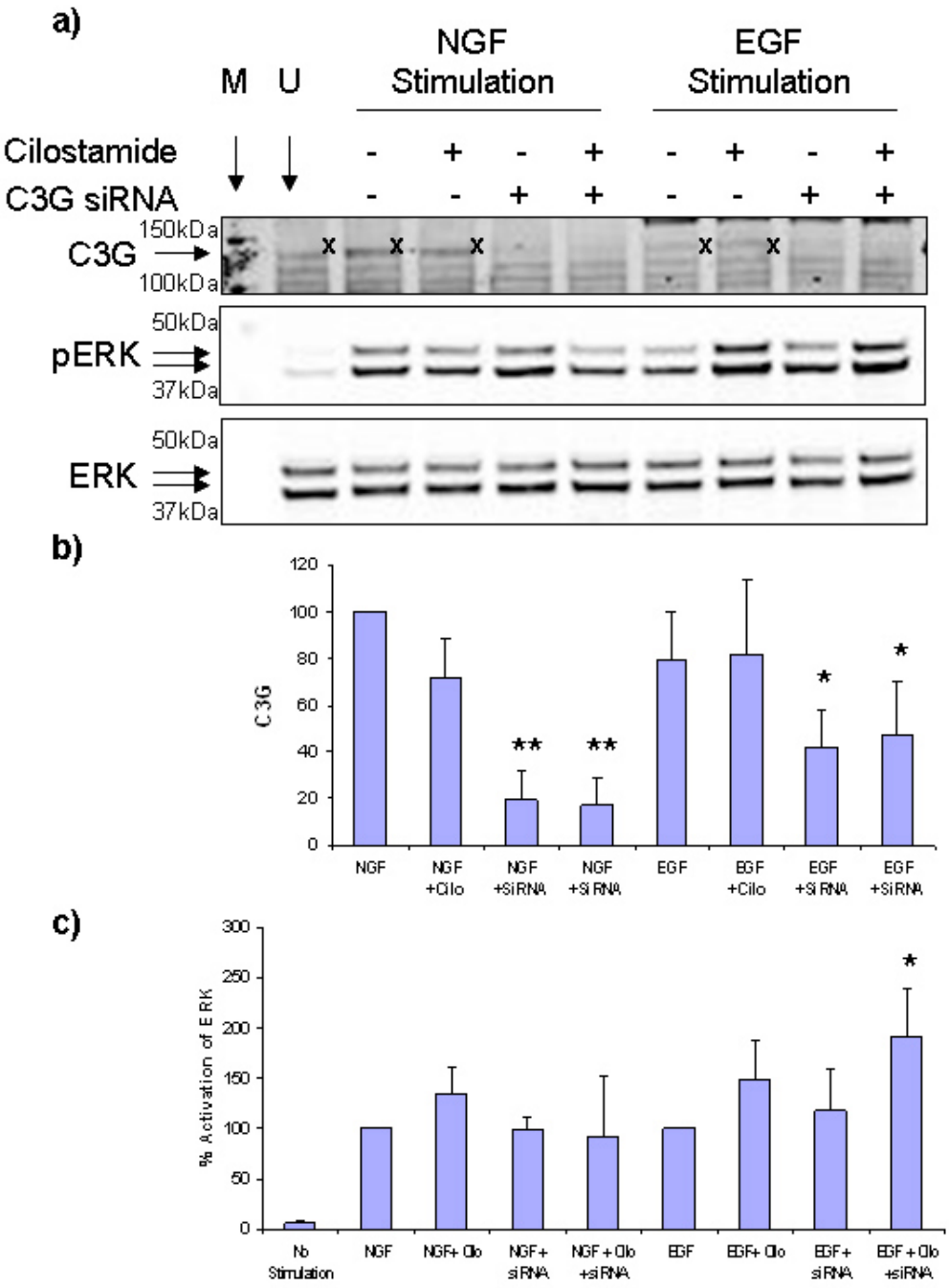


Figure 5. 4: C3G knockdown in PC12 cells. a) PC12 cells were transfected with or without C3G pool of four SiRNAs using Lipofectamine™ 2000 for 6 hours in Opti-MEM® I Reduced Serum Medium. They were incubated for 72 hours in normal medium. The cells were serum starved for at least 3 hours and then they were treated or not with Cilostamide (10 μM) for 10 min before being stimulated for 10 min with NGF (100 ng/ml) or for 5 min with EGF (100 ng/ml). The lysates were analysed by Western Blotting and the membranes were probed for C3G, phospho-ERK and ERK as loading control. M stands for protein marker and U for untreated cells. b) The graph represents the percentage of the depletion of C3G (the bands corresponding to C3G are indicated with an x symbol). c) The graph represents the percentage of the ratio between pERK/ERK. The values shown are the mean of 3 different experiments ± S.D. Each effect of the different treatment was compared to their respective control, * $p < 0.05$ and ** $p < 0.01$.

5.2.3 Raf-1 binds to AKAPs

It is now commonly accepted that PKA inhibits Raf-1 *in vitro* (Hafner et al., 1994) and *in vivo* (Wu et al., 1993) by phosphorylating directly Raf-1 on serines 43, 233, 259 and 621 (Dhillon et al., 2002b; Dumaz et al., 2002; Sidovar et al., 2000). As serines 233, 259 and 621 are 14-3-3 binding sites, and 14-3-3 needs to be dissociated from Raf-1 for efficient activation by Ras (Dhillon, 2002) PKA phosphorylation of Raf-1 will promote its binding to 14-3-3 and blocking of Raf-1 recruitment to the plasma membrane (Dumaz and Marais, 2003). The subcellular localisation of PKA is due to anchoring to AKAPs through the RII regulatory subunits of PKA. Therefore AKAPs target PKA to distinct subcellular localisations to confine PKA phosphorylation activity to specific sets of potential substrates (Carnegie and Scott, 2003; Jarnæss and Taskén, 2007; Wong et al., 2004). Every AKAP contains a PKA-anchoring domain and typically is also able to bind to phosphatases as well as kinases and other proteins involved in signal transduction, forming multivalent signal transduction complexes (Baillie et al., 2005; Dodge-Kafka and Kapiloff, 2006; Tasken and Aandahl, 2004; Wong et al., 2004). From these observations has emerged the idea that AKAPs might scaffold a complex between PKA and Raf-1 and possibly B-Raf, which would allow PKA to regulate these Raf kinases directly. This hypothesis was investigated by co-immunoprecipitation assays as described below.

Growing PC12 cells were lysed as described in section 2.1.6 of chapter 2. Different endogenous AKAPs (AKAP79, 82 and 220) were immunoprecipitated from the PC12 cell lysates. These AKAP immunoprecipitates were probed for co-precipitating Raf-1 and B-Raf (Fig. 5.5.a). Reciprocally, Raf-1 and B-Raf were immunoprecipitated from the cell lysates and probed for associated AKAP79 (Fig. 5.5.b). In order to confirm the presence of these AKAPs, the immunoprecipitates were loaded alongside the cell lysates and probed with their respective antibodies (Fig. 5.6). Due to the poor affinity of AKAP220 antibody, no AKAP220 could be detected in the cell lysate or immunoprecipitate (result not shown).

The results show that Raf-1 co-precipitated with all three AKAPs, whereas B-Raf did not co-precipitate with any AKAPs. However, AKAP79 did co-precipitate with B-Raf, suggesting that there might be some selectivity in the interaction of AKAPs and Raf isoforms. It also serves as a good control assuring the specificity of the co-

immunoprecipitation. Because AKAPs bind to the regulatory subunits of PKA (Dodge et al., 2001; Houslay and Adams, 2003; McConnachie et al., 2006; Michel and Scott, 2002) these results are really interesting suggesting the existence of a complex between PKA, AKAP and Raf-1 and a possible mechanism for PKA to regulate Raf-1 and possibly B-Raf.

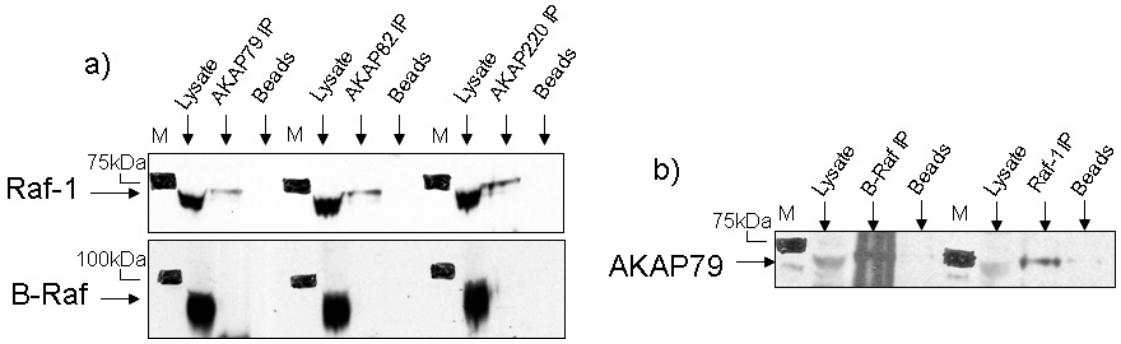


Figure 5. 5: Raf-1 co-precipitates with AKAPs. a) Endogenous proteins AKAP79, AKAP82 and AKAP220 were immunoprecipitated as indicated from PC12 cells lysates. The lysates before immunoprecipitation (as control to prove the presence of Raf-1 and B-Raf in the lysates), the immunoprecipitated proteins and empty protein G sepharose beads (as control to make sure that the beads did not pulldown Raf-1 or B-Raf) were probed for associated Raf-1 and B-Raf. b) Raf-1 and B-Raf were immunoprecipitated and probed for associated AKAP79. M stands for the protein marker. These blots are representative of 3 independent experiments.

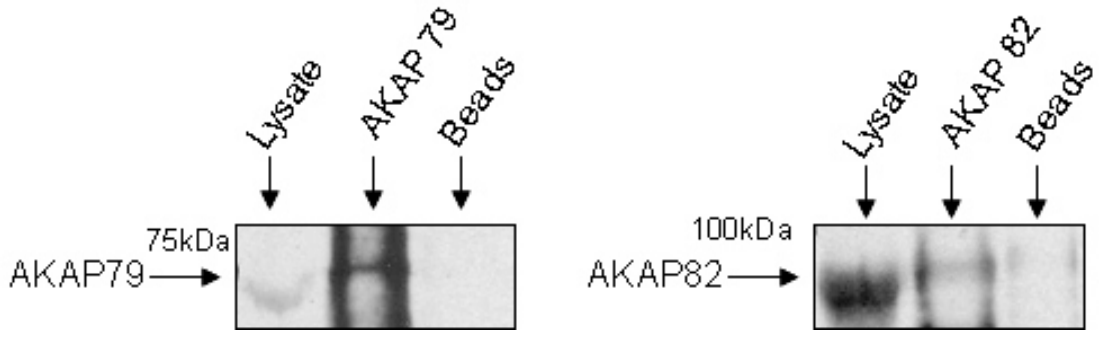


Figure 5. 6: Control of the presence of AKAP79 and AKAP82 in the PC12 cell lysates and immunoprecipitates. Endogenous AKAP79 and AKAP82 were immunoprecipitated from PC12 cells lysates. The lysates before immunoprecipitation (as control to prove the presence of AKAP 79), the AKAP immunoprecipitates and the protein G sepharose beads (as control to make sure that the beads do not pulldown AKAP 79) were probed for AKAP79 and for AKAP82.

5.2.4 Activation status of Raf-1 bound to AKAP79

Since it was demonstrated that AKAP79 co-precipitated with Raf-1, it was logical to investigate the activity of Raf-1 bound to AKAP79 in order to propose a possible molecular mechanism for PKA to inhibit Raf-1.

PC12 cells were plated on 10 cm dishes pre-coated with Poly-L-lysine and incubated at 37ºC until they reached 80% of confluence. Then the cells were treated for 10 min with either cilostamide or forskolin at the indicated concentrations before being lysed in 3T3-lysis buffer. To measure the activation status of Raf-1 bound to AKAP79, AKAP79 was first immunoprecipitated from the cell lysates. Then, the remaining Raf-1 unbound to AKAP79 was immunoprecipitated from the supernatant of the first immunoprecipitation. A 1:2 serial dilution of the Raf-1 immunoprecipitate (IP) was loaded alongside in order to obtain a calibration curve to quantify the percentage of total Raf-1 and the percentage of phosphorylated Raf-1 pulled down in the AKAP79 IPs. The IPs were resolved by electrophoresis and the proteins were transferred onto nitrocellulose membranes by Western Blot. The membranes were probed first for phosphorylated S259 indicating the inactive form of Raf-1, then stripped to be probed for phosphorylated S338 indicating the active form of Raf-1. The membranes were again stripped and probed for Raf-1 to evaluate the total amount of Raf-1 on each membrane (Fig. 5.7). The membranes were analysed using the ECL detection (i.e. section 2.2.3.4). The AKAP IPs and the dilution of Raf-1 IPs were also loaded along to the lysates before immunoprecipitation and probed for AKAP79. There was no AKAP79 detected in the Raf-1 IP, which proved that the totality of AKAP79 was immunoprecipitated before immunoprecipitating Raf-1 (Fig. 5.8).

On the blots two bands around the molecular weight of Raf-1 (74 kD) were detected, both of which correspond to Raf-1. The gels shown were run longer than normal in order to achieve a good separation between the Raf-1 band and the IgG heavy chain bands. As the heavy chains are in vast access they tend to saturate the image and make quantification difficult if they are not far enough away from the bands of interest. When the gels were run normally, then only one Raf-1 band was detected at 74 kDa. This suggested that the two bands detected corresponded to Raf-1.

Each band was quantified using ImageJ software from the National Institutes of Health, which calculates the integrated intensity of band on Western Blots. The values obtained for the 1:2 serial dilution of Raf-1 IPs were analyzed using a linear regression method to obtain a calibration curve. The percentage of unknown Raf-1 pulled down in the AKAP79 IPs was calculated from that curve. In order to compare the amount of phosphorylated Raf on the 3 different blots (that correspond to the different treatments), the percentage of phosphorylated S259 and phosphorylated S338 were normalized to the total amount of Raf-1 in the AKAP79 IPs present on each blot as a ratio phospho-Raf/Raf-1.

According to Dhillon *et al* the phosphorylation of Raf-1 on S259 and on S338 is mutually exclusive and S259 has to be dephosphorylated to allow phosphorylation on S338 and then activation of Raf-1 (Dhillon et al., 2007). The results (Fig. 5.7) show that the two phosphorylated forms of Raf-1 pS259 and pS338 co-exist in resting cells and co-precipitated with AKAP79. This suggests the existence of two different pools of phosphorylated Raf-1 on AKAP79.
When the cells were treated with forskolin (Fig. 5.7.c), the ratio between p259 and Raf-1 was increased by 2.7 fold, suggesting that upon forskolin treatment, the pool of Raf-1 phosphorylated on S259 is enriched. The ratio p338/Raf-1 was also increased but only by 1.4 fold. This suggests that forskolin increased the pool of inactive Raf-1 by stimulating S259 phosphorylation, and also slightly increased the pool of active Raf-1 on AKAP79.
When the cells were treated with cilostamide (Fig. 5.7.b), the p259/Raf-1 ratio was increased by 3 fold compare to the control. However, the p338/Raf-1 ratio was reduced by 4 fold.

This experiement was made only once therefore it has to be repeated before to draw any conclusion. However, the presented results suggest that forskolin and cilostamide have differential effects on the phosphorylation status of Raf-1. Both drugs seem to increase the fraction of inactive Raf-1 (p259) tethered to AKAP79, but only cilostamide seems to reduce the fraction of active Raf-1. This will be discussed further in the discussion part of this chapter.

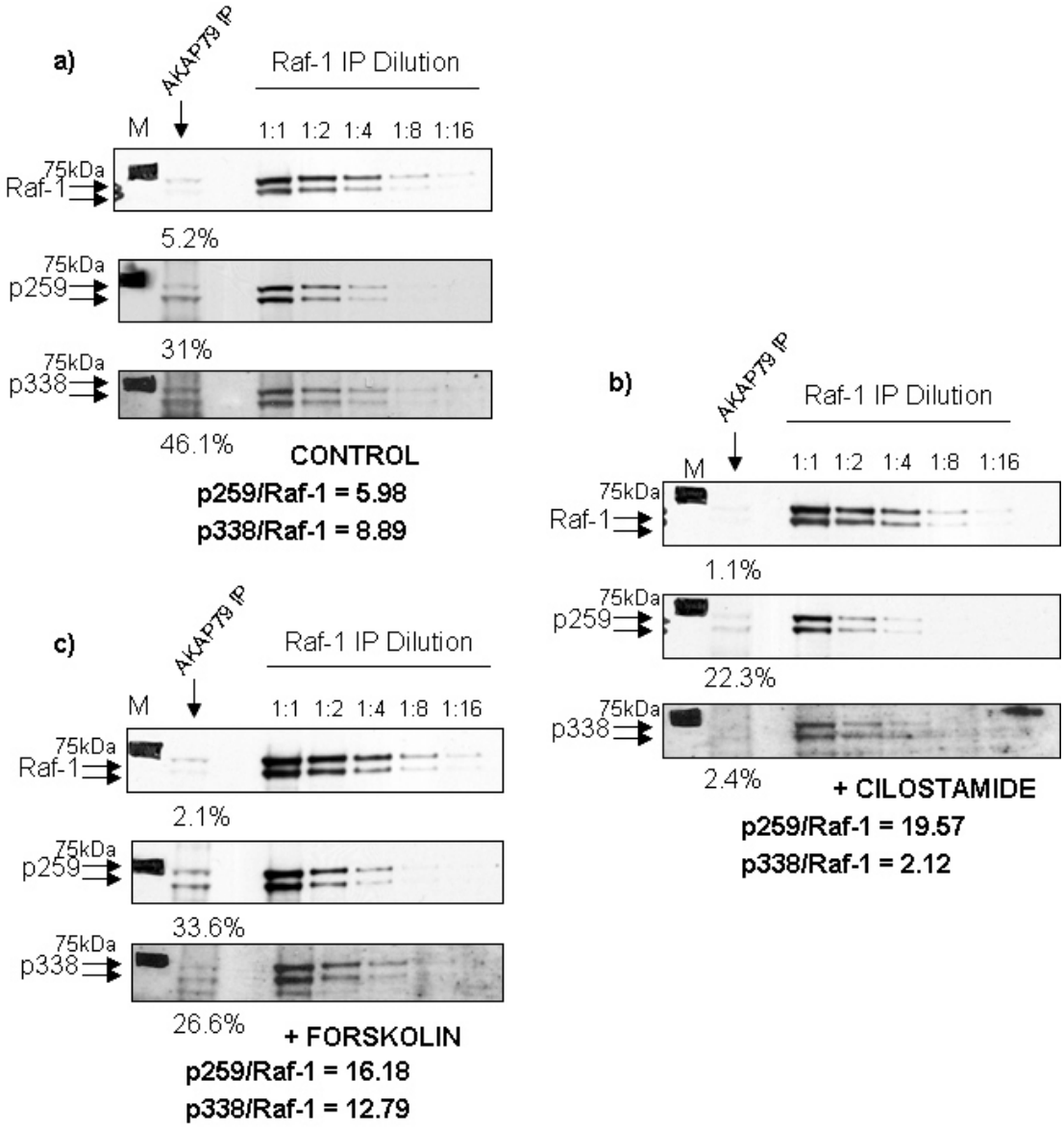


Figure 5. 7: Activation status of Raf-1 bound to AKAP79. a) Growing PC12 cells were treated for 10 min b) with cilostamide (10 μM) or c) with forskolin (100 μM) as indicated. AKAP79 was first immunoprecipitated from the PC12 cell lysates. Then unbound Raf-1 was immunoprecipitated from the lysates. The nitrocellulose membranes were probed for phospho-S259 corresponding to the inactive form of Raf-1, then for phospho-S338 corresponding to the active form of Raf-1 and finally for Raf-1 to evaluate the total amount of Raf-1 pulled down by AKAP79. Each band was quantified using ImageJ software. The Raf-1 IPs were diluted in a 1:2 serial dilution to obtain a calibration curve to determine the percentage of active and inactive Raf-1 bound to AKAP79 displayed under the corresponding band. The relative amount of phospho-Raf was normalised as the ratio between phospho-Raf/Raf-1 (in bold). This experiment was made only once. M stands for protein marker.

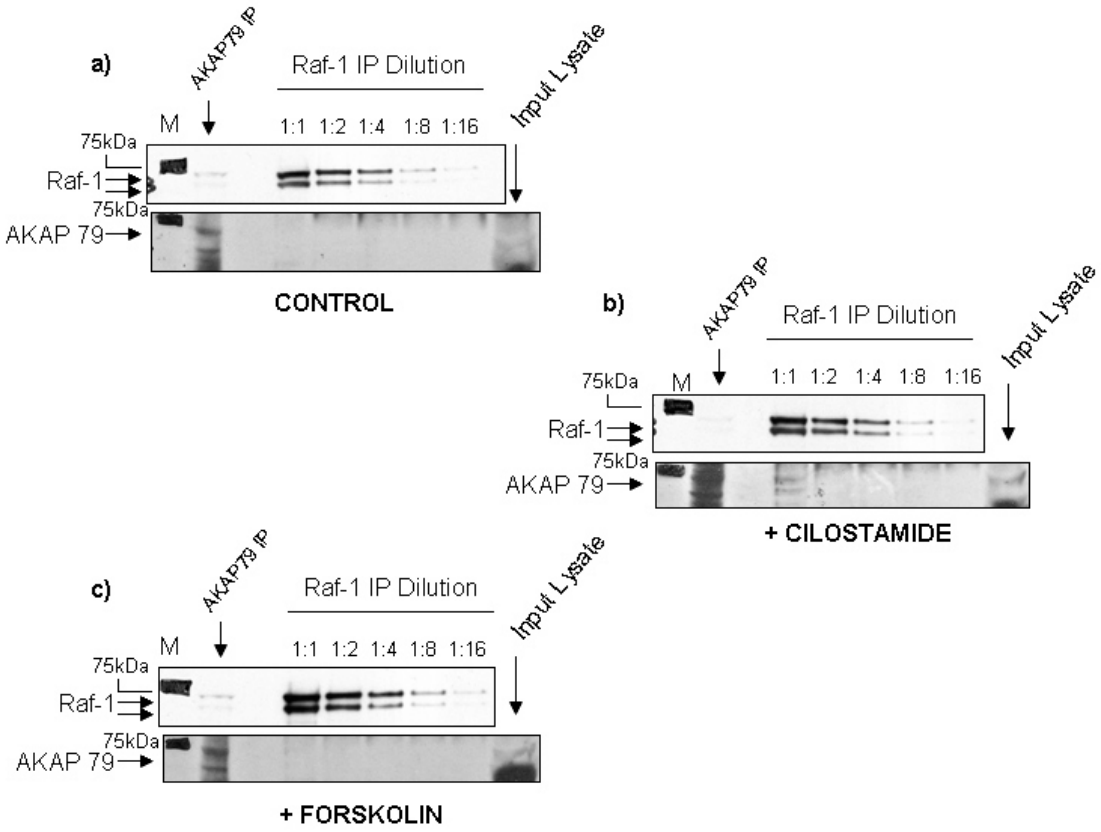


Figure 5. 8: Control that the totality of AKAP79 was immunoprecipitated from the lysates before immunoprecipitating Raf-1. a) Growing PC12 cells were treated for 10 min b) with cilostamide (10 μM) or c) with forskolin (100 μM) as indicated. The AKAP79 IPs and the Raf-1 IP dilutions were loaded along to the lysates before immunoprecipitations (input lysate). The nitrocellulose membranes were probed for Raf-1 and AKAP79. AKAP79 was not detected in the Raf-1 IP dilution, which indicated that AKAP79 was totaly immunoprecipitated from the lysate before the Raf-1 immunoprecipitations.

5.2.5 Over-expressed B-Raf binds to AKAP 79 in HEK293 cells

As shown in the section 5.2.3, although B-Raf did not co-precipitate with AKAP79, AKAP79 co-precipitated with B-Raf in PC12 cells suggesting a weak or indirect interaction between these two proteins. In order to confirm this interaction, B-Raf was co-transfected with AKAP79. Because PC12 cells are difficult to transfect, B-Raf and AKAP79 were transfected in HEK293 cells. Two mutants of B-Raf showing high kinase activity were also transfected in HEK293 cells. Mutations of BRAF (the gene encoding for B-Raf) were identified in about 7% of all cancers. In 90% of these cancers the mutation of BRAF results in the substitution of a valine at the position 600 to a glutamic acid, B-RafV600E (Davies et al., 2002). B-RafV600E has a high kinase activity and induces constitutive ERK signalling through hyperactivation of the MAPK pathway (Dhomen and Marais, 2007). BRAF mutation resulting in the substitution of lysine at the 499 position to a glutamic acid B-RafK499E within the kinase domain, was identified in cardio-facio-cutaneous (CFC) syndrome (Niihori et al., 2006). This B-Raf mutant shows as well high kinase activity, although surprisingly CFC patients do not have a cancer predisposition.

HEK 293 cells were plated onto 10 cm dishes and grown at 37ºC until they reached 50 to 80% confluence. Then, they were co-transfected with AKAP79 and with FLAG-tagged B-Raf or with two FLAG-tagged B-Raf mutants K499E and V600E using PolyFect® transfection reagent from Qiagen. The cells were incubated at 37ºC for 24 to 48 hours. Cells were lysed as described in section 2.1.6 of chapter 2. To investigate possible interactions between AKAP79 and B-Raf, AKAP79 was immunoprecipitated from the cell lysates and probed for co-precipitated B-Raf using anti-FLAG antibody (Fig. 5.9.a). Reciprocally, to confirm the interaction between B-Raf and AKAP79, B-Raf was immunoprecipitated using the anti-FLAG antibody and probed for co-precipitated AKAP79 (Fig. 5.9.b).

The results obtained show that AKAP79 co-precipitated with B-Raf and the B-Raf mutants in HEK293 cells. It seems that the B-Raf mutants had a better affinity for AKAP79 than the wild type B-Raf. This suggests a possible formation of a complex between AKAP79, B-Raf mutants and PKA, and the perspective of a mechanism for the regulation of B-Raf mutants by PKA.

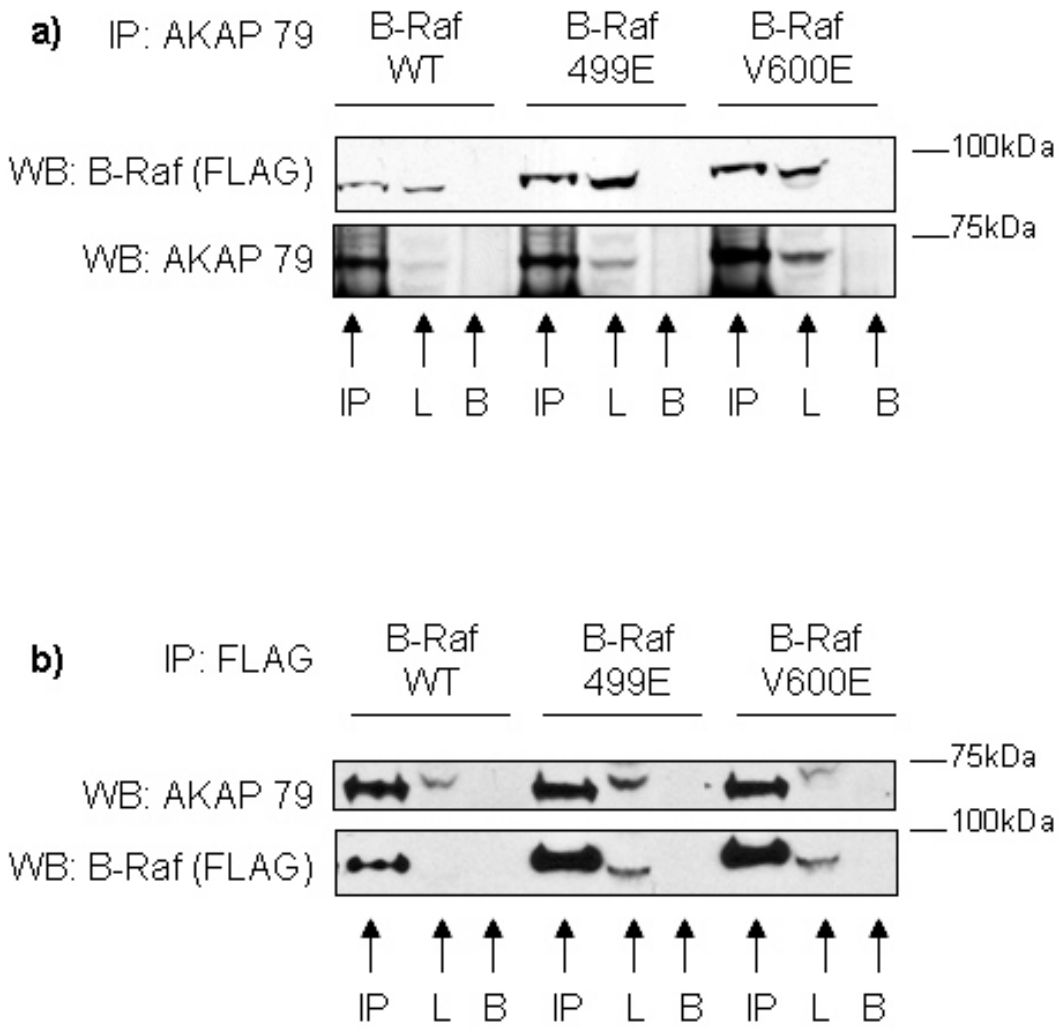


Figure 5. 9: Over-expressed B-Raf mutants bind to AKAP79 in HEK293 cells. HEK 293 cells were co-transfected with AKAP79 and with FLAG-tagged B-Raf, FLAG-tagged B-RafK499E or FLAG-tagged B-RafV600E. a) AKAP79 was immunoprecipitated and immunoblotted for the presence of transfected B-Raf mutants using an anti-FLAG antibody. b) Inversely, the different B-Rafs were immunoprecipitated using an anti-FLAG antibody and immunoblotted for the presence of transfected AKAP79. IP stands for immunoprecipitate, L for lysate and B for beads.

5.3 DISCUSSION

This chapter aimed to investigate deeper the mechanisms through which the extra cellular signal is transduced through the ERK cascade particularly at the level of the Raf kinases. A key experiment would have been to measure the respective activities of Raf-1 and B-Raf in response to mitogen stimulations and to cAMP by performing kinase assays. Unfortunately, as described in the previous chapter (i.e. section 3.4), this kinase assay was not sensitive enough on endogenous proteins. Therefore a siRNA approach was used to knock down Raf-1 and B-Raf respectively in order to determine which kinase is involved in the transduction of the signal. As described in the section 5.2.2, the siRNA targeted against B-Raf failed to deplete B-Raf. Therefore C3G, which is an upstream GEF for Rap1, was targeted instead.

The Raf-1 knockdown experiment revealed that in PC12 Raf-1 might not be essential for the transduction of the signal in response to NGF, and suggests that Ras might mediate the signal through B-Raf upon EGF stimulation. This is supported by the work of Kao *et al*., who measured the activity of Raf-1 and B-Raf upon NGF and EGF (Kao et al., 2001). Both growth factors activated Raf-1 and B-Raf simultaneously. Upon stimulation by either growth factor, the activation of Raf-1 was transient. Upon EGF, the activation of B-Raf was also transient whereas it was sustained upon NGF stimulation. They also demonstrated that most of the activation of ERK is due to B-Raf and that the contribution of Raf-1 to activate ERK is only 10%. Furthermore, in the previous chapter, it was demonstrated that EGF only activates Ras and was unable to activate Rap1. Therefore upon EGF stimulation, the pathway should be switched off. The data show that the signal is still transduced, suggesting that it is mediated through another route. Taken together, these results confirm that Ras might activate B-Raf to compensate the loss of Raf-1. When cilostamide was applied, cAMP has still an enhancing effect on the phosphorylation of ERK upon both NGF and EGF stimulation. This suggests that cAMP might strengthen the mitogen signal by stimulating the hypothesised Rap1/B-Raf pathway, which is consistent with the affinity pulldown assays demonstrating that Rap1 is sensitised and activated in response to high levels of cAMP. However, as demonstrated in figure 5.3, Raf-1 was not totally depleted. Therefore it might be possible that the remaining Raf-1was enough to fully activate ERK in response to mitogen stimulation.

The C3G knockdown experiment has to be interpreted cautiously. C3G is upstream of Rap1 and links Rap1 to the growth factor receptors (Zwartkruis and Bos, 1999). As just described above, Ras is able to activate B-Raf, thus when C3G is depleted this should not interfere with the activation of B-Raf by Ras. Furthermore, C3G is not the only GEF for Rap1, for example Epac is also a specific GEF for Rap1 (de Rooij et al., 1998). Therefore, Rap1 is still subject to be activated by Epac or other activators particularly when the levels of cAMP are elevated. Then this experiment cannot be compared to a direct B-Raf knockdown experiment. This experiment reveals that the depletion of C3G did not affect the activation of ERK upon NGF stimulation. However C3G seems to be important for the activation of ERK by cAMP as the activation of ERK was slightly reduced when C3G was knocked down and cilostamide was applied. Therefore, C3G might be involved in the activation of ERK by cAMP. However, the results also show that the C3G knockdown has absolutely no effect on the activation of ERK upon EGF stimulation with or without the treatment with cilostamide. In order to confirm that the enhancing effect of cAMP on the phosphorylation of ERK is mediated through Rap1/B-Raf via Epac, it will be interesting to inhibit Epac. So far there is no compound available to inhibit Epac. An siRNA approach could be considered instead.

In chapter 4, it was demonstrated that PKA agonist had an inhibiting effect on the activation of ERK upon EGF stimulation (section 4.2.2). It was suggested that the alteration of the activation of ERK was due to the inhibition of Raf-1 by PKA. As described in section 5.1.3 of this chapter, PKA is anchored to AKAPs to be directed to specific subcellular localisations and subset of cAMP pools, coordinating the phosphorylation of specific substrates by PKA. Here, it is the first time that an interaction between AKAPs and Raf-1 has been demonstrated. Although the interaction between AKAP and PKA had not been demonstrated here, it is likely that PKA is tethered to AKAP79. Therefore, the fact that Raf-1 can bind to AKAP79 (section 5.2.3) suggests that AKAP79 scaffolds a complex including PKA and Raf-1 and participates to the regulation of Raf-1 by PKA. This is consistent and supported by data from Dumaz *et al* (Dumaz and Marais, 2003). Indeed their data show that Raf-1 co-precipitates with the PKA RII subunits and that PKA participates to the phosphorylation of Raf-1 on S43, S233 and S259.

In rat cell extracts, Dodge *et al* (Dodge et al., 2001) demonstrated that both RII subunit of PKA and PDE4D co-immunoprecipitate with mAKAP (muscle-selective A-kinase

anchoring protein). They also demonstrated that the activity of PKA is enhanced by the inhibition of mAKAP-associated PDE4 activity, suggesting that active PDE4 can down regulate PKA (Fig. 5.10). Moreover, the phosphorylation of PDE4D3 by PKA enhances the affinity of PDE4D3 to mAKAP, suggesting the activation of mAKAP-associated PKA enhances the recruitment of PDE4, allowing quicker signal termination by PDE4 (Carlisle Michel et al., 2004). In previous research it was observed that PKA can phosphorylate PDE4 (Houslay and Adams, 2003; Sette and Conti, 1996) and also PDE3 (Murthy et al., 2002) in order to enhance their PDE activity. These findings suggest that AKAPs play a role in the regulation of cAMP, so that PKA activity is attenuated by PDE activity in a negative-feedback loop. It was hypothesised that under basal conditions, mAKAP-anchored PDE4D maintains local cAMP level at a low level insufficient to activate PKA. Upon hormonal stimulation, increased levels of cAMP overcome the PDE activity and cAMP can activate PKA that releases its active C subunit from the AKAP complex. The C subunits can in turn phosphorylate PDE4D, which becomes more active and lowers the level of cAMP back to basal levels, favouring the reformation of the inactive PKA holoenzyme.

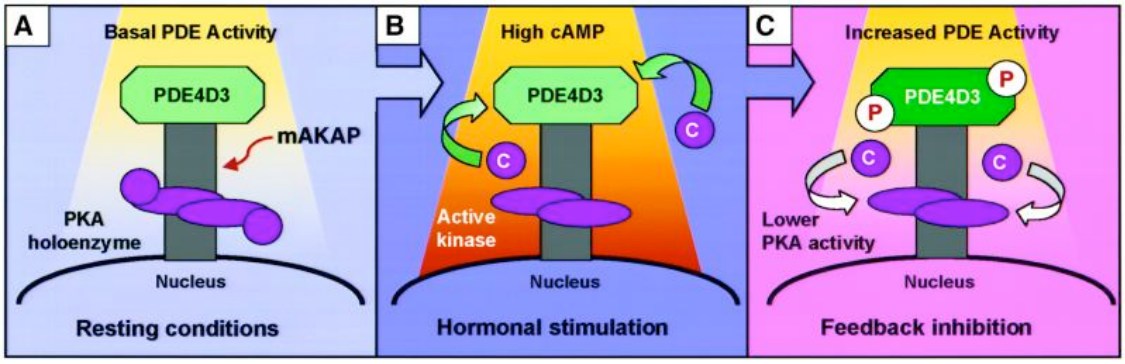


Figure 5. 10: Dodge *et al*'s proposal for the feedback inhibition of PKA by PDE4D3 anchored to mAKAP. A) In resting conditions the basal PDE activity prevent PKA to be activated by cAMP. B) Increasing level of cAMP activates PKA that phosphorylates and activates PDE4D3. C) The increasing PDE activity lowers the level of cAMP and PKA holoenzyme is reformed and inactivated (Dodge et al., 2001).

In this context and taking these observations together, we propose a molecular mechanism for the inhibition of Raf-1 by PKA (Fig. 5.11). Under basal conditions, Raf-1 bound to AKAP79 can be in an active form phosphorylated on S338 and in an inactive form phosphorylated on S259. As the phosphorylation of S259 and S338 is mutually exclusive (Dhillon et al., 2007), the inactive and active forms of Raf-1 must be

two separate pools. When forskolin was applied increasing the level of cAMP, Raf-1 showed an increase in both S259 and S338 phosphorylation. This suggests that forskolin might increase the pool of inactive Raf-1 by stimulating S259 phosphorylation, and also the pool of active Raf-1. This might be a mechanism to trap active Raf-1 away from its substrate. Cilostamide had a similar effect although it seems to deplete the pool of active Raf-1 tethered to AKAP79 probably by promoting its dissociation from AKAP79. This suggests that cilostamide might be less efficient in promoting Raf-1 inhibition by PKA than forskolin. This is consistent with cilostamide being most efficient to hyperstimulate ERK pathway activation in response to both EGF and NGF. Furthermore, these results may indicate that the event which causes Raf-1 to dissociate from AKAP79 is not due to S259 phosphorylation, but may be attributable to one of the other PKA phosphorylation sites in Raf-1, such as S43, S233 or S621. It will be interesting to test such point mutants for AKAP79 binding and dissociation.

The apparent lack of cilostamide having inhibitory effects on Raf-1 may be explained if PKA would phosphorylate PDE3 preferentially over Raf-1, which would increase PDE3 activity and lower the level of cAMP. While cAMP returns to basal, then PKA holoenzyme would reform preventing the inhibition of Raf-1.

More work needs to be undertaken in order to confirm this hypothesis. First, it will be crucial to investigate a possible interaction between AKAP79 and PDE3 (or another PDE) or at least if they co-localise as both AKAP79 and PDE3B are membrane targeted. Then, it will be also interesting to investigate the phosphorylation status and kinase activities of Raf-1 anchored to AKAP79 under different conditions of mitogen stimulations and high levels of cAMP.

The data suggest that cilostamide might reduce the fraction of Raf-1 phosphorylated on S338 and increase the fraction of Raf-1 phosphorylated on S259. It was hypothesized above that PKA phosphorylating Raf-1 might dissociate Raf-1 from AKAP79. Another hypothesis would be that a phosphatase might be recruited to AKAP79 and would dephosphorylate S338, allowing PKA to phosphorylate Raf-1 on S259. However this possible phosphatase remains to be determined.

Another hypothesis that will be consistent with Dumaz's model described in the introduction in section 5.1.2 (Dumaz and Marais, 2003), will be that the constitutively inactive Raf-1 in resting cells, would be recruited to AKAP79 upon high levels of

cAMP, where PKA would phosphorylate S233 displacing 14-3-3 from S621 to S233 changing the conformation of Raf-1 into a closed and non-activatable conformation. To confirm this hypothesis, it will be interesting to investigate the phosphorylation of S233.

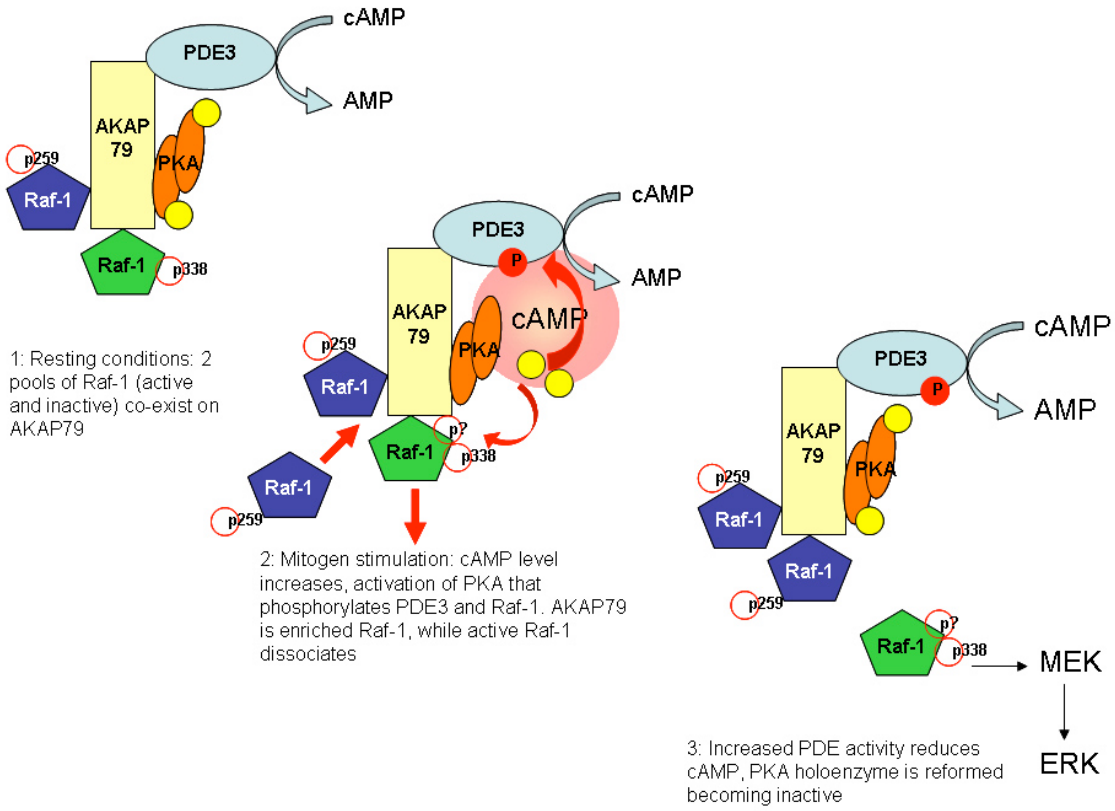


Figure 5. 11: Proposed mechanisms for the inhibition of Raf-1 by PKA anchored to AKAP79. 1: In resting conditions, the basal PDE activity regulating cAMP to basal level prevents PKA from being active. Active and inactive Raf-1 co-exist on AKAP79 2: Upon forskolin treatment, the level of cAMP increases activating PKA, which might phosphorylate Raf-1 stimulating the recruitment of inactive Raf-1 and stimulating the dissociation of active Raf-1 from AKAP79. PKA also might phosphorylate and activate PDE3. 3: Increased PDE activity reduces the level of cAMP, PKA holoenzyme reforms and PKA becomes inactive.

CHAPTER 6
GENERAL DISCUSSION

Biological processes are controlled by complex cellular signalling pathways that relay extracellular stimuli into the nucleus where specific genes are activated. The cellular signal is tightly regulated and is the result of crosstalk between different signalling pathways in order to deliver the correct response. In PC12 cells the ERK and the cAMP signalling pathways are both involved in the determination of the fate of the cell. While the ERK pathway controls cell proliferation and differentiation, the cAMP pathway promotes the differentiation of PC12 cells. It is becoming apparent that cAMP induces cell differentiation by activating the ERK pathway.

So far to study the crosstalk between these two pathways, the common strategy has been to activate adenylyl cyclase using hormones or an activating agent. Here a novel approach was undertaken by targeting the degradation of cAMP. Indeed, phosphodiesterases are key regulators of the intracellular concentration of cAMP. PDE isoforms are organised in different subsets to delimitate compartments of cAMP within subcellular microdomains to control distinct downstream effects. The phosphodiesterase inhibitors assayed for this research showed a great ability to elevate the level of cAMP within the cells, thus they were suitable to study the crosstalk between the ERK and the cAMP signalling pathways. The advantage was that instead of elevating the general content of cAMP, here the inhibition of selective PDEs allowed the accumulation of cAMP within specific subcellular compartments as the different PDE isoforms control distinct pools of cAMP. Therefore, using the appropriate PDE inhibitors might reduce crosstalk between the cAMP pathway with other pathways than the ERK signalling pathway. In PC12 cells, the accumulation of cAMP induced by cilostamide was responsible for the increase in the activation of ERK upon both NGF and EGF stimulation, and therefore identifying PDE3 to be controlling the pool of cAMP surrounding the ERK pathway. Moreover, cilostamide had an enhancing effect on the differentiation of PC12 cells and induced more robust and longer neurite outgrowth upon NGF stimulation. The most surprising result was that the combination of cilostamide with rolipram dramatically caused cell differentiation upon EGF stimulation. The data presented proved that modifying the intracellular concentration of cAMP can totally disturb and deregulate the mitogen signal and even turn the proliferation effect of EGF into a differentiation effect.

The level and duration of ERK signalling are tightly regulated at several levels throughout the pathway to ensure the appropriate biological response. The main regulation mechanisms occur at the receptor level where specific complex formation of adaptor proteins and GEFs are recruited to stimulate specific small G-proteins that link the receptors to the ERK pathway. Scaffolding proteins regulate the duration of the signalling by stabilising the formation of these complexes. The termination of the signal is as well controlled by negative feedback loops. The data presented here show that NGF can stimulate Rap1 contrary to EGF. This is consistent with previous research that demonstrated that the sustained activation of ERK upon NGF stimulation correlated with the activation of Rap1 through the long-life Crk/C3G complex that is stabilised by FRS2 only at the NGF receptor. It was also demonstrated that accumulated cAMP can sensitise Rap1, which become active upon EGF stimulation, which correlated with the activation of ERK. It is highly probable that the sensitisation of Rap1 is independent from the activation by Crk/C3G, which was confirmed by the result obtained when C3G was depleted and upon EGF stimulation. Therefore, cAMP might be able to activate the Rap1/B-Raf pathway independently from the activation of the receptor tyrosine kinases that would strengthen the activation of ERK. Upon NGF stimulation, it seems that C3G remains important for the activation of ERK by cAMP as it mediates the sustained activation of ERK.

The specificity of the signal is also ensured by the expression level of the different Raf kinase isoforms. In cells that do not express B-Raf, cAMP induces profound inhibition of the ERK pathway and inhibition of cell growth. It is generally accepted that PKA is responsible for the inhibition of Raf-1 by cAMP. In cells expressing B-Raf such as PC12 cells, cAMP promotes cell growth and proliferation. B-Raf can be activated by different small G-proteins. It is transiently activated by Ras, while its activation is sustained when it is activated by Rap1 in response to NGF stimulation. Recent studies have demonstrated that the GEFs Epac1 and Epac2 can also specifically activate Rap1. The major difficulty in the study of this crosstalk is that the effects of cAMP on the ERK pathway counteract with each other: while cAMP inhibits Raf-1, it activates B-Raf. In previous research it was demonstrated that B-Raf contributes to 90% of the activation of ERK, therefore the whole effect of cAMP on the activation of ERK is due to B-Raf. Here cAMP analogues were used to specifically activate either PKA or Epac in order to investigate the routes of the regulation of ERK by cAMP. The data clearly

showed that the Epac agonist reproduced the effects of cilostamide upon both NGF and EGF stimulations confirming that the activation of ERK by cAMP is mediated mainly through Epac. However the PKA agonist reduced the activation of ERK upon EGF stimulation, which confirmed that PKA could inhibit Raf-1. Upon NGF stimulation the PKA agonist had not effects on the phosphorylation of ERK, which can be conceivable since the majority of the signal is transduced through B-Raf. But the effect of the PKA agonist abrogated the enhancing effect of the Epac agonist on the activation of ERK upon NGF stimulation. It seems that PKA by inhibiting Raf-1 protects the ERK pathway from high levels of cAMP.

Finally an interaction between Raf-1 and AKAP79 has been demonstrated for the first time. This finding might provide a molecular mechanism by which AKAP would participate to the regulation of ERK by cAMP. AKAP would target PKA near the ERK pathway to inhibit Raf-1 and trap Raf-1 in an inactive conformation upon high levels of cAMP. It would be interesting to investigate further the role of AKAP and PKA in the regulation of ERK by cAMP by using for example the targeting peptide Ht31 that competes with the PKA RII subunit for the binding with AKAP. It would be also interesting to find out the interaction sites of Raf-1 and AKAP79 using peptide array and mutagenesis techniques.

The work presented in this thesis is a contribution to elucidate the mechanisms of the crosstalk between the ERK and the cAMP signalling pathways. This work provides new insights into this particular field with the finding that the activation of ERK by growth factors is deregulated by the inhibition of specific PDEs. The ERK pathway is hyperstimulated in 30% of all human cancers. Studying the crosstalk between the ERK and the cAMP signalling pathways allow a better understanding on how the ERK pathway is regulated. The fact that cAMP is involved in the regulation of the ERK pathway opens the possibility to find new drug targets in order to improve cancer therapy. For example, potent PDE inhibitors could be used in the treatment of certain types of cancers to elevate the intracellular concentration of cAMP and therefore to suppress constitutive oncogenic Ras activity.

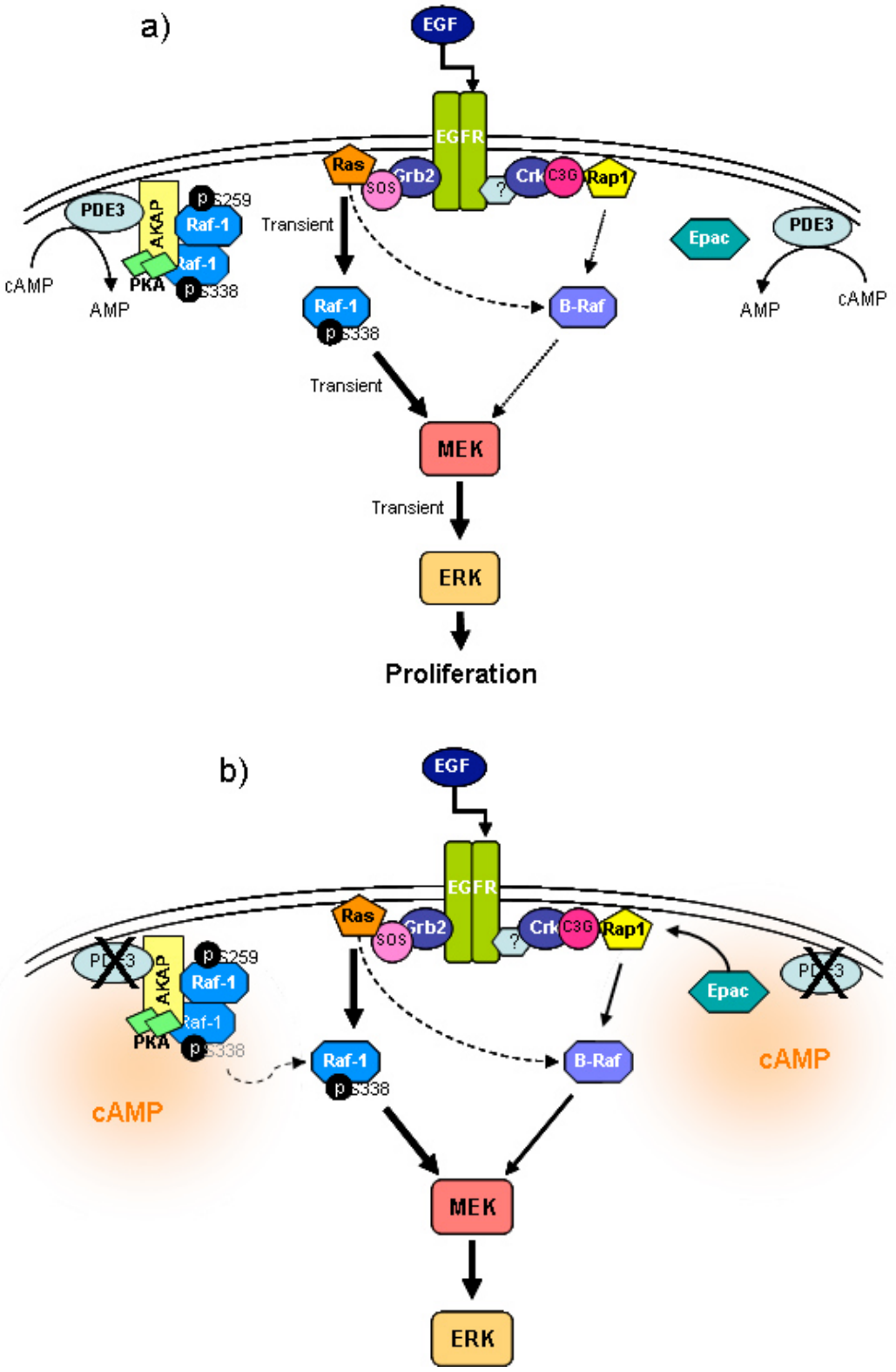


Figure 6. 1: Proposed mechanism for the effect of cAMP on the ERK signalling pathway upon EGF stimulation. a) Upon EGF stimulation, the signal is transiently mediated through Ras/Raf-1. b) The accumulation of cAMP due to PDE inhibitor (cilostamide) induced stronger and delayed phosphorylation of ERK. This might be due to the activation of Rap1/B-Raf through the activation of Rap1 by Epac. The effect of cilostamide and rolipram were additive and lead to the differentiation of PC12 cells.

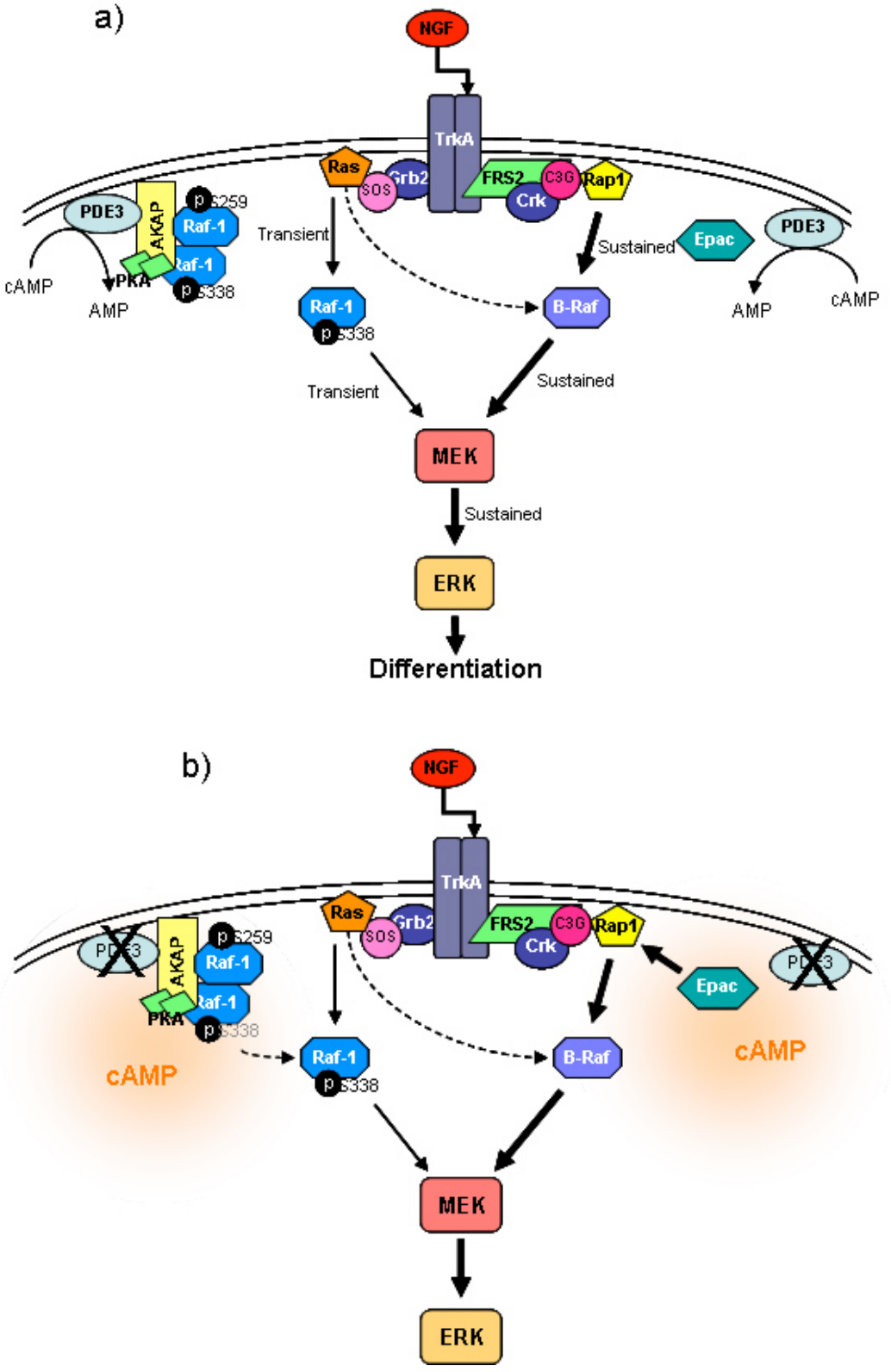


Figure 6. 2: Proposed mechanism for the effect of cAMP on the ERK signalling pathway upon NGF stimulation. a) Upon NGF stimulation, the signal is transiently mediated through Ras/Raf-1 pathway and the signal is sustained through the Rap1/B-Raf pathway due to the FRS2/Crk/C3G complexe at the receptor level. b) Accumulation of cAMP due to PDE inhibitor causes stronger phosphorylation of ERK. The effect of cAMP seems to be mediated through Epac.

APPENDIX

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Computational modelling reveals feedback redundancy within the epidermal growth factor receptor/extracellular-signal regulated kinase signalling pathway

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Abstract: The epidermal growth factor receptor (EGFR) activated extracellular-signal regulated kinase (ERK) pathway is a central cell signalling pathway that mediates many biological responses including cell proliferation, transformation, survival and motility. Deregulation of the pathway either through mutation of components or overexpression of EGFRs is associated with several forms of cancer. Under normal conditions, EGF stimulates a rapid but transient activation of ERK as the signal is rapidly shutdown, whereas under cancerous conditions, the ERK signal cannot be shutdown and is sustained. Computational modelling techniques have been used to investigate the signalling dynamics of the EGFR/ERK pathway, focusing on identifying the key processes involved in signal termination and what role the ERK to son of sevenless (SOS) negative feedback loop plays in generating a transient response. This model predicts that this negative feedback loop is not needed to achieve a transient activation of ERK as the process of receptor degradation alone is enough to terminate the signal. Importantly, the behaviour and predictions of this model are verified with laboratory data, as is essential for modern systems biology approaches. Further analysis showed that the feedback loop and receptor degradation were both redundant processes, as each could compensate for the absence of the other. This led to the prediction that in the case of a receptor which is not degraded, such as the insulin receptor, the negative feedback loop to SOS will actually be essential for a transient response to be achieved. Overall, the results shed new light on the role of negative feedback in EGF receptor signalling and suggest that different receptors are dependent on different features within the ERK pathway when relaying their signals.

1 Introduction

The extracellular-signal regulated kinase (ERK) pathway is one of the principal intracellular signalling pathways linking the activation of cell surface receptors to cytoplasmic and nuclear effectors. The core ERK pathway consists of the three kinases Raf, MAPK/ERK kinase (MEK) and ERK, which are activated by a diverse range of receptors such as the epidermal, nerve, fibroblast and platelet-derived growth factor receptors as well as the insulin receptor [1, 2]. Upon ligand binding, cell surface receptors such as the epidermal growth factor receptor (EGFR) change into their active dimeric form thereby juxtaposing the intracellular kinase domains for mutual trans-phosphorylation on several tyrosine residues [3]. These phospho-tyrosine residues act as docking sites that enable receptors to recruit a large number of cytoplasmic signalling proteins [4] including adaptor proteins such as Shc and Grb2, which can

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subsequently recruit the guanosine nucleotide exchange factor SOS (Fig. 1). This recruitment brings SOS into close proximity to its membrane bound target, the small G-protein Ras, where it can activate Ras through the loading of guanosine triphosphatase (GTP). Ras-GTP is then able to bind Raf-1 with high affinity, translocating it from the cytoplasm to the cell membrane where Raf-1 activation ensues. Active Raf-1 can then dual-phosphorylate and activate MEK, which in turn dual-phosphorylates and activates ERK [1, 2]. Activated ERK can phosphorylate over 160 substrates in the cytoplasm and nucleus [5] and can regulate gene expression by directly phosphorylating transcription factors such as Ets, Elk and Myc [6]. In addition, activated ERK is able to phosphorylate SOS both directly and indirectly, via p90 RSK-2, resulting in the dissociation of SOS from Grb2 and thus forming a negative feedback loop [7–11].

How distinct receptors can utilise the same pathways to relay different signals and generate distinct cellular outcomes has long fascinated many researchers. However, recent work with the rat pheochromocytoma PC12 cell line has greatly increased our understanding of this biological phenomenon. In PC12 cells, EGF stimulates a transient activation of ERK leading to cellular proliferation, whereas nerve growth factor (NGF) stimulates a sustained activation of ERK leading to neuronal differentiation. There is now compelling evidence that the duration of the ERK signal governs whether PC12 cells proliferate or withdraw from the cell cycle and differentiate into a neuronal phenotype [12–14]. Differences in ERK signal duration can affect cellular outcome via gene expression, as the sustained activation of ERK by NGF results in its translocation to the nucleus [15] where it can activate nuclear targets such as nuclear transcription factors. In contrast, far less translocation of ERK is observed during its transient activation by EGF [15]. Furthermore, more recent work has suggested that the duration of ERK signalling is regulated by a dynamic restructuring of feedback loops in response to EGF and NGF [16], and that the differing kinetics are interpreted in the nucleus not so much through the differential expression of immediate-early genes, but rather due to differential stabilisation of the early gene products [17]. Although the PC12 system has been well studied, it is still not completely clear how different ERK dynamics can be robustly controlled by different upstream receptors. In the case of EGF and NGF, it is not exactly known how the two receptors utilise the same pathway to generate different ERK responses and what the critical differences between the two receptor systems are. However, there are currently a number of theories to explain this, such as differences in the strength of feedback loops between receptors [16, 18] and differences in the adaptor proteins that can bind to the receptor [14].

Over recent years, the computational modelling of biological systems has become increasingly valuable. As the EGFR-activated ERK (EGFR/ERK) pathway is such an important signalling pathway, which is implicated in various forms of cancer [19], it has become a popular topic for computational modelling strategies. Currently, there are a wide variety of models of the EGFR/ERK pathway available which have led to some novel insights and interesting predictions as to how this system functions [6]. Three of the most recent and popular models of the EGFR/ERK pathway are the Brightman [18], Schoeberl [20] and Brown [21] models. We recreated and analysed these three models to investigate in more detail how the EGFR/ERK pathway behaved and noticed that these models contradict one another in some salient aspects. Specifically, the Brightman and Brown models predict that the negative feedback loop from ERK to SOS is essential for a transient activation of ERK to be achieved; deleting the feedback loop in these models results in a sustained activation of ERK (Fig. 2). In contrast, the Schoeberl model implies that the negative feedback loop is not

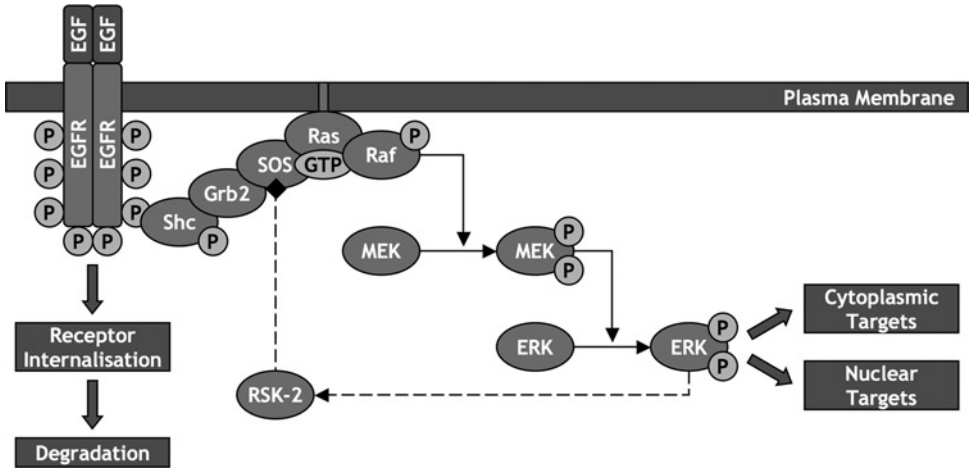

Figure 1 *Basic diagram of the EGFR/ERK signalling pathway*

This is a basic diagram of the EGFR/ERK signalling pathway which depicts the major proteins and interactions involved in the transduction of the signal from the plasma membrane to cell nucleus; see the above text for a detailed overview of the pathway

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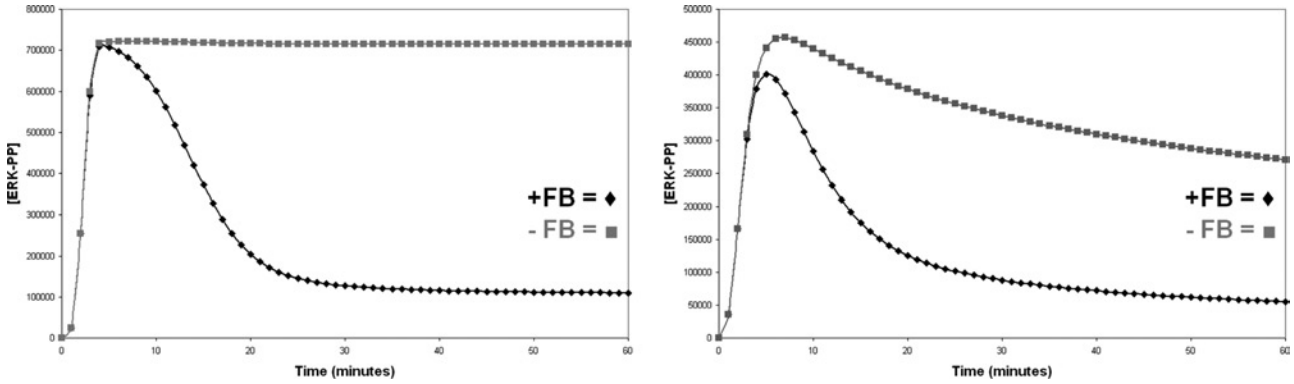

Figure 2 *Simulations of the Brightman and Brown models with and without the SOS negative feedback loop present*

This figure contains simulations from the Brightman model (left chart) and Brown model (right chart). In both cases, the *x*-axis represents time in minutes, the *y*-axis represents the concentration of activated ERK (ERK-PP) in molecules/cell, and the black (+FB, diamonds) and grey (−FB, squares) lines represent the simulated levels of ERK-PP with and without the SOS negative feedback loop present, respectively. As can be seen in both models, knocking out the negative feedback loop has a dramatic effect with the ERK-PP signal switching from a transient to a sustained response, suggesting that the feedback loop is essential for the transient response and efficient signal termination

required for the transient activation of ERK because it is not even considered in the original model. However, we also noticed a number of biological inaccuracies and oversimplifications within the Schoeberl model which, when amended, could possibly resolve this apparent contradiction.

Therefore we used computational modelling techniques to investigate whether or not the negative feedback loop from ERK to SOS was actually needed for the transient activation of ERK induced by EGF. If the feedback loop was found to be redundant, we aimed to identify the critical biological processes involved in signal termination, and validate any predictions made from the modelling with experimental data. Thus, we aimed for a better, more general and system level understanding of the EGFR/ERK pathway by identifying the key processes involved in generating a transient rather than sustained response. Furthermore, as the cause of the transient response is a fundamental feature, it was hoped that this investigation could also shed some light on how different upstream receptors can generate different ERK activation dynamics.

2 Methods

2.1 Computational modelling

As the Schoeberl model contradicted both the Brightman and Brown models, we decided to investigate the Schoeberl model further and use it as a base to develop from and investigate the role of the ERK to SOS negative feedback loop. The Schoeberl model is an ordinary differential equation-based model of the EGFR-activated ERK pathway that consists of 125 reactions involving 94 species [20]. The model was originally used to investigate the effects of receptor internalisation on the ERK pathway, and also the signal–response relationship between the binding of EGF to its receptor at the cell surface and the activation of downstream proteins in the signalling pathway. Interestingly, the original Schoeberl model did not even consider the ERK to SOS negative feedback loop suggesting that it was not needed for the transient response and efficient signal shutdown. An updated version of the Schoeberl model was later made available by the authors online (http://www.mpi-magdeburg.mpg.de/en/research/projects/1010/1022/1017) which did include the ERK to SOS feedback loop, as well as a number of additional degradation reactions. However, the addition of these reactions had little effect on the behaviour of the model (Supplementary Fig. 1).

Examination of the Schoeberl model reveals a number of biological inaccuracies and oversimplifications. First, GAP must bind to the receptor before any other adaptor protein can bind. This is incorrect as all adaptor proteins, such as Shc and Grb2, can bind directly to various receptor phospho-tyrosine residues through SH2 and PTB domains and there is no requirement for GAP to be present [4, 22–24]. In actual fact, GAP directly competes with some adaptor proteins for binding and indirectly competes with others due to the close proximity of the phospho-tyrosine residues [4, 23, 24]. Furthermore, this assumption creates a stoichiometric bottleneck in the model as the pre-binding of GAP limits the number of activated receptors that can actually activate the downstream ERK pathway, as there are 50 000 receptors but only 12 000 molecules of GAP. Secondly, there is a form of Ras called Ras-GTP*, which is formed after the activation of Raf-1 and dissociation of the Ras-GTP/Raf-1 complex. In the model, Ras-GTP* is activated but cannot bind to its effector Raf-1, thus limiting the number of Raf molecules that Ras-GTP can activate to one. However, there is no evidence that the number of Raf molecules that Ras-GTP can activate is limited as Raf binding to Ras-GTP is not a static process but subjected to dynamic turnover [6, 25]. Therefore the introduction of Ras-GTP* is artificial as there is no

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evidence that a form of activated Ras exists that cannot interact with effectors. Thirdly, Ras-GTP* can only be deactivated and recycled back to Ras-GDP by binding to receptor complexes containing SOS. However, Ras is deactivated by GAP and there is no evidence that Ras deactivation can only occur in an EGFR complex that contains both GAP and SOS. Overall, simulations of the original Schoeberl model show that the vast majority of Ras (~97%) rapidly builds up and remains in the Ras-GTP* form (Supplementary Fig. 2) which is an intermediate with no function. Essentially, this means that the activation (GTP loading) of Ras is in fact sustained in the original Schoeberl model. However, the GTP loading of Ras in response to EGF stimulation is well known to be transient without the sustained accumulation of Ras-GTP [14], especially in PC12 cells. Indeed, we have our own laboratory data that shows the EGF stimulated GTP loading of Ras is rapidly transient in nature (Supplementary Fig. 3). Therefore we constructed a new model of the EGFR/ERK pathway to investigate the role of feedback, by using the original Schoeberl model as a template. We amended the three biological inaccuracies described above by detaching GAP binding, deleting the Ras-GTP* species, and enabling Ras-GTP to be deactivated by all GAP complexes, respectively, and also added the ERK to SOS feedback loop. In addition, there were also a number of minor errors in the original Schoeberl model, such as receptor degradation reactions also degrading any bound adaptor proteins, which is incorrect and was also corrected. As we had fundamentally changed some aspects of the model, we used parameter estimation techniques to re-estimate the parameters relating to GAP binding and the deactivation of Ras-GTP which had been previously estimated in the original Schoeberl model.

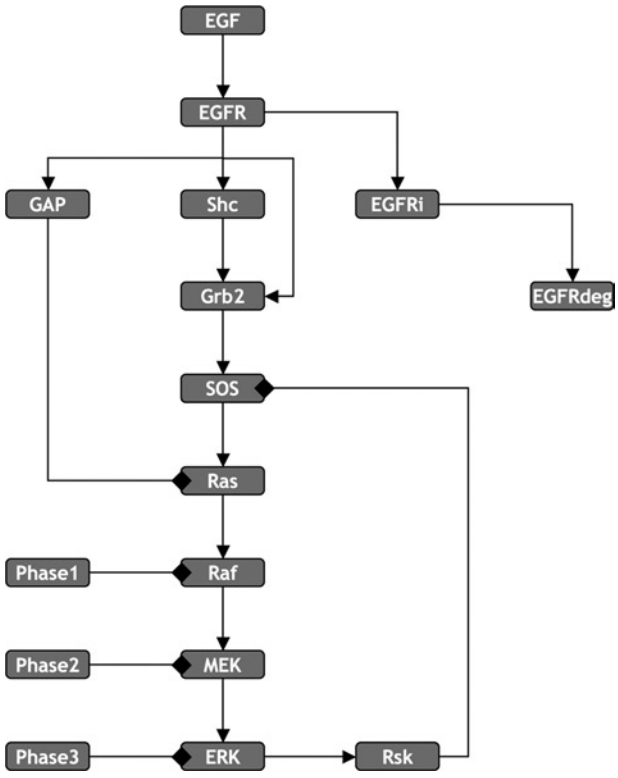

Figure 3 *Block diagram of our EGFR/ERK pathway model*

This is a block diagram of our model of the EGFR/ERK pathway which depicts the basic structure of the model and the basic relationships between the main species involved; positive activating relationships end with arrows, whereas negative deactivating relationships end with diamonds

Technically, the biochemical simulation tool Gepasi [26] was used for model construction, simulation and parameter estimation. A sensitivity analysis of the model was also performed by writing a custom script and simulating the model in MATLAB (Mathworks Inc.; http://www.mathworks.com/); sensitivity analysis is a general technique for establishing the contribution of individual parameter values to the overall performance of a complex system [27]. To accomplish this, we simulated the model under normal parameter conditions for 3600 s (1 h), monitoring the concentration of activated ERK at each second giving 3600 data points. We then calculated the area underneath the activated ERK curve by simply summing up all the data points. We then took each reaction rate parameter in turn and increased it by 10%, re-simulated the model and re-calculated the area underneath the activated ERK curve. The sensitivity (S) of the system to the change in the parameter (P) with respect to our selected system output, activated ERK (E), could then be calculated as $S = (\Delta E/E)/(\Delta P/P)$ where ΔE is the change in E caused by the change in $P(\Delta P)$. A similar process was used to calculate the sensitivity of the system to changes in initial species concentrations in order to identify the critical species involved in the signal's transduction.

2.2 Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's minimal (DMEM) medium (Sigma) supplemented with 10% (v/v) horse serum, 5% (v/v) newborn calf serum (Sigma), 1% (v/v) glutamax, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Paisley, UK) at 5% CO_2. For treatment with EGF, PC12 cells were plated on poly-lysine coated six-well plates at 50–60% confluency and deprived of serum for 8 h in DMEM medium supplemented with 1% (v/v) glutamax, 100 U/mL penicillin and 100 μg/mL streptomycin. Then, EGF (Promega) was added to a final concentration of 50 ng/mL and the cells were incubated at 37°C, 5% CO_2 for the desired time interval. The treatment was stopped by washing the cells with ice-cold PBS and immediate flash-freezing in dry ice. To inhibit MEK1/2, U0126 (Cell Signalling Technologies) was added to a final concentration of 25 μM 1 h prior to EGF treatment.

2.3 Western blotting

Cells were lysed at 4°C in 3T3-cell lysis buffer (25 mM HEPES pH 7.4, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with protease inhibitors (Protease

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Inhibitor cocktail, Roche) and phosphatase inhibitors (2 mM NaF, 0.2 mM NaP_2O_5, 0.5 mM sodium orthovanadate, 10 mM β-glycerophosphate). Lysates were cleared by centrifugation at 16 000 × g at 4°C for 15 min. Protein concentrations were quantified by a modified Lowry assay (Bio-Rad Dc protein assay; Bio-Rad Laboratories). The samples were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis using NOVEX 4–12% BisTris gradient gels (Invitrogen) and transferred to nitrocellulose membranes (Schleicher and Schuell). After blocking with TBS-Tween buffer (150 mM NaCl, 50 mM TrisHCl pH 7.4, 0.1% Tween-20) containing 5% non-fat dry milk for 1 h at room temperature, the membranes were probed with primary phosho-ERK1/2 (1:1000), ERK1/2 (1:1000), phospho-MEK1/2 (1:1000) and MEK1/2 antibodies (Cell Signalling Technologies) diluted in TBS-Tween plus 5% non-fat dry milk, as indicated. The phospho-specific antibodies specifically detect the activating phosphorylations and therefore can be used to assess activation of MEK and ERK. The blots were then incubated with the appropriate secondary antibodies conjugated with LI-COR IRDye© 680 nm and the band intensities were quantified by immunofluorescence using an Odyssey scanner (LICOR). Total amounts of MEK or ERK remained constant under these experimental conditions.

3 Results

We now present a new model of the EGFR/ERK pathway consisting of 123 reactions involving 16 distinct protein species, which form 83 different complexes (Fig. 3). Our model is essentially an amended version of the original Schoeberl model and therefore utilises a receptor-complex strategy, which comprehensively includes the processes of receptor internalisation and degradation, and also includes the Shc-dependent and independent pathways leading to the activation of Ras. We have validated the behaviour of this new model and its predictions with experimental data showing that our model better reflects the true biology and dynamics of the EGFR/ERK pathway. We have made a Systems Biology Markup Language (SBML; [28]) version of our model available at http://www.dcs.gla.ac.uk/~rorton/egf, as well as the original Schoeberl, Brightman and Brown models.

Initially, we validated the behaviour of our model by comparing the simulation traces of activated ERK (ERK-PP) from the model to our own laboratory data of activated ERK after EGF stimulation in PC12 cells. The phospho-ERK antibody detects the activating phosphorylation sites, and therefore ERK-PP levels directly correspond to the levels of activated ERK. As can be seen in Fig. 4*a*, there is a reasonable fit between the simulation and laboratory data as both sets show a rapid transient activation with ERK-PP levels peaking at around 5 min and returning to basal levels around 20 min. This also conforms well with previously published data of ERK activation in PC12 cells [14, 29]. In addition, the activation (GTP loading) of Ras in our model is very much transient, unlike the original Schoeberl model where Ras built up in RasGTP* form (Supplementary Fig. 2).

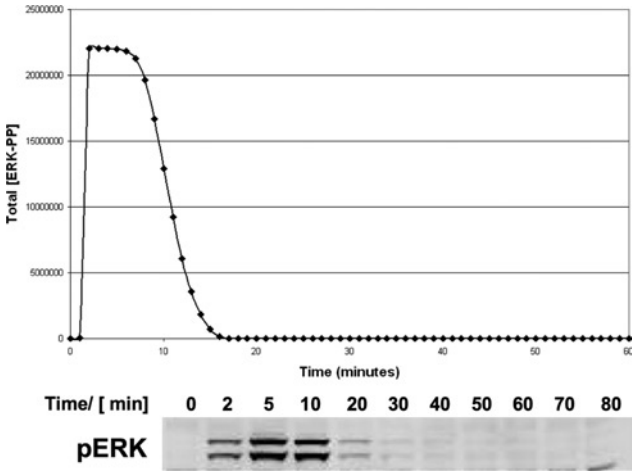

Figure 4 *Comparison of simulation and laboratory data for ERK activation*

At the top is a chart of the simulated levels of total activated ERK (ERK-PP) from the model where the *x*-axis represents time in minutes and the *y*-axis represents the total concentration of ERK-PP in molecules/cell. At the bottom are the corresponding laboratory data where cell lysates from PC12 cells stimulated with EGF for the indicated periods were Western blotted and probed for activated ERK using anti-phospho-ERK antibodies. There is a reasonable agreement between the simulated and laboratory data with activated ERK levels being transient, peaking at 5 min, remaining high at 10 min, and returning to basal levels at around 20 min. Total ERK levels remained constant (data not shown)

We were specifically interested in investigating whether or not the negative feedback loop from ERK to SOS was actually needed for the transient ERK response and efficient signal shutdown. Therefore our first experiment was to knock out the feedback loop by deleting all the corresponding reactions from the model. As can be seen in Fig. 5, knocking out the feedback loop has only a small effect on the behaviour of the model. ERK activation proceeds with the same kinetics and reaches the same peak amplitude and is only slightly prolonged. MEK phosphorylation also occurs with the same velocity, but reaches a higher and slightly later peak with the overall activation only slightly extended. Therefore our model clearly predicts that the feedback loop is not needed for the transient ERK phosphorylation and efficient signal shutdown. To test this prediction experimentally, we stimulated PC12 cells with EGF with or without U0126 present and monitored both phospho-MEK and phospho-ERK levels. U0126 is a small molecule inhibitor that binds to MEK with high affinity resulting in a conformational change that does not interfere with the activating phosphorylation of MEK, but prevents MEK from binding to and activating its substrate ERK [30–32]. As U0126 blocks the activation of ERK, it blocks the ERK to SOS negative feedback loop. One can therefore investigate the role of the negative

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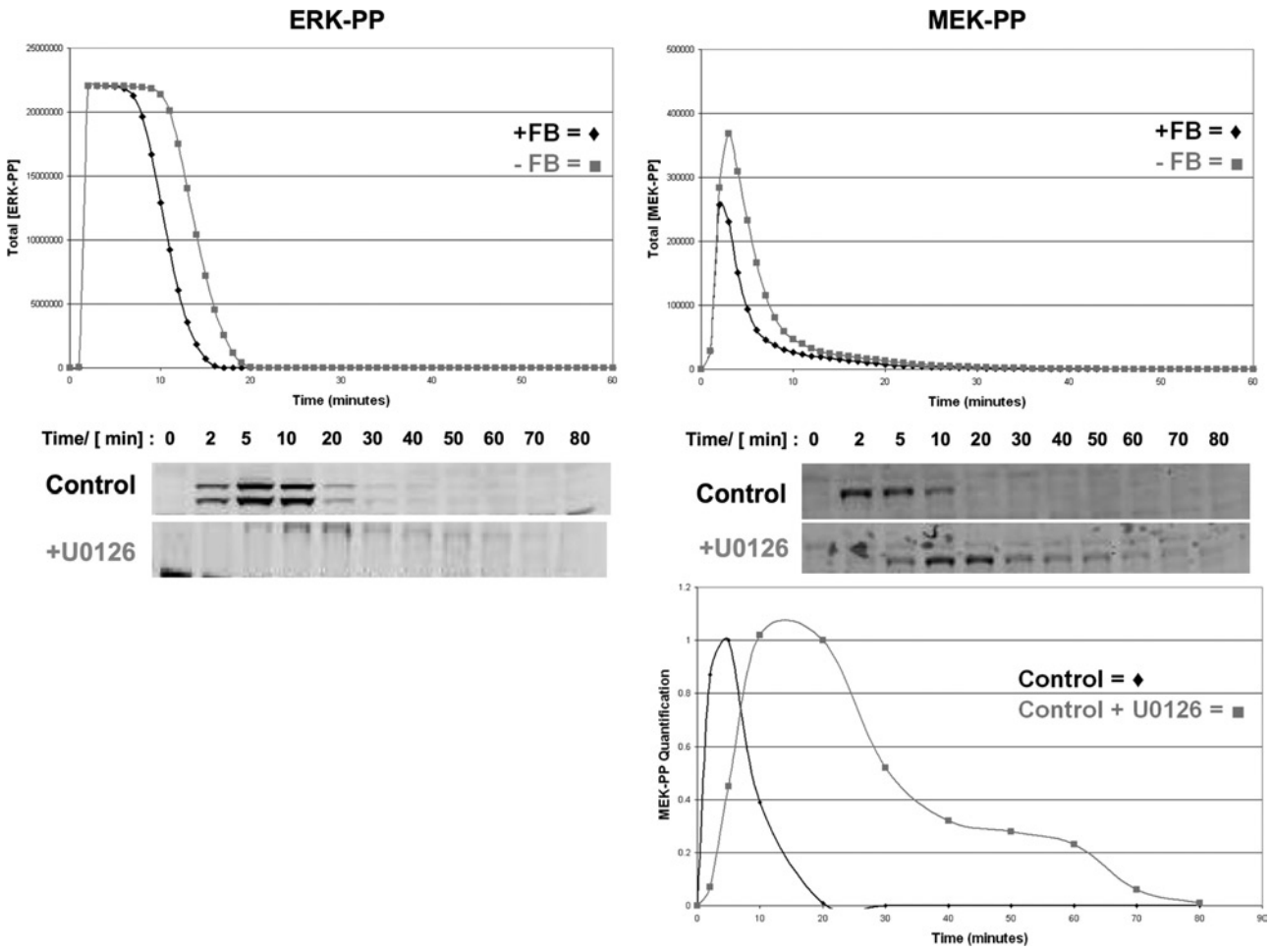

Figure 5 *Comparison of simulation and laboratory data for feedback loop knockout*

At the top are the model simulations for ERK-PP (left) and MEK-PP (right). In both cases, the *x*-axis represents time in minutes, the *y*-axis represents the corresponding total species concentration in molecules/cell, the black line represents the simulations from the normal feedback intact model (+FB, diamonds), whereas the grey line represents simulations from the feedback knockout model (−FB, squares). Knocking out the feedback loop has only a small effect on activated MEK or ERK levels which, although slightly prolonged, still remain transient. At the bottom are the corresponding laboratory data for ERK-PP (left) and MEK-PP (right); control represents normal EGF stimulation conditions where the feedback loop is intact (compare to +FB) whereas +U0126 represents treatment with U0126 prior to EGF stimulation in order to knock the feedback loop out (compare to −FB). When the feedback loop is knocked out by U0126 treatment of cells, the activation of ERK by MEK is blocked and therefore no active ERK is observable. However, the signal can still be monitored at the level of MEK phosphorylation which is not prevented by U0126. The experiment shown was repeated four times and the data shown is a representation of four independent experiments. A quantification of the MEK-PP blots is also shown, as can be seen the MEK-PP signal, although slightly prolonged, is still very much transient showing that feedback is not needed for signal termination

feedback loop by comparing the levels of phospho-MEK with and without feedback to examine if the signal switches from transient to sustained after the feedback loop is abolished; obviously, ERK activation is experimentally not measurable under these conditions as it is prevented by U0126. As can be seen in Fig. 5, knocking out the feedback loop through U0126 treatment results in a slightly prolonged but still very much transient phosphorylation of MEK; therefore validating our model prediction that feedback is not needed for a transient response. However, an apparent delay in the activation of MEK after U0126 treatment is observed in the experimental results, which is much more exaggerated than in the model. We believe that this is a result of U0126 interference, as U0126 stops MEK-PP from binding to ERK and the formation of MEK-PP/ERK complexes is therefore slowed down. As MEK-PP/ERK complexes actually protect MEK-PP from being deactivated by phosphatases, the activation of MEK is slower due to the increased vulnerability to phosphatase activity. It is important to note that no delay is observed in the computational model because the feedback loop was simply deleted without explicitly taking the intricacies of U0126 into account. The purpose of the experiment rather was to test the model's prediction that the feedback loop was not needed for signal termination. This is clearly the case, and in this respect the results of this experiment validate the prediction from the model showing that the ERK to SOS feedback loop is dispensable for the transient response and efficient signal shutdown.

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We next investigated why the negative feedback loop from ERK to SOS appears to be redundant, and what the key processes involved in generating the transient response actually were. Analyses of the model showed that there are two key processes involved in signal termination and generating the transient response, namely the process of receptor degradation and the negative feedback loop from ERK to SOS itself. The model predicts that these two processes are individually redundant as each can compensate for the absence of the other. This is effectively demonstrated in the model, as individually deleting the feedback loop or the process of receptor degradation has little effect on the transient nature of ERK activation (Fig. 6). However, deleting both the feedback loop and receptor degradation has a dramatic effect with the ERK signal switching from a transient to a sustained response (Fig. 6). Therefore the model predicts that in the case of the EGF receptor, the negative feedback loop from ERK to SOS is redundant because the process of receptor degradation alone is sufficient for the transient response and efficient signal shutdown. Consistent with our model, Hendriks *et al.* [33] observed that EGFR mutations that delay receptor internalisation had no effect on the kinetics of ERK activation, but extended Akt signalling.

The above analysis of our model also led to another interesting prediction, namely that in the case of a receptor which is not degraded, the negative feedback loop from ERK to SOS will in fact become essential for a transient response. A case of such a receptor is the insulin receptor, which is more efficiently recycled back to the plasma membrane rather than being degraded, but still only gives a transient rather than sustained ERK signal [8, 34]. To investigate this, we removed the process of receptor degradation from the model and ran simulations with or without the feedback loop present. As can be seen in Fig. 6, in the absence of receptor degradation, knocking out the negative feedback loop from ERK to SOS now causes the ERK signal to switch from a transient to a sustained response. Therefore our model predicts that the ERK to SOS negative feedback loop is essential for the transient response in the case of receptors which are not degraded, such as the insulin receptor. Interestingly, this prediction has already been validated by a previous experimental study where, as in our experiment, the feedback loop was knocked out using an MEK inhibitor and its effect on the signal flowing through the pathway after insulin stimulation was monitored upstream [8]. In this case, it was found that knocking out the feedback loop resulted in the signal switching from a transient to a sustained response of Ras activity (see Fig. 3 in [8]), thus showing that the feedback loop was essential for the insulin-induced transient response and further validating our model and its predictions.

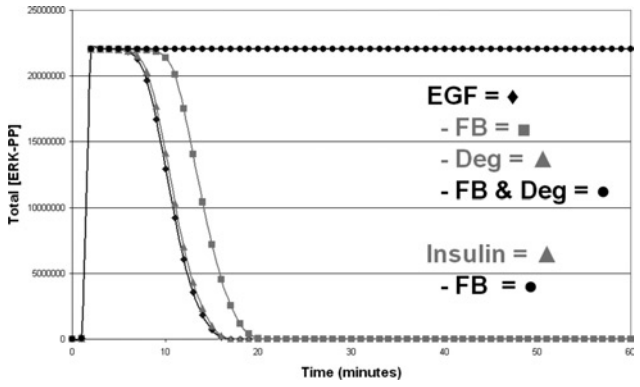

Figure 6 *Model simulations with feedback and degradation knockouts*

This is a chart of simulated ERK-PP levels under normal EGF stimulation conditions (EGF, black line, diamonds), after the feedback loop has been knocked out (−FB, grey line, squares), after receptor degradation has been knocked out (−Deg, grey line, triangles), and after both the feedback loop and receptor degradation have been knocked out (−FB & Deg, black line, circles); the *x*-axis represents time in minutes and the *y*-axis represents the concentration of total ERK-PP in molecules/cell. In addition, this chart also contains the simulated ERK-PP levels under normal insulin stimulation conditions (insulin, grey line, triangles), and after the feedback loop has been knocked out (−FB, black line, circles); this is because the only difference between the EGF and insulin systems is the lack of receptor degradation in the case of insulin

We next performed a classical sensitivity analysis of the model as an additional way to identify the key reactions involved in generating the transient ERK response. Sensitivity analysis can be used to identify the model parameters, and therefore reactions, which have the greatest influence on the ERK signal. A small change to a critical parameter will have a big effect on the ERK signal and therefore parameters with the highest sensitivity have the largest influence on the system output being considered. The parameter sensitivity analysis showed that the ERK signal was clearly most sensitive to the parameters concerned with the activation of Ras by SOS and the deactivation of Ras by GAP (Fig. 7). Furthermore, the species sensitivity analysis showed that the ERK signal was most sensitive to changes in the initial concentrations of GAP, closely followed by Ras and then EGFR (Supplementary Fig. 4). Taken together, this highlights the critical role of the Ras protein, which represents the bridge between the activated receptors at the membrane and the cytoplasmic ERK cascade. Furthermore, it also highlights the importance of the GAP protein which was found to be the most sensitive protein, whereas the deactivation of Ras by GAP was found to be the most sensitive reaction parameter. The process of Ras deactivation by GAP is obviously a critical step in ERK signal termination, as Ras essentially dictates what happens to the cytoplasmic ERK cascade. If Ras remains active, it will continue to stimulate the ERK cascade resulting in pathologies such as cancer, whereas if it is deactivated the stimulation of the ERK cascade stops and the kinases Raf, MEK and ERK are simply deactivated by their respective phosphatases. It is therefore no surprise that one of the most common

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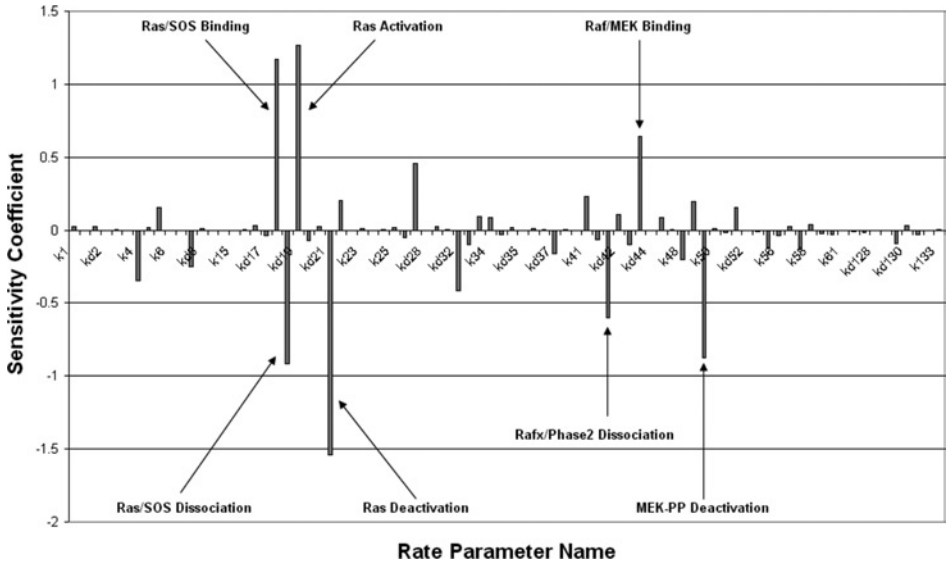

Figure 7 *Sensitivity coefficients of reaction rate parameters*

This chart displays the sensitivity coefficients of the 82 reaction rate parameters with respect to the overall ERK-PP signal. All parameters with a sensitivity coefficient higher than 0.5 are labelled with a brief description of their corresponding reaction

mutations found in cancer is a Ras mutation at residue 12 (glycine to valine) which renders Ras insensitive to inactivation by GAP and thus locked in the 'on' state [35].

4 Discussion

In this study, we have used computational modelling techniques to investigate the signalling dynamics of the EGFR/ERK pathway, focusing on identifying the key processes involved in signal termination and determining what role the ERK to SOS negative feedback loop plays in generating a transient response. Our model predicted that the negative feedback loop is in fact redundant and not required to achieve a transient activation of ERK as the process of receptor degradation alone was enough to terminate the signal. Further analysis showed that the feedback loop and receptor degradation were both individually redundant processes, as each could compensate for the absence of the other. This led to the prediction that in the case of a receptor which is not degraded, such as the insulin receptor, the feedback loop will actually be essential for a transient response to be achieved. In actual fact, if one examines Fig. 6 in more detail, one could argue that receptor degradation is more redundant than the feedback loop, as knocking out receptor degradation has less of an effect on the ERK-PP signal. Indeed, both our computational models and previous experimental work by Waters *et al.* [8] show that the receptor system is able to function perfectly well without receptor degradation and still achieve a transient response, as in the case of the insulin receptor.

It is important to note that receptor degradation and the ERK to SOS negative feedback loop are not the only important processes concerned with signal termination and the transient response. The activities of GAP as well as all the phosphatases are essential for the signal to be fully terminated. If any one of them were absent, or severely impaired, their respective target would remain active leading to an uncontrolled sustained response. Indeed, the results from the sensitivity analysis showed that the deactivation of Ras by GAP was perhaps the most critical step in ERK signalling. The deactivation of Ras is not a trivial process, as GAP must first bind to an activated receptor (in order to get into close proximity to its membrane bound target), but as receptors are rapidly degraded, GAP has only a short time window in which to bind to receptors and deactivate Ras to terminate the signal. Furthermore, in the initial stages of signalling, GAP must not interfere with the other adaptor proteins binding to the receptor, such as Shc and Grb2 which subsequently recruit SOS to activate Ras and initiate the signal. Overall, this therefore suggests that the regulation of GAP binding and activity is a critical and perhaps highly regulated process. Indeed, recent studies have highlighted a potential role in the regulation of GAP for SHP-2, which has been shown to dephosphorylate the GAP binding sites on activated EGF receptors as well as on the docking protein Gab1 [36, 37]. Furthermore, studies on the related Torso receptor in *Drosophila melanogaster* showed that the SHP-2 homologue Corkscrew was able to bind directly to the receptor, dephosphorylate the binding site for Ras-GAP, while also acting as an adaptor protein for binding the Grb2 homologue DRK [38].

The ERK pathway is a core cell signalling module that is utilised by numerous cell surface receptors to relay different signals. Although ligand specific, these receptors are all similar and can recruit the same family of adaptor proteins, such as Shc and Grb2, to activate the downstream ERK pathway. Our results suggest that the ERK pathway has

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evolved to become a complex and multi-functional pathway that can be robustly utilised by different receptors with different properties to generate the same ERK response. Although certain features of the pathway are essential for signalling from one receptor, the same feature may be redundant for signalling from a different receptor. For instance, the negative feedback loop from ERK to SOS appears essential for the transient signalling stimulated by insulin, but not for the transient signalling stimulated by EGF. This could therefore suggest that other features within the ERK pathway are redundant under specific signalling conditions. Perhaps, different receptors rely on different features of the ERK pathway to relay their signal properly. However, it is also important to note that although EGF and insulin both stimulate a similar transient activation of ERK, they lead to very different cellular responses: EGF primarily stimulates cells to proliferate, whereas insulin primarily has metabolic effects. Therefore this suggests that is not just the ERK signal that governs the cellular response. Additional pathways are also likely to be important, as receptors are able to bind other adaptor proteins which can initiate other pathways. For example, the insulin receptor also binds and activates IRS-1 (insulin receptor substrate one) which initiates the IRS-1/PI3K/Akt pathway which can activate the transcription factor NFκB thus affecting gene expression [39–42]. The fact that our model does not consider such additional pathways could also explain a potential contradiction between our results and previous studies on mutated EGFR receptors that report when EGFR internalisation (the first step towards degradation) is impaired, ERK signalling is sustained [43]; the sustained signal from these mutated receptors could well be passing down one of these additional pathways not considered in our model such as the Rap1 pathway. Furthermore, although we suspect the observed experimental delay in MEK activation (Fig. 5) is caused by the interference of U0126, an alternative explanation could be the presence of a positive feedback loop involving ERK, or a downstream target, which leads to increased activation of Raf-1 or MEK; an example of such a positive feedback loop is the Raf kinase inhibitor protein (RKIP) feedback loop [44, 45].

Our initial investigation into the role of the negative feedback loop was initially inspired by comparisons of three existing models of the EGFR/ERK pathway. Our results suggest that both the Brightman and Brown models are in fact good representations of the insulin, rather than EGF, activated ERK pathway. This is because they both lack the process of receptor degradation which therefore makes the presence of the ERK to SOS negative feedback loop essential for a transient response to be achieved. However, it is important to emphasise that a model is not a real or exact representation of a biological system. Rather, it is a simplified description to assist in analysis and to help us to better understand the real by making computational predictions about the system that are testable in the laboratory. Our biochemical understanding of the EGFR/ERK pathway is by no means complete and is constantly improving and evolving over time. Therefore computational models can be used to suggest interesting new hypotheses and explanations for the observed data that challenge our current understanding. However, it is important to note that any predictions from a model must be verified experimentally in the laboratory. Like our knowledge of the pathway, models too can evolve over time. This is demonstrated in our study, as we have used the original Schoeberl model as a basis for the development of a new model of the pathway which we believe better reflects the pathway and the true dynamics of its behaviour. Although we have validated the behaviour of our model and its predictions with experimental data, it is important to remember that it is still very much a simplification. For example, our model does not consider true spatial aspects such as the translocation of ERK to the nucleus, ERK's effect on gene expression, scaffold proteins, additional adaptor proteins that can bind to the receptor such as SHP-2 or the Rap1/b-Raf pathway. Therefore in the years to come, our model may be proven to be incomplete or even incorrect in certain aspects and may need to be expanded or evolved in the future to explain additional laboratory data. In conclusion, it would seem fitting to end with a quote from the statistician George E. P. Box, who once wrote 'essentially, all models are wrong, but some are useful' [46].

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6 References

[1] WELLBROCK C., KARASARIDES M., MARAIS R.: 'The RAF proteins take centre stage', *Nat. Rev. Mol. Cell Biol.*, 2004, **5**, pp. 875–885

[2] KOLCH W.: 'Coordinating ERK/MAPK signalling through scaffolds and inhibitors', *Nat. Rev. Mol. Cell Biol.*, 2005, **6**, pp. 827–837

[3] WARD C.W., LAWRENCE M.C., STRELTSOV V.A., ADAMS T.E., McKern N.M.: 'The insulin and EGF receptor structures: new insights into ligand-induced receptor activation', *Trends Biochem. Sci.*, 2007, **32**, pp. 129–137

[4] SCHULZE W.X., DENG L., MANN M.: 'Phosphotyrosine interactome of the ErbB-receptor kinase family', *Mol. Syst. Biol.*, 2005, **1**, 2005.0008

[5] YOON S., SEGER R.: 'The extracellular signal-regulated kinase: multiple substrates regulate diverse cellular functions', *Growth Factors*, 2006, **24**, pp. 21–44

© The Institution of Engineering and Technology 2008

[6] ORTON R.J., STURM O.E., VYSHEMIRSKY V., CALDER M., GILBERT D.R., KOLCH W.: 'Computational modelling of the receptor-tyrosine-kinase-activated MAPK pathway', *Biochem. J.*, 2005, **392**, pp. 249–261

[7] LANGLOIS W.J., SASAOKA T., SALTIEL A.R., OLEFSKY J.M.: 'Negative feedback regulation and desensitization of insulin- and epidermal growth factor-stimulated'p21ras activation', *J. Biol. Chem.*, 1995, **270**, pp. 25320–25323

[8] WATERS S.B., HOLT K.H., ROSS S.E., *ET AL.*: 'Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex', *J. Biol. Chem.*, 1995, **270**, pp. 20883–20886

[9] DONG C., WATERS S.B., HOLT K.H., PESSIN J.E.: 'SOS phosphorylation and disassociation of the Grb2-SOS complex by the ERK and JNK signaling pathways', *J. Biol. Chem.*, 1996, **271**, pp. 6328–6332

[10] CORBALAN-GARCIA S., YANG S.S., DEGENHARDT K.R., BAR-SAGI D.: 'Identification of the mitogen-activated protein kinase phosphorylation sites on human Sos1 that regulate interaction with Grb2', *Mol. Cell Biol.*, 1996, **16**, pp. 5674–5682

[11] DOUVILLE E., DOWNWARD J.: 'EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2', *Oncogene*, 1997, **15**, pp. 373–383

[12] TRAVERSE S., SEEDORF K., PATERSON H., MARSHALL C.J., COHEN P., ULLRICH A.: 'EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor', *Curr. Biol.*, 1994, **4**, pp. 694–701

[13] MARSHALL C.J.: 'Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation', *Cell*, 1995, **80**, pp. 179–185

[14] KAO S., JAISWAL R.K., KOLCH W., LANDRETH G.E.: 'Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells', *J. Biol. Chem.*, 2001, **276**, pp. 18169–18177

[15] TRAVERSE S., GOMEZ N., PATERSON H., MARSHALL C., COHEN P.: 'Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor', *Biochem. J.*, 1992, **288 (Pt 2)** pp. 351–355

[16] SANTOS S.D., VERVEER P.J., BASTIAENS P.I.: 'Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate', *Nat. Cell Biol.*, 2007, **9**, pp. 324–330

[17] MURPHY L.O., MACKEIGAN J.P., BLENIS J.: 'A network of immediate early gene products propagates subtle differences in mitogen-activated protein kinase signal amplitude and duration', *Mol. Cell Biol.*, 2004, **24**, pp. 144–153

[18] BRIGHTMAN F.A., FELL D.A.: 'Differential feedback regulation of the MAPK cascade underlies the quantitative differences in EGF and NGF signalling in PC12 cells', *FEBS Lett.*, 2000, **482**, pp. 169–174

[19] DHILLON A.S., HAGAN S., RATH O., KOLCH W.: 'MAP kinase signalling pathways in cancer', *Oncogene*, 2007, **26**, pp. 3279–3290

[20] SCHOEBERL B., EICHLER-JONSSON C., GILLES E.D., MULLER G.: 'Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors', *Nat. Biotechnol.*, 2002, **20**, pp. 370–375

[21] BROWN K.S., HILL C.C., CALERO G.A., *ET AL.*: 'The statistical mechanics of complex signaling networks: nerve growth factor signaling', *Phys. Biol.*, 2004, **1**, pp. 184–195

[22] BATZER A.G., ROTIN D., URENA J.M., SKOLNIK E.Y., SCHLESSINGER J.: 'Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor', *Mol. Cell Biol.*, 1994, **14**, pp. 5192–5201

[23] ODA K., MATSUOKA Y., FUNAHASHI A., KITANO H.: 'A comprehensive pathway map of epidermal growth factor receptor signaling', *Mol. Syst. Biol.*, 2005, **1**, 2005.0010

[24] JONES R.B., GORDUS A., KRALL J.A., MACBEATH G.: 'A quantitative protein interaction network for the ErbB receptors using protein microarrays', *Nature*, 2006, **439**, pp. 168–174

[25] DHILLON A.S., KOLCH W.: 'Untying the regulation of the Raf-1 kinase', *Arch. Biochem. Biophys.*, 2002, **404**, pp. 3–9

[26] MENDES P.: 'GEPASI: a software package for modelling the dynamics, steady states and control of biochemical and other systems', *Comput. Appl. Biosci.*, 1993, **9**, pp. 563–571

[27] IHEKWABA A.E., BROOMHEAD D.S., GRIMLEY R.L., BENSON N., KELL D.B.: 'Sensitivity analysis of parameters controlling oscillatory signalling in the NFκB pathway: the roles of IKK and IκBα', *Syst. Biol. (Stevenage)*, 2004, **1**, pp. 93–103

[28] HUCKA M., FINNEY A., SAURO H.M., *ET AL.*: 'The systems biology markup language (SBML): a medium for representation and exchange of biochemical network models', *Bioinf.*, 2003, **19**, pp. 524–531

[29] SASAGAWA S., OZAKI Y., FUJITA K., KURODA S.: 'Prediction and validation of the distinct dynamics of transient and sustained ERK activation', *Nat. Cell Biol.*, 2005, **7**, pp. 365–373

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doi: 10.1049/iet-syb:20070066

[30] FAVATA M.F., HORIUCHI K.Y., MANOS E.J., *ET AL.*: 'Identification of a novel inhibitor of mitogen-activated protein kinase kinase', *J. Biol. Chem.*, 1998, **273**, pp. 18623–18632

[31] KOHNO M., POUYSSEGUR J.: 'Pharmacological inhibitors of the ERK signaling pathway: application as anticancer drugs', *Prog. Cell Cycle Res.*, 2003, **5**, pp. 219–224

[32] OHREN J.F., CHEN H., PAVLOVSKY A., *ET AL.*: 'Structures of human MAP kinase kinase 1 (MEK1) and MEK2 describe novel noncompetitive kinase inhibition', *Nat. Struct. Mol. Biol.*, 2004, **11**, pp. 1192–1197

[33] HENDRIKS B.S., GRIFFITHS G.J., BENSON R., *ET AL.*: 'Decreased internalisation of erbB1 mutants in lung cancer is linked with a mechanism conferring sensitivity to gefitinib', *IEE Proc. Syst. Biol.*, 2006, **153**, pp. 457–466

[34] VASTRIK I., D'EUSTACHIO P., SCHMIDT E., *ET AL.*: 'Reactome: a knowledge base of biologic pathways and processes', *Genome Biol.*, 2007, **8**, p. R39

[35] BOS J.L.: 'Ras oncogenes in human cancer: a review', *Cancer Res.*, 1989, **49**, pp. 4682–4689

[36] AGAZIE Y.M., HAYMAN M.J.: 'Molecular mechanism for a role of SHP2 in epidermal growth factor receptor signaling', *Mol. Cell Biol.*, 2003, **23**, pp. 7875–7886

[37] MONTAGNER A., YART A., DANCE M., PERRET B., SALLES J.P., RAYNAL P.: 'A novel role for Gab1 and SHP2 in epidermal growth factor-induced Ras activation', *J. Biol. Chem.*, 2005, **280**, pp. 5350–5360

[38] CLEGHON V., FELDMANN P., GHIGLIONE C., *ET AL.*: 'Opposing actions of CSW and RasGAP modulate the strength of Torso RTK signaling in the Drosophila terminal pathway', *Mol. Cell*, 1998, **2**, pp. 719–727

[39] OZES O.N., MAYO L.D., GUSTIN J.A., PFEFFER S.R., PFEFFER L.M., DONNER D.B.: 'NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase', *Nature*, 1999, **401**, pp. 82–85

[40] ROMASHKOVA J.A., MAKAROV S.S.: 'NF-κB is a target of AKT in anti-apoptotic PDGF signalling', *Nature*, 1999, **401**, pp. 86–90

[41] BUROW M.E., WELDON C.B., MELNIK L.I., *ET AL.*: 'PI3-K/AKT regulation of NF-κB signaling events in suppression of TNF-induced apoptosis', *Biochem. Biophys. Res. Commun.*, 2000, **271**, pp. 342–345

[42] ROUDABUSH F.L., PIERCE K.L., MAUDSLEY S., KHAN K.D., LUTTRELL L.M.: 'Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells', *J. Biol. Chem.*, 2000, **275**, pp. 22583–22589

[43] LYNCH T.J., BELL D.W., SORDELLA R., *ET AL.*: 'Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib', *N. Engl. J. Med.*, 2004, **350**, pp. 2129–2139

[44] YEUNG K., SEITZ T., LI S., *ET AL.*: 'Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP', *Nature*, 1999, **401**, pp. 173–177

[45] CHO K.H., SHIN S.Y., KIM H.W., WOLKENHAUER O., MCFERRAN B., KOLCH W.: 'Mathematical modeling of the influence of RKIP on the ERK signaling pathway'. CMSB'03, 2003

[46] BOX G.E.P., DRAPER N.R.: 'Empirical model-building and response surfaces' (Wiley, 1987)

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REFERENCES

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T. and Saltiel, A.R. (1995) PD 098059 Is a Specific Inhibitor of the Activation of Mitogen-activated Protein Kinase Kinase in Vitro and in Vivo. *J. Biol. Chem.,* **270**, 27489-27494.

Avruch, J., Khokhlatchev, A., Kyriakis, J.M., Luo, Z., Tzivion, G., Vavvas, D. and Zhang, X.-F. (2001) Ras Activation of the Raf Kinase: Tyrosine Kinase Recruitment of the MAP Kinase Cascade. *Recent Prog Horm Res,* **56**, 127-156.

Balan, V., Leicht, D.T., Zhu, J., Balan, K., Kaplun, A., Singh-Gupta, V., Qin, J., Ruan, H., Comb, M.J. and Tzivion, G. (2006) Identification of Novel In Vivo Raf-1 Phosphorylation Sites Mediating Positive Feedback Raf-1 Regulation by Extracellular Signal-regulated Kinase. *Mol. Biol. Cell,* **17**, 1141-1153.

Basu, T., Warne, P.H. and Downward, J. (1994) Role of Shc in the activation of Ras in response to epidermal growth factor and nerve growth factor. *Oncogene,* **9**, 3483-3491.

Beavo, J.A. (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol. Rev.,* **75**, 725-748.

Bibel, M. and Barde, Y.-A. (2000) Neurotrophins: key regulators of cell fate and cell shape in the vertebrate nervous system. *Genes Dev.* **14**, 2919-2937.

Bos, J.L. (1989) ras Oncogenes in Human Cancer: A Review. *Cancer Res,* **49**, 4682-4689.

Bos, J.L. (1998) All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *Embo J,* **17**, 6776-6782.

Bos, J.L. (2003) EPAC: A NEW cAMP TARGET AND NEW AVENUES IN cAMP RESEARCH. *Nature Reviews Molecular Cell Biology,* **4**, 733-738.

Brostrom, M.A., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) A cyclic 3',5'-amp-stimulated protein kinase from cardiac muscle. *Adv Enzyme Regul,* **8**, 191-203.

Brott, B.K., Alessandrini, A., Largaespada, D.A., Copeland, N.G., Jenkins, N.A., Crews, C.M. and Erikson, R.L. (1993) MEK2 is a kinase related to MEK1 and is differentially expressed in murine tissues. *Cell Growth Differ,* **4**, 921-929.

Butcher, R.W. and Sutherland, E.W. (1962) Adenosine 3',5'-Phosphate in Biological Materials. I. PURIFICATION AND PROPERTIES OF CYCLIC 3',5'-NUCLEOTIDE PHOSPHODIESTERASE AND USE OF THIS ENZYME TO CHARACTERIZE ADENOSINE 3',5'-PHOSPHATE IN HUMAN URINE. *J. Biol. Chem.,* **237**, 1244-1250.

Carlisle Michel, J.J., Dodge, K.L., Wong, W., Mayer, N.C., Langeberg, L.K. and Scott, J.D. (2004) PKA-phosphorylation of PDE4D3 facilitates recruitment of the mAKAP signalling complex. *Biochem J,* **381**, 587-592.

Catling, A.D., Schaeffer, H.J., Reuter, C.W., Reddy, G.R. and Weber, M.J. (1995) A proline-rich sequence unique to MEK1 and MEK2 is required for raf binding and regulates MEK function. *Mol. Cell. Biol.*, **15**, 5214-5225.

Chambers, R.J., Abrams, K., Garceau, N.Y., Kamath, A.V., Manley, C.M., Lilley, S.C., Otte, D.A., Scott, D.O., Sheils, A.L., Tess, D.A., Vellekoop, A.S., Zhang, Y. and Lam, K.T. (2006) A new chemical tool for exploring the physiological function of the PDE2 isozyme. *Bioorganic & Medicinal Chemistry Letters*, **16**, 307-310.

Chong, H. and Guan, K.-L. (2003) Regulation of Raf through Phosphorylation and N Terminus-C Terminus Interaction. *J. Biol. Chem.*, **278**, 36269-36276.

Chong, H., Lee, J. and Guan, K.L. (2001) Positive and negative regulation of Raf kinase activity and function by phosphorylation. *Embo J*, **20**, 3716-3727.

Christensen, A.E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.G. and Doskeland, S.O. (2003) cAMP Analog Mapping of Epac1 and cAMP Kinase: DISCRIMINATING ANALOGS DEMONSTRATE THAT Epac AND cAMP KINASE ACT SYNERGISTICALLY TO PROMOTE PC-12 CELL NEURITE EXTENSION. *J. Biol. Chem.* **278**, 35394-35402.

Cook, S.J. and McCormick, F. (1993) Inhibition by cAMP of Ras-dependent activation of Raf. *Science,* **262**, 1069-1072.

de Rooij, J., Zwartkruis, F.J., Verheijen, M.H., Cool, R.H., Nijman, S.M., Wittinghofer, A. and Bos, J.L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*, **396**, 474-477.

Degerman, E., Belfrage, P. and Manganiello, V.C. (1997) Structure, Localization, and Regulation of cGMP-inhibited Phosphodiesterase (PDE3). *J. Biol. Chem.*, **272**, 6823-6826.

Dessauer, C.W., Scully, T.T. and Gilman, A.G. (1997) Interactions of Forskolin and ATP with the Cytosolic Domains of Mammalian Adenylyl Cyclase. *J. Biol. Chem.* **272**, 22272-22277.

Deutsch, P.J. and Sun, Y. (1992) The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J. Biol. Chem.*, **267**, 5108-5113.

Dhanasekaran, N. and Premkumar Reddy, E. (1998) Signaling by dual specificity kinases. *Oncogene*, **17**, 1447-1455.

Dhillon, A.S. and Kolch, W. (2002) Untying the regulation of the Raf-1 kinase. *Archives of Biochemistry and Biophysics*, **404**, 3-9.

Dhillon, A.S., Meikle, S., Yazici, Z., Eulitz, M. and Kolch, W. (2002a) Regulation of Raf-1 activation and signalling by dephosphorylation. *Embo J,* **21**, 64-71.

Dhillon, A.S., Pollock, C., Steen, H., Shaw, P.E., Mischak, H. and Kolch, W. (2002b) Cyclic AMP-Dependent Kinase Regulates Raf-1 Kinase Mainly by Phosphorylation of Serine 259. *Mol. Cell. Biol.*, **22**, 3237-3246.

Dhillon, A.S., von Kriegsheim, A., Grindlay, J. and Kolch, W. (2007) Phosphatase and feedback regulation of Raf-1 signaling. *Cell Cycle*, **6**, 3-7.

Dhomen, N. and Marais, R. (2007) New insight into BRAF mutations in cancer. *Curr Opin Genet Dev*, **17**, 31-39.

Diaz, B., Barnard, D., Filson, A., MacDonald, S., King, A. and Marshall, M. (1997) Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. *Mol. Cell. Biol.*, **17**, 4509-4516.

Dougherty, M.K., Muller, J., Ritt, D.A., Zhou, M., Zhou, X.Z., Copeland, T.D., Conrads, T.P., Veenstra, T.D., Lu, K.P. and Morrison, D.K. (2005) Regulation of Raf-1 by direct feedback phosphorylation. *Mol Cell*, **17**, 215-224.

Dumaz, N., Light, Y. and Marais, R. (2002) Cyclic AMP Blocks Cell Growth through Raf-1-Dependent and Raf-1-Independent Mechanisms. *Mol. Cell. Biol.* **22**, 3717-3728.

Dumaz, N. and Marais, R. (2003) Protein Kinase A Blocks Raf-1 Activity by Stimulating 14-3-3 Binding and Blocking Raf-1 Interaction with Ras. *J. Biol. Chem.*, **278**, 29819-29823.

Dumaz, N. and Marais, R. (2005) Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on The Anniversary Prize of the Gesellschaft für Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. *FEBS Journal*, **272**, 3491-3504.

Eckstein, F., Romaniuk, P.J., Heideman, W. and Storm, D.R. (1981) Stereochemistry of the mammalian adenylate cyclase reaction. *J. Biol. Chem.*, **256**, 9118-9120.

Erhardt, P., Troppmair, J., Rapp, U.R. and Cooper, G.M. (1995) Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells. *Mol. Cell. Biol.*, **15**, 5524-5530.

Fabian, J.R., Daar, I.O. and Morrison, D.K. (1993) Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol. Cell. Biol.*, **13**, 7170-7179.

Fischmeister, R., Castro, L.R., Abi-Gerges, A., Rochais, F., Jurevicius, J., Leroy, J. and Vandecasteele, G. (2006) Compartmentation of cyclic nucleotide signaling in the heart: the role of cyclic nucleotide phosphodiesterases. *Circ Res*, **99**, 816-828.

Francis, S.H. and Corbin, J.D. (1994) Structure and function of cyclic nucleotide-dependent protein kinases. *Annu Rev Physiol*, **56**, 237-272.

Francis, S.H., Turko, I.V. and Corbin, J.D. (2001) Cyclic nucleotide phosphodiesterases: relating structure and function. *Prog Nucleic Acid Res Mol Biol*, **65**, 1-52.

Friedman, W.J. and Greene, L.A. (1999) Neurotrophin signaling via Trks and p75. *Exp Cell Res*, **253**, 131-142.

Greene, L.A. and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci U S A*, **73**, 2424-2428.

Hafner, S., Adler, H.S., Mischak, H., Janosch, P., Heidecker, G., Wolfman, A., Pippig, S., Lohse, M., Ueffing, M. and Kolch, W. (1994) Mechanism of inhibition of Raf-1 by protein kinase A. *Mol. Cell. Biol.*, **14**, 6696-6703.

Hekman, M., Fischer, A., Wennogle, L.P., Wang, Y.K., Campbell, S.L. and Rapp, U.R. (2005) Novel C-Raf phosphorylation sites: serine 296 and 301 participate in Raf regulation. *FEBS Lett*, **579**, 464-468.

Helmbrecht, K., Zeise, E. and Rensing, L. (2000) Chaperones in cell cycle regulation and mitogenic signal transduction: a review. *Cell Prolif*, **33**, 341-365.

Hernandez-Alcoceba, R., del Peso, L. and Lacal, J.C. (2000) The Ras family of GTPases in cancer cell invasion. *Cell Mol Life Sci*, **57**, 65-76.

Hirsh, L., Dantes, A., Suh, B.-S., Yoshida, Y., Hosokawa, K., Tajima, K. and Kotsuji, F. (2004) Phosphodiesterase inhibitors as anti-cancer drugs. *Proceedings from the 6th and 7th international conferences, Signal Transduction 2004 and Chromatin 2004*, **68**, 981-988.

Houslay, M.D. (2001) PDE4 cAMP-specific phosphodiesterases. *Prog Nucleic Acid Res Mol Biol*, **69**, 249-315.

Houslay, M.D. and Adams, D.R. (2003) PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization. *Biochem J*, **370**, 1-18.

Houslay, M.D. and Kolch, W. (2000) Cell-Type Specific Integration of Cross-Talk between Extracellular Signal-Regulated Kinase and cAMP Signaling. *Mol Pharmacol*, **58**, 659-668.

Houslay, M.D. and Milligan, G. (1997) Tailoring cAMP-signalling responses through isoform multiplicity. *Trends Biochem Sci*, **22**, 217-224.

Huang, E.J. and Reichardt, L.F. (2003) Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem*, **72**, 609-642.

Huang, L.J.-s., Wang, L., Ma, Y., Durick, K., Perkins, G., Deerinck, T.J., Ellisman, M.H. and Taylor, S.S. (1999) NH2-Terminal Targeting Motifs Direct Dual Specificity A-Kinase-anchoring Protein 1 (D-AKAP1) to Either Mitochondria or Endoplasmic Reticulum. *J. Cell Biol.* **145**, 951-959.

Hubbard, S.R. and Miller, W.T. (2007) Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr Opin Cell Biol*, **19**, 117-123.

Hurley, J.H. (1999) Structure, Mechanism, and Regulation of Mammalian Adenylyl Cyclase. *J. Biol. Chem.* **274**, 7599-7602.

Jaiswal, R.K., Weissinger, E., Kolch, W. and Landreth, G.E. (1996) Nerve growth factor-mediated activation of the mitogen-activated protein (MAP) kinase cascade involves a signaling complex containing B-Raf and HSP90. *J Biol Chem*, **271**, 23626-23629.

Jarnæss, E. and Taskén, K. (2007) Spatiotemporal control of cAMP signalling processes by anchored signalling complexes. *Biochemical Society Transactions* **035**, 931-937.

Jeon, Y.H., Heo, Y.S., Kim, C.M., Hyun, Y.L., Lee, T.G., Ro, S. and Cho, J.M. (2005) Phosphodiesterase: overview of protein structures, potential therapeutic applications and recent progress in drug development. *Cell Mol Life Sci*, **62**, 1198-1220.

Jing, S., Tapley, P. and Barbacid, M. (1992) Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron*, **9**, 1067-1079.

Johnson, D.A., Akamine, P., Radzio-Andzelm, E., Madhusudan, M. and Taylor, S.S. (2001) Dynamics of cAMP-dependent protein kinase. *Chem Rev*, **101**, 2243-2270.

Juilfs, D.M., Fulle, H.J., Zhao, A.Z., Houslay, M.D., Garbers, D.L. and Beavo, J.A. (1997) A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway. *Proc Natl Acad Sci U S A*, **94**, 3388-3395.

Kakkar, R., Raju, R.V., Rajput, A.H. and Sharma, R.K. (1996) Inhibition of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by deprenyl. *Life Sci*, **59**, PL337-341.

Kao, S.-c., Jaiswal, R.K., Kolch, W. and Landreth, G.E. (2001) Identification of the Mechanisms Regulating the Differential Activation of the MAPK Cascade by Epidermal Growth Factor and Nerve Growth Factor in PC12 Cells. *J. Biol. Chem.*, **276**, 18169-18177.

Kennelly, P.J. and Krebs, E.G. (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.*, **266**, 15555-15558.

Kiermayer, S., Biondi, R.M., Imig, J., Plotz, G., Haupenthal, J., Zeuzem, S. and Piiper, A. (2005) Epac activation converts cAMP from a proliferative into a differentiation signal in PC12 cells. *Mol Biol Cell*, **16**, 5639-5648.

King, A.J., Sun, H., Diaz, B., Barnard, D., Miao, W., Bagrodia, S. and Marshall, M.S. (1998) The protein kinase Pak3 positively regulates Raf-1 activity through phosphorylation of serine 338. *Nature*, **396**, 180-183.

Kolch, W. (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J*, **351 Pt 2**, 289-305.

Kubicek, M., Pacher, M., Abraham, D., Podar, K., Eulitz, M. and Baccarini, M. (2002) Dephosphorylation of Ser-259 Regulates Raf-1 Membrane Association. *J. Biol. Chem.*, **277**, 7913-7919.

Lazarovici, P., Jiang, H. and Fink Jr, D. (1998) The 38-Amino-Acid Form of Pituitary Adenylate Cyclase-Activating Polypeptide Induces Neurite Outgrowth in PC12 Cells that Is Dependent on Protein Kinase C and Extracellular Signal-Regulated Kinase but not on Protein Kinase A, Nerve Growth Factor Receptor Tyrosine Kinase, p21ras G protein, and pp60c-src Cytoplasmic Tyrosine Kinase. *Mol Pharmacol*, **54**, 547-558.

Li, S., Janosch, P., Tanji, M., Rosenfeld, G.C., Waymire, J.C., Mischak, H., Kolch, W. and Sedivy, J.M. (1995) Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins. *Embo J*, **14**, 685-696.

MacNicol, M.C., Muslin, A.J. and MacNicol, A.M. (2000) Disruption of the 14-3-3 Binding Site within the B-Raf Kinase Domain Uncouples Catalytic Activity from PC12 Cell Differentiation. *J. Biol. Chem.* **275**, 3803-3809.

Manallack, D.T., Hughes, R.A. and Thompson, P.E. (2005) The next generation of phosphodiesterase inhibitors: structural clues to ligand and substrate selectivity of phosphodiesterases. *J Med Chem*, **48**, 3449-3462.

Manganiello, V.C. and Degerman, E. (1999) Cyclic nucleotide phosphodiesterases (PDEs): diverse regulators of cyclic nucleotide signals and inviting molecular targets for novel therapeutic agents. *Thromb Haemost*, **82**, 407-411.

Marais, R., Light, Y., Paterson, H.F. and Marshall, C.J. (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *Embo J*, **14**, 3136-3145.

Marais, R., Light, Y., Paterson, H.F., Mason, C.S. and Marshall, C.J. (1997) Differential Regulation of Raf-1, A-Raf, and B-Raf by Oncogenic Ras and Tyrosine Kinases. *J. Biol. Chem.*, **272**, 4378-4383.

Marshall, C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179-185.

Martinez, S.E., Wu, A.Y., Glavas, N.A., Tang, X.B., Turley, S., Hol, W.G. and Beavo, J.A. (2002) The two GAF domains in phosphodiesterase 2A have distinct roles in dimerization and in cGMP binding. *Proc Natl Acad Sci U S A*, **99**, 13260-13265.

Mason, C.S., Springer, C.J., Cooper, R.G., Superti-Furga, G., Marshall, C.J. and Marais, R. (1999) Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *Embo J*, **18**, 2137-2148.

Matheny, S.A., Chen, C., Kortum, R.L., Razidlo, G.L., Lewis, R.E. and White, M.A. (2004) Ras regulates assembly of mitogenic signalling complexes through the effector protein IMP. *Nature*, **427**, 256-260.

Maurice, D.H., Palmer, D., Tilley, D.G., Dunkerley, H.A., Netherton, S.J., Raymond, D.R., Elbatarny, H.S. and Jimmo, S.L. (2003) Cyclic Nucleotide Phosphodiesterase Activity, Expression, and Targeting in Cells of the Cardiovascular System. *Mol Pharmacol* **64**, 533-546.

Mehats, C., Andersen, C.B., Filopanti, M., Jin, S.L. and Conti, M. (2002) Cyclic nucleotide phosphodiesterases and their role in endocrine cell signaling. *Trends Endocrinol Metab*, **13**, 29-35.

Meller, N., Liu, Y.C., Collins, T.L., Bonnefoy-Berard, N., Baier, G., Isakov, N. and Altman, A. (1996) Direct interaction between protein kinase C theta (PKC theta) and 14-3- 3 tau in T cells: 14-3-3 overexpression results in inhibition of PKC theta translocation and function. *Mol. Cell. Biol.*, **16**, 5782-5791.

Mikula, M., Schreiber, M., Husak, Z., Kucerova, L., Ruth, J., Wieser, R., Zatloukal, K., Beug, H., Wagner, E.F. and Baccarini, M. (2001) Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. *Embo J*, **20**, 1952-1962.

Mor, A. and Philips, M.R. (2006) Compartmentalized Ras/MAPK signaling. *Annu Rev Immunol*, **24**, 771-800.

Morrison, D.K., Heidecker, G., Rapp, U.R. and Copeland, T.D. (1993) Identification of the major phosphorylation sites of the Raf-1 kinase. *J. Biol. Chem.*, **268**, 17309-17316.

Movsesian, M.A. (2002) PDE3 cyclic nucleotide phosphodiesterases and the compartmentation of cyclic nucleotide-mediated signalling in cardiac myocytes. *Basic Res Cardiol*, **97 Suppl 1**, I83-90.

Murthy, K.S., Zhou, H. and Makhlouf, G.M. (2002) PKA-dependent activation of PDE3A and PDE4 and inhibition of adenylyl cyclase V/VI in smooth muscle. *Am J Physiol Cell Physiol* **282**, C508-517.

Niihori, T., Aoki, Y., Narumi, Y., Neri, G., Cave, H., Verloes, A., Okamoto, N., Hennekam, R.C., Gillessen-Kaesbach, G., Wieczorek, D., Kavamura, M.I., Kurosawa, K., Ohashi, H., Wilson, L., Heron, D., Bonneau, D., Corona, G., Kaname, T., Naritomi, K., Baumann, C., Matsumoto, N., Kato, K., Kure, S. and Matsubara, Y. (2006) Germline KRAS and BRAF mutations in cardio-facio-cutaneous syndrome. *Nat Genet*, **38**, 294-296.

Nikolaev, V.O. and Lohse, M.J. (2006) Monitoring of cAMP Synthesis and Degradation in Living Cells. *Physiology*, **21**, 86-92.

Nioche, P., Liu, W.-Q., Broutin, I., Charbonnier, F., Latreille, M.-T., Vidal, M., Roques, B., Garbay, C. and Ducruix, A. (2002) Crystal structures of the SH2 domain of grb2: highlight on the binding of a new high-affinity inhibitor. *Journal of Molecular Biology*, **315**, 1167-1177.

Nosaka, Y., Arai, A., Miyasaka, N. and Miura, O. (1999) CrkL Mediates Ras-dependent Activation of the Raf/ERK Pathway through the Guanine Nucleotide Exchange Factor C3G in Hematopoietic Cells Stimulated with Erythropoietin or Interleukin-3. *J. Biol. Chem.* **274**, 30154-30162.

Obara, Y., Labudda, K., Dillon, T.J. and Stork, P.J.S. (2004) PKA phosphorylation of Src mediates Rap1 activation in NGF and cAMP signaling in PC12 cells. *J Cell Sci*, **117**, 6085-6094.

Papin, C., Denouel, A., Calothy, G. and Eychene, A. (1996) Identification of signalling proteins interacting with B-Raf in the yeast two-hybrid system. *Oncogene*, **12**, 2213-2221.

Park, S., Yeung, M.L., Beach, S., Shields, J.M. and Yeung, K.C. (2005) RKIP downregulates B-Raf kinase activity in melanoma cancer cells. *Oncogene*, **24**, 3535-3540.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.-e., Karandikar, M., Berman, K. and Cobb, M.H. (2001) Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions. *Endocr Rev* **22**, 153-183.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. and Piwnica-Worms, H. (1997) Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science*, **277**, 1501-1505.

Piiper, A., Dikic, I., Lutz, M.P., Leser, J., Kronenberger, B., Elez, R., Cramer, H., Muller-Esterl, W. and Zeuzem, S. (2002) Cyclic AMP Induces Transactivation of the Receptors for Epidermal Growth Factor and Nerve Growth Factor, Thereby Modulating Activation of MAP Kinase, Akt, and Neurite Outgrowth in PC12 Cells. *J. Biol. Chem.*, **277**, 43623-43630.

Pritchard, C.A., Bolin, L., Slattery, R., Murray, R. and McMahon, M. (1996) Post-natal lethality and neurological and gastrointestinal defects in mice with targeted disruption of the A-Raf protein kinase gene. *Curr Biol*, **6**, 614-617.

Pritchard, C.A., Samuels, M.L., Bosch, E. and McMahon, M. (1995) Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. *Mol. Cell. Biol.*, **15**, 6430-6442.

Qiao, J., Mei, F.C., Popov, V.L., Vergara, L.A. and Cheng, X. (2002) Cell Cycle-dependent Subcellular Localization of Exchange Factor Directly Activated by cAMP. *J. Biol. Chem.* **277**, 26581-26586.

Qiu, W., Zhuang, S., von Lintig, F.C., Boss, G.R. and Pilz, R.B. (2000) Cell Type-specific Regulation of B-Raf Kinase by cAMP and 14-3-3 Proteins. *J. Biol. Chem.* **275**, 31921-31929.

Reinhardt, R.R., Chin, E., Zhou, J., Taira, M., Murata, T., Manganiello, V.C. and Bondy, C.A. (1995) Distinctive anatomical patterns of gene expression for cGMP-inhibited cyclic nucleotide phosphodiesterases. *J Clin Invest*, **95**, 1528-1538.

Robinson, D.R., Wu, Y.M. and Lin, S.F. (2000) The protein tyrosine kinase family of the human genome. *Oncogene*, **19**, 5548-5557.

Robinson, G.A., Butcher, R.W. and Sutherland, E.W. (1968) Cyclic AMP. *Annual Review of Biochemistry*, **37**, 149-174.

Roy, S., McPherson, R.A., Apolloni, A., Yan, J., Lane, A., Clyde-Smith, J. and Hancock, J.F. (1998) 14-3-3 Facilitates Ras-Dependent Raf-1 Activation In Vitro and In Vivo. *Mol. Cell. Biol.*, **18**, 3947-3955.

Rubin, C.S., Rangel-Aldao, R., Sarkar, D., Erlichman, J. and Fleischer, N. (1979) Characterization and comparison of membrane-associated and cytosolic cAMP-dependent protein kinases. Physicochemical and immunological studies on bovine cerebral cortex protein kinases. *J. Biol. Chem.*, **254**, 3797-3805.

Sadhu, K., Hensley, K., Florio, V.A. and Wolda, S.L. (1999) Differential Expression of the Cyclic GMP-stimulated Phosphodiesterase PDE2A in Human Venous and Capillary Endothelial Cells. *J. Histochem. Cytochem.*, **47**, 895-906.

Schaeffer, H.J. and Weber, M.J. (1999) Mitogen-Activated Protein Kinases: Specific Messages from Ubiquitous Messengers. *Mol. Cell. Biol.*, **19**, 2435-2444.

Schmitt, J.M. and Stork, P.J.S. (2001) Cyclic AMP-Mediated Inhibition of Cell Growth Requires the Small G Protein Rap1. *Mol. Cell. Biol.* **21**, 3671-3683.

Schneider, R. and Schweiger, M. (1991) A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors. *Oncogene*, **6**, 1807-1811.

Segal, R.A. and Greenberg, M.E. (1996) Intracellular signaling pathways activated by neurotrophic factors. *Annu Rev Neurosci*, **19**, 463-489.

Shabb, J.B. (2001) Physiological substrates of cAMP-dependent protein kinase. *Chem Rev*, **101**, 2381-2411.

Shakur, Y., Takeda, K., Kenan, Y., Yu, Z.-X., Rena, G., Brandt, D., Houslay, M.D., Degerman, E., Ferrans, V.J. and Manganiello, V.C. (2000) Membrane Localization of Cyclic Nucleotide Phosphodiesterase 3 (PDE3). TWO N-TERMINAL DOMAINS ARE REQUIRED FOR THE EFFICIENT TARGETING TO, AND ASSOCIATION OF, PDE3 WITH ENDOPLASMIC RETICULUM. *J. Biol. Chem.*, **275**, 38749-38761.

Sidovar, M.F., Kozlowski, P., Lee, J.W., Collins, M.A., He, Y. and Graves, L.M. (2000) Phosphorylation of Serine 43 Is Not Required for Inhibition of c-Raf Kinase by the cAMP-dependent Protein Kinase. *J. Biol. Chem.* **275**, 28688-28694.

Skalhegg, B.S. and Tasken, K. (2000) Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA. *Front Biosci*, **5**, D678-693.

Spada, A., Lania, A., Mantovani, S. and Ballare, E. (1997) Cyclic AMP and calcium in the transduction of hypothalamic neurohormone action in human pituitary tumors. *Horm Res*, **47**, 235-239.

Stancato, L.F., Silverstein, A.M., Owens-Grillo, J.K., Chow, Y.H., Jove, R. and Pratt, W.B. (1997) The hsp90-binding antibiotic geldanamycin decreases Raf levels and epidermal growth factor signaling without disrupting formation of signaling complexes or reducing the specific enzymatic activity of Raf kinase. *J Biol Chem*, **272**, 4013-4020.

Stein, J.C., Farooq, M., Norton, W.T. and Rubin, C.S. (1987) Differential expression of isoforms of the regulatory subunit of type II cAMP-dependent protein kinase in rat neurons, astrocytes, and oligodendrocytes. *J. Biol. Chem.*, **262**, 3002-3006.

Stork, P.J.S. and Schmitt, J.M. (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends in Cell Biology*, **12**, 258-266.

Storm, S.M., Brennscheidt, U., Sithanandam, G. and Rapp, U.R. (1990) raf oncogenes in carcinogenesis. *Crit Rev Oncog*, **2**, 1-8.

Sunahara, R.K. and Taussig, R. (2002) Isoforms of Mammalian Adenylyl Cyclase: Multiplicities of Signaling. *Mol. Interv.*, **2**, 168-184.

Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., Kishimoto, T. and Nagasawa, T. (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*, **393**, 591-594.

Tang, W.-J. and Hurley, J.H. (1998) Catalytic Mechanism and Regulation of Mammalian Adenylyl Cyclases. *Mol Pharmacol*, **54**, 231-240.

Tasken, K. and Aandahl, E.M. (2004) Localized Effects of cAMP Mediated by Distinct Routes of Protein Kinase A. *Physiol. Rev.*, **84**, 137-167.

Thorson, J.A., Yu, L.W.K., Hsu, A.L., Shih, N.-Y., Graves, P.R., Tanner, J.W., Allen, P.M., Piwnica-Worms, H. and Shaw, A.S. (1998) 14-3-3 Proteins Are Required for Maintenance of Raf-1 Phosphorylation and Kinase Activity. *Mol. Cell. Biol.*, **18**, 5229-5238.

Traverse, S., Cohen, P., Paterson, H., Marshall, C., Rapp, U. and Grand, R.J. (1993) Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of ras-transformed retinal cells. *Oncogene*, **8**, 3175-3181.

Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem J*, **288 (Pt 2)**, 351-355.

Tzivion, G., Luo, Z. and Avruch, J. (1998) A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature*, **394**, 88-92.

Vandecasteele, G., Rochais, F., Abi-Gerges, A. and Fischmeister, R. (2006) Functional localization of cAMP signalling in cardiac myocytes. *Biochem Soc Trans*, **34**, 484-488.

Vossler, M.R., Yao, H., York, R.D., Pan, M.G., Rim, C.S. and Stork, P.J. (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell*, **89**, 73-82.

Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) An Adenosine 3',5'-Monophosphate-dependant Protein Kinase from Rabbit Skeletal Muscle. *J. Biol. Chem.*, **243**, 3763-3765.

Wang, H.G., Takayama, S., Rapp, U.R. and Reed, J.C. (1996) Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1. *Proc Natl Acad Sci U S A*, **93**, 7063-7068.

Wei, S., Kasuya, Y., Yanagisawa, M., Kimura, S., Masaki, T. and Goto, K. (1997) Studies on endothelium-dependent vasorelaxation by hydralazine in porcine coronary artery. *Eur J Pharmacol*, **321**, 307-314.

Wojnowski, L., Zimmer, A.M., Beck, T.W., Hahn, H., Bernal, R., Rapp, U.R. and Zimmer, A. (1997) Endothelial apoptosis in Braf-deficient mice. *Nat Genet*, **16**, 293-297.

Wu, C., Lai, C.-F. and Mobley, W.C. (2001) Nerve Growth Factor Activates Persistent Rap1 Signaling in Endosomes. *J. Neurosci.*, **21**, 5406-5416.

Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M.J. and Sturgill, T.W. (1993) Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science*, Vol. 262, pp. 1065-1069.

Yaffe, M.B. (2002) How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett*, **513**, 53-57.

Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J. and Cantley, L.C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*, **91**, 961-971.

Yamamori, B., Kuroda, S., Shimizu, K., Fukui, K., Ohtsuka, T. and Takai, Y. (1995) Purification of a Ras-dependent Mitogen-activated Protein Kinase Kinase Kinase from Bovine Brain Cytosol and Its Identification as a Complex of B-Raf and 14-3-3 Proteins. *J. Biol. Chem.*, **270**, 11723-11726.

Yamashita, H., Avraham, S., Jiang, S., Dikic, I. and Avraham, H. (1999) The Csk Homologous Kinase Associates with TrkA Receptors and Is Involved in Neurite Outgrowth of PC12 Cells. *J. Biol. Chem.* **274**, 15059-15065.

Yan, K.S., Kuti, M., Yan, S., Mujtaba, S., Farooq, A., Goldfarb, M.P. and Zhou, M.-M. (2002) FRS2 PTB Domain Conformation Regulates Interactions with Divergent Neurotrophic Receptors. *J. Biol. Chem.* **277**, 17088-17094.

Yao, H., York, R.D., Misra-Press, A., Carr, D.W. and Stork, P.J.S. (1998) The Cyclic Adenosine Monophosphate-dependent Protein Kinase (PKA) Is Required for the Sustained Activation of Mitogen-activated Kinases and Gene Expression by Nerve Growth Factor. *J. Biol. Chem.* **273**, 8240-8247.

Yarden, Y. and Sliwkowski, M.X. (2001) Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*, **2**, 127-137.

Yeung, K., Janosch, P., McFerran, B., Rose, D.W., Mischak, H., Sedivy, J.M. and Kolch, W. (2000) Mechanism of Suppression of the Raf/MEK/Extracellular Signal-Regulated Kinase Pathway by the Raf Kinase Inhibitor Protein. *Mol. Cell. Biol.* **20**, 3079-3085.

Yip-Schneider, M.T., Miao, W., Lin, A., Barnard, D.S., Tzivion, G. and Marshall, M.S. (2000) Regulation of the Raf-1 kinase domain by phosphorylation and 14-3-3 association. *Biochem. J.* **351**, 151-159.

Yoon, S. and Seger, R. (2006) The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors*, **24**, 21 - 44.

York, R.D., Yao, H., Dillon, T., Ellig, C.L., Eckert, S.P., McCleskey, E.W. and Stork, P.J.S. (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. *Nature*, **392**, 622-626.

Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S.J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell*, **87**, 619-628.

Zheng, C.F. and Guan, K.L. (1994) Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. *Embo J*, **13**, 1123-1131.

Zufall, F., Shepherd, G.M., Barnstable, C.J., Brostrom, M.A., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1997) Cyclic nucleotide gated channels as regulators of CNS development and plasticity

A cyclic 3',5'-amp-stimulated protein kinase from cardiac muscle. *Curr Opin Neurobiol*, **7**, 404-412.

Zwartkruis, F.J., Wolthuis, R.M., Nabben, N.M., Franke, B. and Bos, J.L. (1998) Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. *Embo J*, **17**, 5905-5912.

Zwartkruis, F.J.T. and Bos, J.L. (1999) Ras and Rap1: Two Highly Related Small GTPases with Distinct Function. *Experimental Cell Research*, **253**, 157-165.