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Distinct roles of Gi/o protein subunits in signaling by dopamine D2S receptors in rat pituitary adenoma cells

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To:

My parents, Parvin and Ahmad,

For all their encouragement and sacrifice,

My brother, Amir,

For all his unconditional support,

And my brother, Behnam.

Abstract:

In GH4ZR7 lactotroph cells dopamine-D2S receptor activation inhibited forskolin-induced cAMP production, BayK8644-activated calcium influx, basal B-raf kinase and blocked TRH-mediated c-Raf activity, phosphorylation of MEK1/2 and p42/44-MAPK. These actions were blocked by pretreatment with PTX, indicating mediation by Gi/o proteins. Following PTX treatment, D2S receptor signaling was rescued in cells stably transfected with individual PTX-insensitive Gα mutants. Inhibition of adenylyl cyclase (AC) was partly rescued by Gαi2 or Gαi3. By contrast, Gαo alone completely reconstituted D2S-mediated inhibition of L-type calcium channels. Gαo and Gαi3 mediated D2S inhibition of p42/44-MAPK. In cells transfected with GRK-carboxylterminal domain to inhibit Gβγ signaling, only D2S-mediated inhibition of calcium influx was blocked, but not inhibition of adenylyl cyclase or MAPK. These results indicate that the dopamine-D2S receptor couples to distinct Gi/o proteins depending on the pathway addressed, and suggest a novel Gαi3/Gαo-dependent inhibition of MAPK that is independent of Gβγ signaling.

Abbreviations:

Abbreviation	Full name
aa	Amino acid
AC	Adenylyl cyclase
cAMP	Cyclic adenosine monophosphate
CHO	Chinese hamster ovary *cell line*
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
CTX	Cholera toxin
DA	Dopamine
DAG	Diacylglycerol
DOPA	Dihydroxy phenylalanine
DR	Dopamine receptor
EGF	Epidermal growth factor
ERK	Extracellular-signal regulated kinase
GAP	GTPase activating protein
G protein	Guanine nucleotide binding protein
GPCR	G protein coupled receptor
GRK	G protein-coupled receptor kinase
5HT	5-hydroxytryptamine
HVA	Homovanillic acid
IP3	Inositol triphosphate

KDa	Kilo Dalton
Kb	Kilobase
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MKP	MAPK phosphatase
PGE1	Prostaglandin E1
PH domain	Pleckstrin homology domain
PIP2	Phosphatidyl inositol (4,5) bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PRL	Prolactin
PTP	Phoshotyrosine phosphatase
PTX	Pertussis toxin
RGS	Regulator of G protein signaling
TM	Transmembrane
TRH	Thyrotropin-releasing hormone
VIP	Vasoactive intestinal peptide
VMAT	Vesicular monoamine transporter

Table of contents:

List of Figures:

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CHAPTER I

General Introduction

Dopaminergic System:

History:

Dopamine (DA) is an important catecholamine neurotransmitter in mammalian central nervous system (CNS), that is involved in a variety of functions including locomotion, cognition, emotion, reward and positive reinforcement, endocrine regulation and food intake. This catecholamine also plays prominent roles in the periphery as a modulator of catecholamine release, cardiovascular function, hormone secretion, vascular tone (indirectly), renal function and gastrointestinal motility. DA and dopaminergic system in the brain have been studied extensively since several pathological conditions including Parkinson's disease, Tourette's syndrome, schizophrenia and hyperprolactinemia are linked to dysregulation of dopaminergic neurotransmission.

The pharmacological activity of DA has been known since the early years of the twentieth century. However, it was in 1957 that Blaschko suggested a discrete physiological function for DA that considered a role for DA in the CNS (Hornykiewicz, 1966). In the 1960's DA was detected biochemically in the brain and its intraneuronal localization was histochemically demonstrated (reviewed in (Lindvall and Bjorklund, 1978). The first evidence that DA receptors are present in the CNS came from biochemical studies in 1972, in which DA was shown to stimulate adenylyl cyclase (AC)

activity in brain membrane preparations. Later in the 1970's, based on pharmacological evidence of differences in dopaminergic regulation of AC, two discrete populations of DA receptors were proposed (Spano et al., 1978). In fact, it was shown that in the pituitary DA inhibited prolactin secretion but did not stimulate adenosine 3', 5'-cyclic monophosphate (cAMP) formation (Caron et al., 1978). A year later, in 1979, Kebabian and Calne (Kebabian and Calne, 1979) summarized these observations and suggested to name the receptors that stimulate AC as D1 and those that did not stimulate AC as D2.

Biosynthesis and metabolism:

Tyrosine is the precursor for DA biosynthesis and this amino acid (aa) is derived from dietary proteins or from dietary phenylalanine, which is converted to tyrosine in the liver by phenylalaninehydroxylase .The tyrosine in blood is taken up into brain by a low-affinity amino acid transport system at the blood-brain barrier and then from the extracellular fluid into dopaminergic neurons by high- and low- affinity amino acid transporters. Once tyrosine has entered the neuron, its conversion to dihydroxy phenylalanine (L-DOPA), driven by the cytosolic enzyme tyrosine hydroxylase, is normally the rate limiting step in DA biosynthesis and can be blocked by α-methyl tyrosine. Subsequently, L-DOPA is decarboxylated to DA by dopa decarboxylase (aromatic amino acid decarboxylase).

In dopaminergic neurons, the neurotransmitter is transported from the cytoplasm to specialized storage vesicles. Here the amine is concentrated to approximately 0.1M by the vesicular monoamine transporter (VMAT2) in rat brain (Henry et al., 1994). Upon the arrival of an action potential and following influx of calcium ions, calcium-dependent

mechanisms cause vesicle fusion with the neuronal membrane and release DA in the synapse by exocytosis (Kelly, 1993).

The released DA in the synapse is either taken up again into the nerve terminal or is subjected to a series of enzymatic metabolic conversion by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT). In dopaminergic nerve terminals there is a specific sodium-dependent transporter that actively pumps DA back into nerve terminal from synaptic cleft up to 100- to 1000-fold concentration gradient.

Glia and nondopaminergic neurons can take up DA to a limited extent. The cytosolic DA can be recycled or can be degraded by MAO, which is located on the outer mitochondrial membrane. There are two subtypes of MAO: MAO-A and MAO-B. MAO-A is present in dopaminergic and noradrenergic neurons and MAO-B is found in glia and serotonergic neurons (Kandel et al., 1991). COMT is mostly present in glia and postsynaptic membrane. After a series of enzymatic degradation, DA is converted to 3, 4-dihydroxyphenylacetic acid (DOPAC) and later to homovanillic acid (HVA) in CNS. DOPAC is the major metabolite in the rat brain, while in human brain HVA is the major one (Elsworth and Roth, 1997) (Kandel et al., 1991).

Dopaminergic system in the CNS:

In the CNS, dopaminergic neurons form an organized system. Dopaminergic neurons are 3-4 times more abundant than noradrenergic neurons in brain. These neurons are localized in four major systems in the brain: tubero-infundibular, nigro-striatal, mesolimbic, and mesocortical systems.

Tubero-infundibular system: The cell bodies of dopaminergic neurons of this pathway are located in the arcuate nucleus and a portion of the periventricular nucleus of the hypothalamus. Their axons project to the median eminence of hypothalamus, secreting DA into the hypophyseal portal blood stream. Then DA is transferred to the anterior and intermediate lobes of pituitary gland. This system provides the major tonic inhibitory regulation of prolactin secretion, which influences fertility and milk secretion.

Nigro-striatal system: In this system, neurons located in the substantia nigra (pars compacta) and its axons extend to the putamen, caudate nucleus and globus pallidus (striatum). This system has an important role in controlling locomotion. Impairment in this pathway due to loss of nigral dopaminergic neurons is mainly responsible for Parkinson's disease and extrapyramidal side effects of antipsychotic drugs.

Mesolimbic system: These neurons originate from ventral tegmental area and send their projections to the nucleus accumbens, the nuclei of the stria terminalis, parts of the amygdala and hippocampus, the lateral septal nuclei, and the mesofrontal and entorhinal cortex. This system is important in the regulation of emotion, motivation and reward and is involved in schizophrenia and instability of thought and perception. This pathway is also the site of antipsychotic actions of neuroleptics.

Mesocortical system: This system originates from the ventral tegmental area and projects to the neocortex and prefrontal cortex. This pathway is involved in planning, motivation and behavior. It is believed that alterations in this pathway are involved in the negative symptoms of schizophrenia.

Other systems: Periglomerular system and retinal dopaminergic system are other dopaminergic pathways that have been described in many mammalian species.

Dopamine receptors:

The diverse dopaminergic functions are mediated through the interaction of DA with a variety of dopamine receptors. Following the recognition of DA as a neurotransmitter in 1972, the first biochemical assay was stimulation of AC (reviewed in (Missale et al., 1998). In 1979, based on the biochemical and pharmacological studies, two separate receptors, D1 and D2, were proposed. The idea of the presence of two receptors was utilized for more than a decade in DA receptor research.

A new era in DA receptor research came from the application of gene-cloning approaches to receptor biology in the 1980's. Based on the structural similarity of G-protein coupled receptors (GPCRs), the hamster β2 adrenergic receptor cDNA (Dixon et al., 1986) was used as a probe to screen a rat genomic library under low stringency hybridization conditions. This approach led to the cloning of the first DA receptor cDNA in 1988, the rat D2 receptor (Bunzow et al., 1988).

Later on, other groups cloned the D1 receptor (Dearry et al., 1990); (Monsma et al., 1990); (Sunahara et al., 1990); (Zhou et al., 1990), by using low stringency cloning approaches. The dual receptor concept served as the foundation for the study of DA receptors for many years. However, by using these gene-cloning procedures, three other novel DA receptor subtypes have been characterized. These have been named D3 (Sokoloff et al., 1990), D4 (Van Tol et al., 1992), and D5/D1b (Sunahara et al., 1991).

Detailed structural, pharmacological and biochemical studies have shown that all DA receptor subtypes fall into one of the two initially recognized receptor categories.

D5 is very similar structurally to D1, while D3 and D4 receptors resemble the D2 receptor. Pharmacological analysis confirmed that the D1 and D5 receptors are indeed functionally similar and both stimulate AC to increase cAMP production. On the other hand, the structurally similar D2 family of DA receptors, including D2, D3 and D4, exhibit similar pharmacology and they all couple negatively to AC (Civelli et al., 1993).

Thus D1/D2 classification concept developed in the late 1970s is still valid as D1-like and D2-like receptors. The mammalian D1b receptor, originally named on the basis of its high homology with the D1 receptor, is now commonly referred to as the D5 receptor (Missale et al., 1998).

The primary evaluation of the structure of DA receptors revealed that these receptors are members of the GPCR family with seven transmembrane domains (TMs), an extracellular N-terminal, an intracellular C-terminal, and three intracellular and three extracellular loops. D1-like and D2-like receptors have some similarities and some differences, structurally and functionally (Missale et al., 1998), that will be reviewed in the following sections.

D1-like receptor family:

The D1-like family was first classified according to its pharmacological properties that cause increase in cAMP production by coupling positively to AC. The D1-like receptor genes contain no introns in their protein coding regions. The D1-like receptors have a large C-terminal tail and a small third intracellular (i3) loop, typical of many Gs-coupled receptors. This family consists of two subtypes, D1 and D5 which share ~80% similarity in their TM domains (Missale et al., 1998); (Civelli et al., 1993).

Gene structure and distribution:

D1 receptor: D1 receptor contains 466 aa with >90% homology in their aa sequence among mammalian species. The human D1 receptor gene (DRD1) is located on chromosome 5 (Grandy et al., 1990). These receptors have been detected in forebrain with the highest binding density in the caudate-putamen complex, nucleus accumbens and olfactory tubercle. D1 receptor can also be detected in prefrontal cortex and areas of neocortex, choroid plexus and retina (Tiberi et al., 1991) (Meador-Woodruff et al., 1992).

D5 receptor: The human D5 receptor has 477 aa in its coding region which shows ~60% aa homology with D1 receptor. The gene for D5 receptor is located on long arm of chromosome 4 (Missale et al., 1998). Until now, there is no ligand to differentiate D5 from D1 receptors, except that the D5 receptor has a ~10-fold higher affinity for dopamine. However, mRNA expression of D5 receptor indicated a high level of receptor transcript in hippocampus, parafascicular nucleus of thalamus and the lateral mammilary nucleus (Tiberi et al., 1991) (Meador-Woodruff et al., 1992).

Signal transduction:

The well characterized signal transduction of D1-like receptor is to stimulate AC. This action goes through the Gs subtype of G proteins that, via activation of Gαs subunit, increase cAMP production in cell (Gao et al., 1987); (Birnbaumer et al., 1990).By using Golf knock-out mice, it is recently shown that Golf is also involved in mediating some of the D1R signaling pathways in striatum (Corvol et al., 2001); (Zhuang et al., 2000) .D1 receptors are found not only in CNS, but also in blood vessels, different regions of kidney and adrenal gland. Activation of D1-like receptors cause vasodilatation in renal arteries, increases filtration rate in glomerulus, inhibits Na^+ reabsorption in proximal and

collecting tubules by inhibiting a Na^+/H^+ exchanger and also stimulates renin secretion (Missale et al., 1998). D1 receptor expressed in GH4C1 cells stimulates AC and increases opening of L-type $Ca2^+$ channels possibly through protein kinase A. In Ltk-cells the D1 receptor activates AC but also stimulates PI turnover, a pathway not observed in pituitary cells (Liu et al., 1992b), but observed in striatum and kidney (see refs in Liu). It has been speculated that another D1-like receptor mediates PI turnover in striatum (Friedman et al., 1997), but no additional dopamine receptors are present in the sequence of the human genome.

D2-like receptor family:

By 1978, pharmacological analysis revealed that there are two distinct groups of DA receptors. The salient characteristics of the D2 receptor, at that time, was that it mediated inhibition of AC (Missale et al., 1998). The D2-like receptor family consists of three subgroups: D2, D3 and D4 receptors. The D2-like receptors contain exons and introns in their genes, which can undergo alternate splicing to produce different isoforms of these receptors. D2-like receptors have seven TM domains with a relatively large i3 loop and short C-terminal tail. This receptor is alternately spliced to include 29 aa in the i3 domain in the D2L, while these aa's are absent in the D2S isoform. These receptors are coupled to Gi/o proteins and inhibit AC (Missale et al., 1998) (Civelli et al., 1993). However, increasing evidence of alternate functions and signaling mechanisms of the D2 receptors has been produced in the last years. These characteristics of the D2 receptors will be explained in more detail below, since this thesis focuses on D2 action.

Gene structure and distribution:

D3 receptor: The rat D3 receptor is 466 aa, with five introns, and has greater than 50% homology to the rat D2 receptor. Meanwhile, the human D3 receptor has >90% homology to the rat D3 receptor, but lacks 46 aa in the i3 loop domain, and has six introns. The human D3 receptor is located on chromosome 3 (Missale et al., 1998). This receptor is primarily expressed in certain limbic areas of the brain such as the nucleus accumbens, olfactory tubercle, ventral pallidum, ventral tegmental area and islands of Calleja. To a lesser extent this receptor is also expressed in striatum, hippocampus and frontal and prefrontal cortex (Ariano et al., 1997); (De Keyser, 1993).

D4 receptor: D4 receptor gene is located on chromosome 11. Its molecular structure is the least similar to other D2-like receptors. In human variants, the D4 receptor exists with several polymorphic repeat insertions in the third intracellular loop. This loop contains repeat sequences of 16 aa. The number of repeats is different in the different forms of receptor. The four-repeat form (D4.4) is the predominant in the human population (60%). The D4.7 variant is present in 14% of the population and the D4.2 in 10% (Van Tol et al., 1992). Less frequent receptor forms with over 10 repeats have also been identified (Missale et al., 1998). The D4 receptor demonstrates a high expression in brain cortex and a restricted distribution in the nucleus accumbens, but not in the striatum (Ariano et al., 1997).

Signal transduction:

Signaling pathway of D2-like receptors is through Gi/o proteins that inhibit AC and can be blocked by pertussis toxin (PTX). All D2-like receptors inhibit AC in brain and various cell lines (Missale et al., 1998). However, the D3 receptor has weaker effect on AC compared to the other D2-like receptors in the same cell line (Robinson and

Caron, 1997). D2-like receptors modulate $[Ca^{2+}]_i$. D3 and D4 receptors causes inhibition of Ca^{2+} current in NG108-15 neuroblastoma and rat pituitary GH4C1 cells, respectively; however, no effect on PI hydrolysis is observed (Seabrook et al., 1994a; Seabrook et al., 1994c). Inhibition of calcium channels is due to direct interaction of the channel with G-protein beta-gamma subunits released upon receptor activation or indirectly through hyperpolarization of the membrane potential by activation of K^+ channels (Missale et al., 1998).

Although D2-like receptors are generally known as "inhibitory" receptors, they also demonstrate a wide range of "stimulatory" properties in a variety of cell lines. It has been shown that the D3 receptor stimulates [^{3}H]-thymidine incorporation in CHO and NG108-15 cells (Chio et al., 1994b); (Pilon et al., 1994).

The D4 receptors potentiate arachidonic acid synthesis in CHO cells (Chio et al., 1994a). Furthermore, when expressed in MN9D cells, both D3 and D4 stimulate neurite outgrowth from these cells (Swarzenski et al., 1994) (Swarzenski et al., 1996).

These findings indicate D2-like receptors couple to multiple signaling pathways, which can be either stimulatory or inhibitory.

Peripheral dopamine receptors:

In addition to expression in the CNS, dopamine receptors are expressed and play important roles in the periphery, in regulation of vasoconstriction and kidney function. Dopamine is co-secreted with adrenaline from the adrenal medulla and mediates dopamine action at these sites.

In blood vessels:

D1 receptors are identified in the renal, mesenteric and splenic arteries, where they cause activation of AC (Missale et al., 1988). It is shown that D1 receptors are more abundant in the medial layer of arteries and insensitive to chemical sympathectomy (Amenta and Ricci, 1990), indicating their post-junctional localization. D2 receptors are located in the adventitial and the adventitial-medial border as well as intimal layer of renal, mesenteric and splenic arteries (Amenta et al., 1990). Here they cause inhibition of AC and are present both pre-junctionally and post-junctionally.

Renin- Angiotensin-Aldostrone system control:

The exact mechanism of physiologic role of dopaminergic system in the regulation of renin secretion is still not well known. It appears that the major effect of DA on renin secretion is stimulatory and is mediated by D1 receptors (reviewed in (Missale et al., 1998). Dopamine inhibits angiotensin II-stimulated aldosterone secretion. This action has been demonstrated to be mediated by D2 receptors located on adrenal glomerolosa cells (Drake et al., 1984).

In the kidney:

It has been shown that dopamine acts at specific dopaminergic receptors in the renal vasculature and renal parenchyma to produce changes in renal function (reviewed in (Goldberg et al., 1978). At low doses, which do not affect systemic hemodynamics, DA produces renal vasodilatation, diuresis and natriuresis (Missale et al., 1998). However, the exact DA receptor subtypes and molecular mechanisms involved in signal transduction in kidney are beyond the scope of this thesis.

Dopamine D2 receptors:

D2 receptors are part of the D2-like family of DA receptors. The rat D2 receptor was the first receptor cloned in the DA receptor family (Bunzow et al., 1988). D2 receptor has many of the common features of D2-like family with seven predicted TM domains, a large i3 loop and a short C-terminal tail. The D2 receptor gene has introns in its gene, which generate a short (D2S) and long (D2L) form of the receptor. The D2 receptors couple to PTX-sensitive G-proteins (i.e. Gi/o subtype) and mediate multiple signaling pathways in different systems.

Gene structure:

The human D2 receptor gene is located on chromosome 11q. The rat D2 receptor gene spans 48 Kb, with seven coding exons and one 5' non-coding exon. The exons are separated by seven introns in which the first intron is usually large (>30Kb) (Grandy et al., 1989). In rat, The D2 receptor has two alternatively spliced forms, D2-long (D2L) and D2-short (D2S) that are 444 aa and 415 aa in length (443 aa and 414 aa, in humans) with a predicted relative molecular mass of 51 kDa and 47 kDa, respectively. The D2L has an extra insertion of 29 aa in its i3 loop (Missale et al., 1998). The two D2 isoforms have indistinguishable pharmacology, and couple similarly to various signaling pathways.

Localization and distribution:

The localization of mRNA transcripts of D2 receptor in brain corresponds well with the localization of D2 receptor binding sites, indicating high expression in caudate-putamen complex, nucleus accumbens, olfactory tubercle, globus pallidus, substantia nigra, pars compacta and ventral tegmental area (Ariano et al., 1997). By using a subtype-

specific antibody, it is shown that the D2S receptor is located in cell bodies and proximal and distal dendrites of DA neurons in midbrain (Khan et al., 1998a). Meanwhile, the D2L isoform is absent in dopaminergic axons and is strongly expressed in GABAergic and large cholinergic postsynaptic neurons of striatum. Moreover, the D2S isoform protein is expressed at a much higher level than the D2L in substantia nigra (Khan et al., 1998a; Khan et al., 1998b). The higher expression of D2S receptor in cell membrane of somatodendritic and axonal dopaminergic neurons indicates a presynaptic autoreceptor function for the D2S isoform in brain. Usiello and colleagues have recently proposed that D2L and D2S have different and probably antagonistic functions *in vivo* (Usiello et al., 2000). On the other hand, presence of D2L isoform in nondopaminergic neurons suggests a primary function as a post-synaptic receptor (Khan et al., 1998a). The presence of D2 receptor in pituitary has been observed since 1970s in the anterior and intermediate lobes of pituitary gland, by using radioligand binding assays. Later on, by cloning the D2 receptors, it was found that D2S and D2L receptor isoforms are expressed in both melanotroph and lactotroph cells, where D2L is more predominant (reviewed in (Missale et al., 1998). Interestingly, a subpopulation of lactotrophs have been identified that express a different ratio of D2S/D2L mRNA level. It is also shown that gonadal steroids can influence D2S/D2L mRNA ratios in vitro, thus providing a possible basis for variation in the density of pituitary D2 receptors during the estrous cycle (Kukstas et al., 1991). The biological significance of these differences between D2S and D2L isoform expression remains unclear.

Signal transduction:

The dopamine D2 receptor couples to Gi/o heterotrimeric G proteins since most actions of this receptor can be blocked by PTX pretreatment (Missale et al., 1998); (Ghahremani et al., 2000), which specifically blocks coupling of these G proteins (Birnbaumer, 1992). The D2 receptor couples to G-protein at i2 and i3 loops, similar to most GPCRs (Robinson and Caron, 1997); (Bourne, 1997a).

The role of i3 loop in D2 receptor signaling has been confirmed using antibodies to various regions of i3 loop in D2L and D2S receptors where the antibodies decrease the agonist high affinity binding for D2 receptors (Boundy et al., 1993); (Plug et al., 1992). There are some reports indicating the important role of the i2 loop of GPCRs in G protein coupling (Albert et al., 1998). It has been shown that a peptide from i2 loop of $\alpha 2$ adrenergic receptor inhibits agonist binding to the receptor (Dalman and Neubig, 1991) and a peptide encoding the i2 loop of 5-HT1A receptor could inhibit AC and mimic receptor interaction with G proteins (Varrault et al., 1994).

A chimera with i3 loop of D2 and i2 loop of D1 was unable to stimulate (D1 effect) or inhibit (D2 effect) AC, however, a D1 chimera containing both i3 and i2 loops from D2 receptor inhibited AC (Kozell et al., 1994). By using a series of D2/D3 chimeras, it has been shown that the i2 loop is a key determinant in the ability to couple sufficiently to AC (Robinson et al., 1994); (Robinson and Caron, 1996). These findings indicate the importance of i2 and i3 loops of D2 receptor in coupling to G proteins.

D2 receptor stimulation activates Gi/o proteins, resulting in dissociation of $G\alpha$ from $G\beta\gamma$ subunits. Activated G protein subunits couple to variety of effectors to initiate different second messenger systems. Gi/o proteins are known as "inhibitory" G proteins because they inhibit AC (Birnbaumer et al., 1990). Since the D2 receptors couple to Gi/o

proteins they are considered "inhibitory" receptors. However, now it is well established that Gi/o proteins (comprised of αi1, αi2, αi3, αoA, αoB and βγ subunits) can be "inhibitory" or "stimulatory" depending on the cell type and the different effectors present (Gudermann et al., 1996); (Hildebrandt, 1997); (Ghahremani et al., 2000). Therefore, D2 receptor can be "inhibitory" and/or "stimulatory" receptor depending on the G protein coupling and the cellular environment. So, categorizing a receptor or G protein as an "inhibitory" or "stimulatory" receptor is very simplistic, given the multiple, complex signaling pathways that can be activated by a receptor or G protein. Thus, the overall function of a receptor or G protein in a cell system cannot be evaluated by its action on a single signaling pathway. Evaluation of D2 receptor signaling in different cell lines has revealed multiple and rather complex-signaling systems for this receptor that will be explained in following sections.

Regarding differences in their structure, D2L and D2S isoforms have shown very similar signal transduction in a variety of cell systems (Robinson and Caron, 1997); (Albert, 1994). Until now the only major difference is in receptor desensitization by protein kinase C (PKC)-induced uncoupling, in which the D2L is more resistant the actions of PKC (Liu et al., 1992a); (Missale et al., 1998).

Adenylyl cyclase:

As early as the 1970s, it was recognized that DA receptors could influence the activity of AC (reviewed in (Missale et al., 1998). Endogenously or exogenously expressed D2 receptor couples negatively to AC in a PTX-sensitive manner. In most cases, the D2 receptor inhibits activated AC resulting in a decrease in cAMP production.

Meanwhile, in some other conditions the D2 receptor inhibits the basal activity of AC. It seems that inhibition of basal cAMP probably depends more on the basal level of cAMP than on cell type (Huff, 1996); (Albert, 1994); (Missale et al., 1998). Since the D2 receptor may interact with any subtype of Gαi/o protein, subtype specificity of signaling has been studied in a variety of cell lines (Huff, 1996); (Albert, 1994); (Robinson and Caron, 1997). In GH4C1 cells, activation of D2 receptor inhibits basal and Gs- or forskolin-stimulated AC (Liu et al., 1994b); (Senogles, 1994a). It has been shown that the different subunits of Gi/o proteins are involved in inhibition of stimulated AC, when it is stimulated by forskolin (direct activation of AC) or VIP (Gs-coupled) (Liu et al., 1994b; Senogles, 1994a). Contradictory results from these studies could be explained by the state-dependent activation of AC. It has also been shown that different Gαi/o subunits are involved in inhibition of AC in Balbc/3T3 cells (Ghahremani et al., 2000). In this regard we also found differences in Gαi subtype selectivity in coupling to inhibition of forskolin-stimulated AC (please see Chapter 2).

Calcium mobilization:

Activation of the dopamine D2 receptors in cells of mesenchymal origin induces an increase in $[Ca^{2+}]_i$. It has been shown that activation of D2S and D2L receptors in Ltk- (Liu et al., 1992a); (Vallar et al., 1990) and D2S in BALB/c-3T3 cells (Ghahremani et al., 2000) stimulate $[Ca^{2+}]$i mobilization in a PTX-sensitive manner. It was also shown that this activation goes through Gαo and Gβγ subunits in BALB/c-3T3 cells (Ghahremani et al., 2000). In CHO cell, both D2S and D2L activation increase $[Ca^{2+}]_i$ mobilization (Hayes et al., 1992). However, no effect of D2 activation on PI turnover was detected in

CHO and MN9D cells (Tang et al., 1994a); (Lajiness et al., 1993).On the other hand, when expressed in neuro-endocrine cells, activation of D2 receptor lowers $[Ca^{2+}]_i$ by decreasing inward calcium current in GH4C1 pituitary cells (Seabrook et al., 1994b); (Vallar et al., 1990), pituitary lactotrophs (Lledo et al., 1992) and NG108-15 cells (Seabrook et al., 1994c). This inhibition was mediated through Go subunits, since depletion of Gαo using different antisense approaches blocked D2-induced inhibition of calcium influx in lactotrophs and GH4C1 cells (Liu et al., 1994b); (Baertschi et al., 1992). In addition, D2 receptor in the pituitary have been shown to inhibit PI metabolism (Canonico et al., 1983); (Enjalbert et al., 1990). Inhibition of calcium current can go through two mechanisms: D2-like receptor-induced activation of potassium currents leading to alterations in membrane potential, and activation of G proteins that directly inhibit some calcium channels (reviewed in (Missale et al., 1998).

The dual effect of D2 receptor on calcium mobilization has been shown to be dependent on expression of a specific subtype of PLC. It has been shown that in Ltk- cells calcium mobilization induced by D2 receptor is dependant on the expression of PLC-β2 (Missale et al., 1998). Interestingly, in GH4C1 cells, which display an inhibition of $[Ca^{2+}]_i$ mobilization, this subtype is not expressed (Missale et al., 1998).

Potassium channels:

Dopamine receptors have been shown to influence the activity of potassium channels by increasing the outward currents leading to membrane hyperpolarization. This effect has been observed in primary culture from substantia nigra, striatal and mesencephalic neurons (reviewed in (Missale et al., 1998), anterior pituitary (Einhorn et

al., 1991), GH4C1 cells (Vallar et al., 1990), NG108-15 neuroblastoma cells (Castellano et al., 1993) and transfected MN9D mesencephalic cells (Tang et al., 1994b). This modulation of potassium currents appears to be mediated by PTX-sensitive G proteins (Liu et al., 1994a); (Liu et al., 1999a). Moreover, treatment of cells with G protein antibodies or antisense oligonucleotides blocks the D2 receptor stimulation of potassium currents. Stimulation of potassium currents appears to be mediated by Gαi3 in pituitary cells, where as in rat mesencephalic cultures, by Gαo (reviewed in (Missale et al., 1998).

Signal transduction pathways involved in mitogenesis:

It is suggested that D2 receptors may be involved in regulation of mitogenesis and cell differentiation. D2 receptors stimulate [^{3}H]-thymidine incorporation in CHO cells (Lajiness et al., 1993) and BALB/c-3T3 cells (Ghahremani et al., 2000). This effect is blocked by PTX and appears to be independent of changes in cAMP levels. Ghahremani et al., have also shown that activation of D2 receptor can cause cell transformation in BALB/c-3T3 cells (Ghahremani et al., 2000). On the other hand, the D2 receptor has also been shown to inhibit cell growth in some cell lines. D2 receptor activation in transfected GH4C1 cells causes a decrease in [^{3}H]-thymidine uptake which is blocked by PTX pretreatment and is accompanied by an increase in phosphotyrosine phosphatase (PTP) activity (Florio et al., 1992). In contrast, Senogles found that in GH4C1 cells transfected with D2S receptor, the effect of D2-dependent inhibition of [^{3}H]-thymidine incorporation was not abrogated with PTX but was blocked by down regulation of PKC and treatment with PKC inhibitors (Senogles, 1994b). So, although the mechanism of cell growth inhibition by D2 receptor in GH4C1 cells remains unclear, it may result from PKC-

mediated activation of a tyrosine phosphatase. The effect of D2 receptor activation on cell growth appears to highly depend on the cell type examined (Missale et al., 1998). Meanwhile, the D2 receptors may also have some role in cell differentiation (Missale et al., 1998).

Dopamine receptors in the pituitary:

In 1970s, using radioligand binding assays, it was shown for the first time that D2 receptors were present in the anterior and intermediate lobes of the pituitary gland (reviewed in (Missale et al., 1998). It has been shown recently that D2 receptors are relatively evenly distributed throughout the adenohypophysis with slightly higher expression in mature than in young rats (Piano and Pogacnik, 2001). The dopamine and especially the D2 receptor is well recognized as a regulator of prolactin (PRL) secretion. Prolactin secretion is under the inhibitory effect of hypothalamic DA, secreted from tuberoinfundibular neurons into the anterior pituitary. Some of the pathways involved in this inhibition have been elucidated. It has been shown that dopamine stimulation can inhibit both cAMP-dependent and cAMP-independent hormone secretion (Vallar et al., 1990). Furthermore, in pituitary cells activation of the D2 receptor initiates opening of K^+ channels and inhibits PI turnover which in turn decreases $[Ca^{2+}]_i$ (Vallar et al., 1990); (Huff, 1996); (Senogles, 1994b). Decreases in $[Ca^{2+}]_i$ is known to inhibit PRL secretion. The combination of D2 actions on these signaling systems in the pituitary provides a variety of mechanisms for D2-mediated inhibition of PRL secretion *in vivo*. Moreover, the activity of Pit1 POU transcription factor, which induces growth hormone (GH) and PRL gene expression (Ingraham et al., 1990), is blocked by activation of D2 receptors in

transfected cell lines (Lew and Elsholtz, 1995); (Elsholtz et al., 1991). This suggests the existence of dopaminergic control on PRL gene expression. The effect of D2 receptor activation in inhibiting PRL secretion of anterior pituitary gland has an important therapeutic application in the treatment of hyperprolactinemia either due to PRL-secreting tumors or functional hypothalamus-pituitary defects. Bromocriptin, a D2-receptor agonist, is one of the most effective pharmacological tools to normalize PRL level in these patients (Missale et al., 1998). Moreover, knockout mice of the dopamine transporter gene, which results in persistent extracellular hyperdopaminergic tone due to lack of reuptake mechanisms, have hypoprolactinemia (Gainetdinov et al., 1999). This indicates a strong negative regulation of PRL secretion by dopamine D2 receptor activation *in vivo*.

Other signaling systems:

The dopamine D2 receptor activates phospholipase-A2 in primary striatal and CHO cells, resulting in potentiation of arachidonic acid release. It appears that this effect is mediated by alteration in PKC activity and is calcium-dependent. This pathway is sensitive to PTX, suggesting that Gi/o proteins are involved (Missale et al., 1998); (Keefe and Gerfen, 1995); (Piomelli et al., 1991); (Di Marzo et al., 1993).

D2 receptor activation also increases extracellular acidification by activation of the amiloride-sensitive Na^+/H^+ exchanger in C6, Ltk-, CHO, and anterior pituitary primary cell cultures (Chio et al., 1994a); (Ganz et al., 1990); (Neve et al., 1992). Extracellular acidification is one of the earliest events triggered by many growth factors (Missale et al., 1998).

Regulatory mechanisms:

Desensitization and regulation of signal transduction of dopamine D2 receptors takes place at the level of both receptor and G protein. At the receptor level, similar to other GPCRs, the D2 receptor displays three stages of desensitization: uncoupling, sequestration and down-regulation. Activation of the receptor by agonist initiates the uncoupling process, which is primarily mediated by two classes of protein kinases. The second messenger-dependent class of kinases mediate agonist-dependent or agonist-independent phosphorylation of the receptor and include PKA (activated by cAMP) and PKC (activated by calcium and diacylglycerol (DAG)). A unique class of serine/threonine protein kinases, known as G protein receptor kinases (GRKs), mediate agonist-dependant phosphorylation (Bohm et al., 1997); (Carman and Benovic, 1998); (Ferguson, 2001). GRKs are either located at the plasma membrane (GRK1 and GRK5), or translocated to the cell surface by binding to free Gβγ subunits (GRK2 and 3) or by C-terminal palmitoylation (GRK4 and 6). Arrestin then binds to hyperphosphorylated sites on the receptor and interrupts coupling to G proteins (Carman and Benovic, 1998); (Ferguson, 2001); (Penn et al., 2000). Uncoupling takes place within a few minutes of receptor activation. Sequestration is the next step, in which the receptors are endocytosed into clathrin-coated pits (Ferguson, 2001); (Gagnon et al., 1998). After this step the receptor can be recycled back to membrane or become down-regulated, i.e. the final stage of the desensitization. It is suggested that internalization of GPCRs contribute to facilitating GPCR activation of the MAPK pathway (Gagnon et al., 1998); (Pierce et al., 2000). Following continuous agonist activation (hours to days), the receptor moves onto

the final step of down-regulation. In this stage, the receptor-containing endosomes are believed to fuse with lysosomes, where the receptor is destroyed (Carman and Benovic, 1998). To reach sustained down-regulation, gene expression is down-regulated to further lower the production of new receptors (Bohm et al., 1997). Meanwhile, GPCR signaling can be desensitized through inactivation of G proteins by GTPase activating proteins such as the regulators of G protein signaling (RGSs) (Cowan et al., 2001).

Guanine nucleotide binding proteins (G proteins):

It has been known for many years that the minimum requirement to send many kinds of signals across the membrane of cells is a receptor, an effector and an intermediate signal transducer (Bourne, 1997a); (Gilman, 1987). Many extracellular stimuli such as hormones, neurotransmitters, chemokines and diverse olfactory or gustatory stimuli mediate their cellular actions through receptors that are coupled to guanine nucleotide binding proteins or G proteins (Gilman, 1987); (Birnbaumer et al., 1990). Heterotrimeric G proteins transduce the signal from these receptors into intracellular effectors which mediate the physiological responses of the cell.

Sutherland et al., reported the first receptor-sensitive signal transduction system in 1956 (reviewed in (Gilman, 1987)). In 1960s, Rodbell and Birnbaumer suggested that the hormone-sensitive adenylyl cyclase must be heterotrimeric complexes consisting of receptor molecules, which are independent from AC molecules (Birnbaumer and Rodbell, 1969); (Rodbell et al., 1970). They found that five different hormones could stimulate a single AC. In this way they demonstrated a complex and poorly understood model for

hormone-mediated signal transduction. They introduced the terms "discriminator" for the receptor, "amplifier" for the enzyme producing cyclic adenosine monophosphate (cAMP) and "transducer" for the role of the intervening process. Meanwhile, it was reported that GTP could also regulate the stimulation of AC (Rodbell et al., 1971b); (Rodbell et al., 1971a). Krishna et al., explained that GTP was not part of the receptor (PGE1 in that case) or the effector (AC), but it was the "transducer" that was influenced by GTP (Krishna and Harwood, 1972). Later reports of GTP modulation of different receptors showed that the role of GTP was not restricted to one type of receptor (Maguire et al., 1976); (Lefkowitz et al., 1976); (Wheeler and Bitensky, 1977); (Ross and Gilman, 1977). Finally, Ross and Gilman isolated an approximately 40-kDa protein with the characteristics of GTP binding protein (Ross and Gilman, 1977). These GTP-binding proteins were called N or G/F proteins at first. Later it was established that G proteins act at two regulatory pathways, i.e. stimulatory (Gs) and inhibitory (Gi) (Birnbaumer, 1990). Gt or transducin was the first G protein reported as heterodimer that was purified from retinal rod cells (Godchaux and Zimmerman, 1979). One year later, it was shown that transducin was in heterotrimeric form, and the subunits were named α, β and γ. At present, based on information obtained from the human genome, 27 α subunits, 5 β subunits and 13 γ subunits have been identified (Venter et al., 2001). Cloning of different family members of G protein α, β and γ subunits disclosed a vast and complex area in signal transduction. Random association of these subunits would generate hundreds of different heterotrimeric proteins. It appears, however, that there are preferred combinations of isoforms that interact to form a more limited number of distinct complexes (Hamm and Gilchrist, 1996). This is partly because of the three-dimensional

structure of the subunits (Lee et al., 1995); (Sondek et al., 1996). Some of the subunits are expressed ubiquitously, where as others have differential, spatial, and/or temporal expression patterns (Hamm and Gilchrist, 1996). Introduction of PTX as a blocker of the inhibitory G proteins, became a valuable tool to study these proteins. PTX was referred to as an islet-activating protein, a blocker of the inhibitory pathway of insulin regulation (Katada and Ui, 1982).

G protein classification:

After the identification of G proteins, this group of proteins was classified based on the functional coupling of the α subunit to AC. Those that stimulated AC were named the Gs family and those that inhibited AC were called the Gi family. The other two groups are Gq, which activates phospholipase C, and the G12/G13 family.

Structural composition:

G proteins are heterotrimeric proteins composed of α, β and γ subunits. G proteins have been classified by virtue of their α subunit. However, later studies showed that both α and βγ subunits participate in a variety of signaling pathways to provide a very dynamic cross-talk between G protein and other signaling pathways to cause well-regulated cellular signal transduction.

α Subunit:

More than 20 distinct α subunits encoded by 17 different genes are known. Based on their sequence homology, they can be divided into four families: the Gs family consists of αs and αolf and their splice variants; Gi family members include αoA, αoB,

αi1, αi2, αi3, αt1, αt2 and αZ; Gq family members are Gαq, α11, α14, α15 and α16; and G12 family is composed of α12 and α13 (Simon et al., 1991).

The Gα subunits are a family of proteins with molecular weights ranging from 39-52 KDa and 45-80% homology to one another (Rens-Domiano and Hamm, 1995). Realizing the crystal structure of Gαt and Gαi1 (Sondek et al., 1994); (Coleman et al., 1994) provided a new dimension in the knowledge of the structure of all Gα subunits. The Gα subunit contains two domains: a GTPase domain that consists of five α helices surrounding a six-stranded β sheet. This domain contains a guanine nucleotide binding domain (that binds to inactive GDP), the Mg^{2+}-binding domain, and the sites for binding to the receptor, downstream effectors and βγ subunits. The other domain of the α subunit is α helical, its function is not completely known, but it seems to act as a "lid" which buries the guanine nucleotide deep between itself and the GTPase domain (Hamm and Gilchrist, 1996).

G protein activation by receptors leads to GTP binding at the Gα subunit. The GTP-mediated switch of the α subunit is a conformational change of three flexible regions designated as switch domains I, II and III. Mutations in the switch regions lower the affinity of Gα for Gβγ and result in dissociation of the trimeric complex (Hamm, 1998).

<u>βγ subunits:</u>

The βγ subunit consists of two polypeptides that can be dissociated only under denaturing conditions. However, it is functionally and biologically a monomer. It serves to increase the affinity of Gα subunit for its receptor and to regulate many of the effectors directly or indirectly. It is also involved in recruitment of GRKs to the membrane (Neer,

1995). At the amino acid level, the five Gβ subunits are highly conserved (sharing 50-83% identity). The approximately 36 KDa Gβ subunit is predicted to contain two types of structures: an amino-terminal region thought to form an amphipathic α helix, followed by seven repeating β sheets of approximately 43 aa each. These repeating units are examples of WD repeats, a motif found in a variety of proteins (Hamm and Gilchrist, 1996).

In contrast to Gβ subunit, the Gγ subunits are more divergent, sharing only 27-75% homology, and because of this, have been suggested to determine the functional specificity of the Gβγ complexes. The Gγ subunits are small proteins of 6-9 kDa (Kalman et al., 1995). The Gγ subunit makes virtually no contact with itself but is entirely interdigitated with the Gβ subunit via a coiled-coil interaction, which explains the difficulty in dissociating the two subunits from each other (Clapham and Neer, 1997).

The Gγ subunit contains a CAAX motif in its C-terminal that targets the protein for isoprenylation (Yamane and Fung, 1993). If isoprenylation of the Gγ subunit is blocked, the Gβγ dimer will form, but the complex will not bind properly to either the lipid membrane or the Gα subunit, nor will it efficiently signal the effector molecules (Higgins and Casey, 1994).

αβγ trimeric complex:

The trimeric complex has three major contacts with the lipid bilayer: the C-terminal part of Gα, which is known to interact with the GPCR; the palmitoylated and/or myristoylated N-terminal of Gα; and isoprenylated C-terminal of Gγ (Lambright et al., 1996). By using a series of point mutations or combinatorial peptides targeted to the C-

terminal of Gα, it is shown that C-terminal region and especially the last five residues is the most important part in determining the specificity of G protein-receptor binding (Sprang, 1997). Further analysis has indicated that the C-terminal domain of i3 loop of receptor binds to this region on C-terminal of Gα (Kostenis et al., 1997). Meanwhile, Gβ subunit enhances the receptor interaction with Gα (Clapham and Neer, 1997). Moreover, it is known that the βγ combination contributes to the G protein-receptor coupling specificity. For example, it has been shown that the β1γ1 dimer supports binding of Gαt to rhodopsin, however the β1γ2 is ineffective (Kisselev et al., 1995).

G protein coupling:

Coupling to G protein coupled receptor (GPCR):

By complete sequencing of the human genome, it is believed that approximately one thousand genes encode serpentine receptors or GPCRs. GPCRs have seven transmembrane (TM) spanning domains with 3 intracellular loops, an extracellular N-terminal and an intracellular C-terminal tail. These receptors can transmit signals specific for each extracellular stimulus across the membrane lipid bilayer by selectively activating different G proteins. Palczewiski et al., reported the first 3D structure of a GPCR at 2.8Á resolution (Palczewski et al., 2000). They also observed that a short stretch of hydrophobic amino acids of the C-terminal of rhodopsin forms an 8^{th} α-helix that runs parallel to the cytoplasmic surface.

These receptors share similarities in their major structural features; however, variations in agonist binding/activation sites and G protein coupling domains provide for a diversity of function in different cellular environments.

Ligand binding induces changes in the relative orientations of TM 3 and 6. These changes are thought to alter the conformation of G protein-interacting intracellular loops of the receptor, which uncovers the previously masked G protein-binding site (Hamm, 1998). In the unstimulated state, a GDP molecule is bound to the α subunit of the G protein trimeric complex. In the GDP-bound state, the α subunit is attached to the βγ subunits and the trimeric complex is in contact with the receptor. Upon binding of the ligand to the receptor, the conformational change in the receptor dictates a structural change in the α subunit. In the GTP-bound state, the α subunit is thought to dissociate from the βγ subunits. The activated state lasts until the GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α subunit. When it is bound to GDP, the G protein returns to its inactive, βγ-bound form. The dissociation of G protein complex initiates the signaling pathway by coupling to corresponding effectors.

Coupling to the effector:

At the beginning, G proteins were categorized based on the functional coupling of their α subunits to AC. Gs (stimulatory to AC) and Gi (inhibitory to AC), that increase and decrease cAMP, respectively. Following the molecular cloning of different family members and their biochemical and functional characteristics, the classification as "stimulatory" or "inhibitory" were superceded by classification based on the distinct effector coupling of each α or βγ subunits. Until the mid-1990's the α subunit of G proteins was considered the primary signaling component, however, in the past few years researchers have started to uncover the function of βγ subunits and have provided new evidence regarding the importance of βγ signaling.

α subunit:

Gs family: Stimulation of AC through GPCRs involves G proteins of the Gs family with two known main members, Gs and Golf. The main difference in these two members is in their localization. While Gαs is ubiquitously expressed, Golf is mainly located in olfactory sensory neurons and specific parts of brain including the striatum (Zhuang et al., 2000). Activation of AC causes increases in cAMP, which acts as a second messenger and activates PKA. The activation of Gαs can also be induced by cholera toxin (CTX) treatment (Neer, 1995). CTX treatment ADP-ribosylates Gαs subunits and constitutively activates Gαs. To date nine subtypes of AC have been identified and it seems that Gαs activates all of them with minor differences in efficiency (Krupinski and Cali, 1998). Gαs also activates voltage gated Ca^{2+} channels (Hamilton et al., 1991); (Yatani et al., 1987). Complete loss of Gαs in mice homozygous for an inactivating Gαs mutation leads to embryonic lethality (Yu et al., 1998). Heterozygote deletion of Gαs gene causes parent of origin specific defects (Yu et al., 2000).

Gq family: Gαq protein couples to phospholipase C-β (PLC-β) isoforms (Exton, 1997). Activated PLC-β hydrolyzes phosphatidyl inositol (4,5) bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 releases Ca^{2+} from intracellular pools, which initiates numerous calcium-dependent signaling cascades, while DAG along with $[Ca^{2+}]_i$, activates PKC. Gαq is expressed ubiquitously and the mice resulting from homozygous inactivating mutations are ataxic and have defects in platelet activation (Offermanns et al., 1997b); (Offermanns et al., 1997a).

Gi/o family: The α subunit of Gi/o family consists mainly of five subtypes forming two subfamilies, i.e. Gαo and Gαi. All of them are PTX sensitive, which means PTX treatment provides a powerful tool to differentiate the role of Gi/o family from other

G proteins. PTX ADP-ribosylates all members in the Gi/o family with the sole exception of Gz (Ho and Wong, 2001). Gαi1&3 are expressed widely and homozygous Gαi1- or Gαi3-deficient mice have no obvious phenotype (Offermanns, 2001). Gαi2 can be found ubiquitously and homozygous inactivation of this gene in mice causes inflammatory bowel disease and mild platelet activation defect (Offermanns, 2001). Gαo is highly expressed in neuronal and neuroendocrine cells and Gαo knockout mice showed no gross morphological abnormalities but have functional CNS defects (Valenzuela et al., 1997); (Jiang et al., 1998).

The K^+ current induced by acetylcholine was one of the first ion channels reported to be regulated by a G protein, which was identified as a Gi/o protein, since the activation of inwardly-rectifying K^+ channels was PTX-sensitive (Breitwieser and Szabo, 1985) (Pfaffinger et al., 1985). Gαi can also activate ATP-sensitive inward rectifying K^+ channels (Wickman and Clapham, 1995). It has been shown that Gαi1, 2, and 3 mimic the GTPγS effect on this channel whereas Gαo and Gs do not (Kirsch et al., 1990). Although there is various amounts of Gi selectivity for coupling to G protein-activated inwardly rectifying potassium channels (GIRK) depending on cell type and different GPCR involved, it seems the regulation of GIRK occurs via direct interaction of Gβγ subunits with channel subunits (Wickman and Clapham, 1995); (Krapivinsky et al., 1995). The Gi proteins regulate Ca^{2+} channels through fast (membrane-delimited) and slow (second messenger-dependent) mechanisms (Hescheler and Schultz, 1994); (Hille, 1994). Using different techniques including antisense oligonucleotides or antibodies to α subunits, Gαo specificity has been shown to mediate inhibition of L- and N-type Ca^{2+} channels following activation of multiple receptors in neurons (Campbell et al., 1993);

(Harris-Warrick et al., 1988). Similar results were found in GH3 and GH4 pituitary cells (Liu et al., 1994b). However, despite the crucial role for the Gαo subunit to connect receptors and N-type channel, it is the Gβγ dimer that interacts directly with the N-type channel α_{1B} subunit to inhibit channel opening. It is also shown that although multiple Gβγ have the potential to couple to the N-type channels, the endogenous receptor-G protein-Ca channel coupling has a preferred combination of Gαβγ (reviewed in (Albert and Robillard, 2002). But the mechanism by which Go regulates L-type channels is not yet well understood.

Gi/o proteins were originally known for their inhibitory effect on AC. In most systems, the inhibition of AC is mainly mediated by Gi subfamilies, compared to Go (Ghahremani et al., 2000). Measuring G protein specificity to AC is complicated by the presence of different subtypes of AC (Albert et al., 1999); (Ghahremani et al., 1999). The specificity of Gαi subunits is depending on the type of receptor activated and the type of AC present, as well as the type of activation of AC. For example, Taussig et al., have shown that Gαi1 inhibits AC V more effectively than AC I (Taussig et al., 1993). It has also been reported that forskolin activates AC I more effectively than AC II, AC V, or AC VI; whereas Gαs preferentially activates AC II compared to others (Sutkowski et al., 1994).

Gi/o coupled receptors have been implicated in regulation of growth related processes including mitogen activating protein kinase (MAPK) activation, DNA synthesis and cell proliferation. These go through several different mechanisms including the roles of receptor and non-receptor tyrosine kinases (Pierce et al., 2001). Mochizuki et al., have shown that an isoform of Rap1 GTPase-activating protein (Rap1GAP), binds

specifically and directly to the α subunit of Gi proteins (especially the constitutively active form) (Mochizuki et al., 1999). Meanwhile, it is reported that Gαo in its resting state can sequester Rap1GAP thereby regulating Rap1 activity and stimulate MAPK activity. Thus, activation of Go could modulate the Rap1 effects (Jordan et al., 1999). Although there is not much information about direct interactions of α subunit with effectors related to growth signaling pathways, the role of βγ subunit has been widely studied (please see the following section).

βγ Subunits:

GPCR activation causes dissociation of α subunit from βγ subunit. It was thought, for a while, that it was simply the Gα subunit that could interact with effectors and βγ subunits could only regulate the Gα subunit signaling. Logothetis et al., showed the first clear evidence that Gβγ could regulate effectors (Logothetis et al., 1987). It has now been shown that Gβγ can directly interact and regulate a wide range of effectors. Extensive research has been done to identify and define the specificity of effectors for different combinations of Gβγ. Using nuclear injection of antisense oligonucleotides in GH3 cells, it is shown that different Gβγ pairs specifically couple to different Gαo isoforms in order to mediate muscarinic and somatostatin receptor-induced inhibition of Ca^{2+} channels (Kleuss et al., 1992). The conformation of free Gβγ does not change after dissociation from αβγ trimeric complex and Gα turns off the ability of Gβγ to activate effectors (Clapham and Neer, 1997). These observations propose that the Gα binding site of the Gβγ subunit overlaps with the effector-coupling site. It is also known that the C-terminal of GRK2, that contains the pleckstrin homology domain (PH) which is thought

to bind to βγ in order to translocate GRK to the membrane, completely blocks βγ coupling to a variety of effectors. This also suggests that the PH domain interaction site of Gβγ overlaps with the effector-binding site (Clapham and Neer, 1997); (Lohse et al., 1996); (Ford et al., 1998).

Gβγ directly activates several ion channels. Gβγ can directly activate a K^+ - selective ion channel (I KACh) (Logothetis et al., 1987), and GIRK (Kunkel and Peralta, 1995); (Krapivinsky et al., 1995). Gβγ subunits also mediate receptor-induced inhibition of calcium channels (De Waard et al., 1997); (Zamponi et al., 1997). This coupling has some level of specificity. For example, by using intranuclear injections with various Gβ1-5 γ3 combinations, a Gβ1/2γ3 preference is identified for coupling to N-type calcium current to enhance prepulse facilitation following α2-adrenergic stimulation, compared to Gβ3-5γ3 that coupled weakly (Garcia et al., 1998). Difference in specificity could be explained by different levels of expression that could lead to insufficient Gβγ for coupling. The transfected Gβγ subunits did not appear to displace endogenous Gβγ to mediate signaling pathways (Ruiz-Velasco and Ikeda, 2000).

Gβγ subunit is also involved in phospholipase C activation. In some cases, GPCR-induced PI turnover can be blocked by PTX, indicating that both PTX-sensitive (Gi/o) and PTX-insensitive (Gq) G proteins participate in this action. Unlike Gq-induced activation of PLC-β, the Gi/o-induced increase in PI turnover is not mediated by the Gα subunit but through Gβγ (Clapham and Neer, 1997). For example, Ghahremani et al., have shown that increase in $[Ca^{2+}]_i$ through activation of dopamine-D2S receptors goes through the βγ subunit of Gi/o proteins (Ghahremani et al., 2000).

At least eight out of the nine isoforms of AC are differentially regulated by Gβγ subunits or by second messenger systems initiated by Gβγ (like Ca^{2+} and phosphorylation) (Sunahara et al., 1996); (Simonds, 1999). AC I, which is mainly expressed in neuronal cells, can be inhibited by Gβγ; however this inhibition is not due to sequestration of Gαs by βγ subunit but rather appears to be mediated by an independent binding site for Gβγ on AC (Taussig and Gilman, 1995). On the other hand Gβγ can stimulate ACII and ACIV when Gαs is activated simultaneously (Simonds, 1999).

Meanwhile, Gβγ subunit is highly involved in GPCR-induced regulation of cell growth and proliferation, that I will explain in following section.

G proteins, cell growth and proliferation:

Growth and cell proliferation are regulated by a complex network of many intracellular and extracellular signals which integrate diverse signaling pathways. The outcome of this network directs the cell toward proliferation, differentiation or cell death. In order to understand cell proliferation, one should identify the pathways that convey extracellular signals to nuclei where regulation of differentiation program and/or induction of cell cycle progression genes are executed. Among the pathways often involved in this regard are mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein kinase (ERK) cascade. These cascades consist of series of kinases that include a MAPK, which is activated, by a MAPK/ERK kinase (MEK), which in turn is activated by a MEK kinase (MEKK). At least six MAPK pathways have been identified, the ERK1/2, the c-jun N-terminal/stress activating protein kinase (JNK/SAPK), p38, p38-like, ERK3 and ERK5 pathways (Robinson and Cobb, 1997).

The ERK1/2 pathway is the first identified and the best-characterized cascade in this family.

It has not been long since it has been shown that besides classical growth factors like epidermal growth factor (EGF), GPCR activation also plays a role in differentiation, proliferation and even cellular transformation (Dhanasekaran et al., 1995); (Gutkind, 1998). Although the role of ERK/MAPK pathway in controlling cell growth and transformation was originally described in non-neuronal cells, the role of ERKs in the regulation of neuronal function (cell death, differentiation and synaptic plasticity) has received increased attention recently (Gutkind, 1998). Activated ERKs have many different potential targets. These range from transcription factors in nucleus (like Elk1 and c-Myc) to membrane-associated proteins (like EGF receptor and phospholipase A2) or cytoskeletal proteins such as neurofilaments (reviewed in (Gutkind, 1998).

Presently, it is well established that most of the GPCRs that initiate a mitogenic response induce activation of ERK1/2 pathway (van Biesen et al., 1996); (Gutkind, 1998). Both Gi-coupled (PTX-sensitive) and Gq-coupled (PTX-insensitive) receptors have been reported to mediate this effect (reviewed in (Gutkind, 1998). The mechanisms of activation of MAPK cascade by GPCR appear to be dependent to cell type, GPCR type and G proteins involved (Rozengurt, 1998). It was shown that Gβγ-dimers couple GPCR to the stimulation of ERK1/2 pathway. Gβγ-dimers stimulate MAPK in situations that constitutively active forms of Gα-subunits do not stimulate ERK2, and under conditions that active forms of Gαs and Gαq, but not Gαi, stimulate ERK1. It is shown that activation of ERK1/2 required a functional Gβγ-dimer and β-subunit or γ-subunit alone do not stimulate ERK1/2 (reviewed in (Schwindinger and Robishaw, 2001). Meanwhile,

inhibition of Gβγ subunit by Gαt (Crespo et al., 1994), Gαo (Ito et al., 1995) or a carboxyl terminal fragment of β-adrenergic receptor kinase (βARK-CT) (Koch et al., 1994) inhibits ERK1/2 stimulation. A few effectors have been proposed for the Gβγ subunit to initiate MAPK activation: PI3K could be activated by Gβγ and then increase the activity of Src family tyrosine kinases (Bondeva et al., 1998). Gβγ may trans-activate receptor or non-receptor tyrosine kinases directly or indirectly (Della Rocca et al., 1999); (Prenzel et al., 1999); (Pierce et al., 2001). Alternately, Gβγ may activate PLCβ which induces PKC- or calcium-dependent MAPK activation (reviewed in (Schwindinger and Robishaw, 2001).

Although the activation of ERK1/2 by GPCRs has been extensively examined, there are few studies regarding G protein-mediated inactivation of MAP kinases, its importance and its signaling pathway.

GH rat pituitary cells: models to study signal transduction

Tashjian et al., was the first group to isolate GH cells from pituitary tumors of rats that had been X-irradiated (Tashjian et al., 1968). Based on growth hormone (GH) secretion, the cell line was originally named GH cells, but later it was identified that these cells also secrete prolactin (PRL), and several associated substances. Since PRL and GH are produced and secreted by the same individual cells, GH cell tumors are suggested to have arisen from transformation of somatomamotrophs, dual-secreting pituitary cells that predominate in the developing pituitary gland (Frawley and Boockfor, 1991). GH4C1 cells were originally subcloned and selected from GH3 cells for high PRL/ low GH production. But it seems that they now secrete in similar levels to those of parental GH3

cells. GH cells differ from normal pituitary cells in several ways. Unlike normal pituitary cells, GH cells are transformed and cause tumors when injected into animals (Tashjian et al., 1968). GH cells have smaller hormone storage and higher basal level hormone secretion, which is probably due to lack of negative regulation, since, unlike normal lactotrophs, GH cells lack inhibitory dopamine-D2 receptors (Tashjian, 1979). Finally, GH cells lack the cyclooxygenase enzyme and do not metabolize arachidonic acid to prostaglandin, but lactotrophs do (Osborne and Tashjian, 1981).

In GH4C1 cells, secretion rate of PRL and GH is increased by releasing hormones such as thyrotropin releasing hormone (TRH) or vasoactive intestinal peptide (VIP) and is decreased by inhibitory hormones such as somatostatin (reviewed in (Albert, 1994). Moreover, several of these hormones can regulate the synthesis and gene transcription of PRL and GH (Tashjian, 1979); (Murdoch et al., 1985); (Farrow and Gutierrez-Hartmann, 1999). GH cells express a variety of receptors and provide a model to study receptor-induced signal transduction mechanisms. Gq-coupled (like TRH, bombesin), Gi/o-coupled (somatostatin, muscarinic, adenosine-A1), and Gs-coupled (VIP, PGE2) receptor families are expressed in GH cells (reviewed in (Albert, 1994). Paulssen et al have shown that GH cells contain various amounts of mRNAs and protein for Gαs, Gαi2, Gαi3, Gαo and Gαz subunits (Paulssen et al., 1991). A low level of Gαi1 expression is reported. GH cells also contain a large number of voltage- and ion-gated channels, and a proportion of cells fire spontaneous action potentials. Evidence from ion flux experiments and electrophysiological studies have demonstrated the presence of different kinds of sodium, potassium and calcium channels in these cells, and the actions of receptor activation on them (Koch and Schonbrunn, 1988).

To study detailed signal transduction pathways of D2S receptors, GH cells were stably transfected by dopamine-D2S receptor, a receptor absent in GH cells but normally present in lactotrophs, to produce GH4ZR7 cell line (Albert et al., 1990b). Dopamine is the primary regulator of PRL secretion and synthesis in lactotrophs *in vivo*. In GH4ZR7 cells, it has been shown that activation of the transfected dopamine D2S receptor inhibited both basal and VIP-stimulated cAMP accumulation, and also inhibited TRH- and VIP-stimulated PRL secretion (Albert et al., 1990a). D2R stimulation caused membrane hyperpolarization and decreased basal $[Ca^{2+}]_i$ in these cells (Vallar et al., 1990). Dopamine also causes inhibition of gene transcription and cell proliferation (Ohmichi et al., 1994a); (Senogles, 1994b). Based on this knowledge, this cell system provides an excellent model to study the specificity of PTX-sensitive G proteins in coupling of D2S receptors to different effectors.

Hypothesis and Approach:

Hypothesis:

Based on this knowledge, we hypothesized that in GH4ZR7 cells (a neuro-endocrine cell line derived from rat pituitary tumor GH4C1 cells) the D2S receptor utilize different Gα and Gβγ subunits to couple to various effectors to inhibit MAPK/ERK pathway, calcium mobilization and adenylyl cyclase inhibition.

Approach:

To assess the role of inhibitory G protein subunits (Gαi2, Gαi3, Gαo and Gβγ) in D2S-induced signaling in GH4ZR7 cells, we used the strategy of PTX-insensitive mutant G proteins. Stable expression of PTX-insensitive mutants of Gαi/o subtypes (Gαo, Gαi2 and Gαi3) individually in GH4ZR7 cells and blocking the endogenous Gi/o protein by PTX treatment will provide a series of cell line to evaluate the dopamine D2S receptor signaling pathway. Furthermore, we have created GRK-CT expressing GH4ZR7 cells, which sequester Gβγ, in order to examine the role of this subunit in several different signaling pathways.

CHAPTER II

Results (Manuscript)

Dopamine-D2S receptor inhibition of calcium influx, adenylyl cyclase and mitogen-activated protein kinase in pituitary cells: distinct Gα and Gβγ requirements.

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Running title: G protein selectivity of dopamine-D2S signaling

The G protein specificity of multiple signaling pathways of the dopamine-D2S (short form) receptor was investigated in GH4ZR7 lactotroph cells. Activation of the dopamine-D2S receptor inhibited forskolin-induced cAMP production, reduced BayK8644-activated calcium influx, and blocked thyrotropin-releasing hormone (TRH)-mediated p42/p44 mitogen-activated protein kinase (MAPK) phosphorylation. These actions were blocked by pretreatment with pertussis toxin (PTX), indicating mediation by $G_{i/o}$ proteins. D2S stimulation also decreased TRH-induced MAPK/ERK kinase (MEK1/2) phosphorylation. TRH induced c-Raf but not B-Raf activation and the D2S receptor inhibited both TRH-induced c-Raf and basal B-Raf kinase activity. Following PTX treatment, D2S receptor signaling was rescued in cells stably transfected with individual PTX-insensitive Gα mutants. Inhibition of adenylyl cyclase was partly rescued by $G\alpha_i2$ or $G\alpha_i3$, but $G\alpha_o$ alone completely reconstituted D2S-mediated inhibition of BayK8644-induced L-type calcium channel activation. $G\alpha_o$ and $G\alpha_i3$ were the main components involved in D2S-mediated p42/44 MAPK inhibition. In cells transfected with the carboxyl-terminal domain of G-protein receptor kinase to inhibit Gβγ signaling, only D2S-mediated inhibition of calcium influx was blocked, but not inhibition of AC or MAPK. These results indicate that the dopamine-D2S receptor couples to distinct $G_{i/o}$ proteins depending on the pathway addressed, and suggest a novel $G\alpha_i3/G\alpha_o$-dependent inhibition of MAPK mediated by c-Raf and B-Raf-dependent inhibition of MEK1/2.

INTRODUCTION

G protein-coupled receptors comprise a large superfamily of receptors that is critical for signaling of a diverse group of ligands to heterotrimeric G proteins (1). Activation of these receptors results in dissociation of Gα and Gβγ subunits, which couple to different effectors in the cell. Both the Gα and Gβγ subunits are capable of transferring receptor signals to effectors. The inhibitory G_i and G_o proteins ($G_{i/o}$ proteins) couple to adenylyl cyclase (AC) and inhibit production of cyclic AMP (cAMP) (2, 3). Pertussis toxin (PTX) selectively blocks $G_{i/o}$ proteins by ADP-ribosylating the $G\alpha_i/G\alpha_o$ subunit (4). Different receptors utilize specific combinations of G protein subunits to elicit distinct responses in different cells (5-7). Understanding the roles of individual G proteins to couple receptors to distinct signaling pathways remains one of the central issues in receptor research (8).

The dopamine D2 receptor belongs to the $G_{i/o}$-coupled family of receptors and inhibits pituitary cell proliferation, transformation, and hormone production, and is implicated neurobiological control of movement and behaviour (9). The D2 receptor contains an alternately spliced exon encoding 29 amino acids, to generate short (D2S) and long (D2L) forms of the receptor that are pharmacologically and functionally similar. By coupling to PTX-sensitive $G_{i/o}$ proteins, the D2S receptor mediates inhibitory or stimulatory cellular responses, depending on the cell type (10, 11). In mesenchymal cells such as BALB/c-3T3, Chinese hamster ovary or C6 glioma cells, the D2 receptor stimulates phospholipase C activity to induce calcium mobilization and activates the mitogen-activated protein kinase (MAPK) cascade. These actions correlate with

enhanced gene transcription, cell proliferation and oncogenic transformation (6, 12-14). In lactotrophs, D2S receptor activation opens potassium channels to hyperpolarize the cell membrane, blocks dihydropyridine-sensitive L-type calcium channels, inhibits cAMP production, and inhibits MAP kinase activation. These inhibitory actions correlate with D2-mediated inhibition of hormone secretion, gene transcription and cell proliferation in lactotrophs (15-20). In light of the cell type-specific coupling of D2S receptors, we hypothesize that different subunits of Gi/o proteins may mediate distinct D2S receptor signaling pathways.

As a model of D2 receptor signaling we have used rat pituitary GH4C1 cells stably-transfected with the D2S receptor (GH4ZR7 clone), a receptor absent in GH4C1 cells but present in normal lactotrophs (10, 15). GH4ZR7 cells express all three known $G\alpha_i$ subunits and two types of $G\alpha_o$, as well as various effectors that are regulated by PTX-sensitive G proteins (10, 17, 21). Thus this cell system provides an excellent model to study the specificity of PTX-sensitive G proteins in coupling of D2S receptors to effectors. To address the contribution of specific $G\alpha$ subunits in D2S signaling pathway, PTX-insensitive mutants of $G\alpha_i2$, $G\alpha_i3$ and $G\alpha_o$, in which the ribosyl acceptor cysteine in carboxyl-terminus region was changed to a non-accepting serine, were stably transfected into GH4ZR7 cells. The Cys to Ser mutation is a structurally conservative change, and the mutant G proteins remain functional following PTX pretreatment (6, 7, 22). The role of $G\beta\gamma$ subunits in D2S signaling was evaluated by using carboxyl terminus of G protein coupled receptor kinase (GRK-ct) as a selective $G\beta\gamma$ scavenger (23). In this study, we focused on the role of $G\alpha_i2$, $G\alpha_i3$, $G\alpha_o$ and $G\beta\gamma$ subunits in inhibition of AC, L-type calcium channels and MAPK pathway in GH4ZR7 cells. Our

results indicate that each of these D2S-induced pathways has distinct requirements for different Gα or Gβγ subunits.

RESULTS

Expression of PTX-insensitive $G\alpha_{i/o}$ subtypes and GRK-ct in GH4ZR7 cells. $G\alpha_i2$ and $G\alpha_i3$ PTX-insensitive mutants were Flag-tagged in N-terminal region and these mutants, $G\alpha_o$-PTX, and GRK-ct cDNA were transfected individually in GH4ZR7 cells. Cell extracts from stably transfected clones and wild-type GH4ZR7 cells were analyzed for G protein expression by Western blot analysis (Fig. 1). Expression of $G\alpha_i2$-PTX and $G\alpha_i3$-PTX was confirmed by using anti-Flag antibody. The Gi2Z23 clone displayed a 2.9-fold increase in $G\alpha_i2$ immunoreactivity versus GH4ZR7 cells (Fig. 1A). Since $G\alpha_i3$-specific antibodies were not available, anti-Flag staining revealed that $G\alpha_i3$ was expressed in two clones at levels similar to the $G\alpha_i2$ clone (Fig. 1B). Staining with anti-$G\alpha_o$ antibody indicated that the GαoZ7 and GαoZ15 clones expressed $G\alpha_o$ approximately 3.1- and 1.9-fold, respectively, compared to GH4ZR7 cells (Fig. 1C). Thus the level of mutant $G\alpha_{i\text{-}o}$ proteins was approximately equivalent to that of endogenous $G\alpha_i2$ or $G\alpha_o$. The expression of GRK-ct was confirmed using an antibody directed against full-length GRK2 (24). GRK2 was detected at the expected molecular size (70 kDa) in all cell lines, whereas GRK-ct was detected at 28 kDa only in the transfected clones. Expression of GRK-ct was about 40% and 50% of the GRK2 level for GRKZ16 and GRKZ17 clones, respectively (Fig. 1D).

D2S-induced inhibition of forskolin-stimulated AC via $G\alpha_{i}2$

Under the present experimental conditions, basal cAMP level in GH4ZR7 was very low and D2S receptor activation did not alter basal cAMP production (Appendix 1). Forskolin (1 μM) increased cAMP levels by 10-fold and this was almost completely blocked by treatment with the D2 receptor agonist apomorphine (1 μM). Apomorphine-induced inhibition was reversed by pretreatment with PTX, indicating the role of $G_{i/o}$ proteins in this signaling pathway. In GH4ZR7 cells stably expressing the Gβγ blocker GRK-ct (GRKZ16 and GRKZ17), apomorphine-induced inhibition of forskolin-stimulated AC was not significantly different from the effect in GH4ZR7 cells, indicating no apparent role for Gβγ subunits in this pathway. The role of specific $G\alpha_{i/o}$ proteins in D2S-induced inhibition of AC was evaluated in cells expressing specific PTX-insensitive Gα subunits (Fig. 2). In all clones apomorphine inhibited forskolin action to increase cAMP level, comparable to original GH4ZR7 cells. PTX pretreatment completely blocked D2S-mediated inhibition of cAMP in both $G\alpha_{o}$-PTX clones (GαoZ7 and GαoZ15) as observed in GH4ZR7 cells. However, in multiple experiments the $G\alpha_{i}3$-PTX (Gi3Z6 and Gi3Z15) and $G\alpha_{i}2$-PTX (Gi2Z23) clones were significantly resistant to PTX by 20±1% and 65% respectively. These results indicate that $G\alpha_{i}2$, and to a lesser extent $G\alpha_{i}3$, mediates D2S-induced inhibition of forskolin-stimulated cAMP production in GH4ZR7 cells, consistent with previous results (18).

$G\alpha_{o}$ and Gβγ transduce inhibition of calcium entry by D2S receptors

Dopamine (10 μM) had no detectable effect on basal $[Ca^{2+}]_i$ in GH4ZR7 cells, but induced an immediate decrease in $[Ca^{2+}]_i$ level following stimulation by the dihydropyridine BayK8644 (an L-type calcium channel agonist) (Fig. 3A), as observed

previously (17). As an indicator of responsiveness cells were challenged with TRH, which induced a PTX-insensitive G_q-mediated mobilization of calcium stores (25). Dopamine-induced inhibition of $[Ca^{2+}]_i$ was completely blocked by PTX pretreatment, indicating signaling through $G_{i/o}$ proteins. Blockade of dopamine action by PTX was not reversed in at least three independent experiments with $G\alpha_i2$-PTX and $G\alpha_i3$-PTX clones (Fig. 3B-D), indicating that $G\alpha_i2$ and $G\alpha_i3$ subunits have negligible role in mediating D2S-induced calcium channel inhibition. By contrast, dopamine-induced inhibition of BayK8644-stimulated $[Ca^{2+}]_i$ level was completely rescued in both $G\alpha_o$-PTX-expressing clones following PTX pretreatment (Fig. 3E and F), indicating a critical role for G_o in D2S-mediated inhibition of L-type calcium channels. The role of $G\beta\gamma$ subunits in dopamine-mediated inhibition of $[Ca^{2+}]_i$ was investigated in GRK-ct-expressing GH4ZR7 clones in which dopamine failed to decrease $[Ca^{2+}]_i$ (Fig. 4). These results indicate that D2S-induced decrease in $[Ca^{2+}]_i$ level in GH4ZR7 cells depends on mobilization of $G\beta\gamma$ subunits from functional G_o heterotrimers.

Inhibition of TRH-induced p42/44 phospho-MAPK upon D2S receptor activation

Activation of MAPK was evaluated by measuring phosphorylation of MAPK, detected by Western blotting using an antibody specific for dual phosphorylated (activated) MAPK. In GH4ZR7 cells no basal phosphorylation of MAPK was detected after 1-hour incubation in FBS-free medium (fig. 5). Activation of the D2S receptor had no detectable effect on basal MAPK phosphorylation. TRH is known to activate MAPK in rat pituitary GH3 cells (26-28). In GH4ZR7 cells, TRH stimulated MAPK phosphorylation (Fig. 5), which was maximal within 7 min. (Appendix 2). Upon

activation of the D2S receptor by apomorphine, TRH-induced MAPK activation was almost completely blocked. This action of the D2S receptor was completely reversed by PTX pretreatment, implicating $G_{i/o}$ proteins. PTX treatment had no detectable effect on basal (data not shown) or TRH-induced level of phospho-MAPK.

$G\alpha_i3$ and $G\alpha_o$ mediate D2S inhibition of TRH-induced phospho-MAPK

The role of different $G_{i/o}$ subunits in D2S inhibition of the MAPK pathway was evaluated in different GH4ZR7 clones expressing PTX-insensitive $G\alpha$ subunits (Fig. 6). Although apomorphine almost completely inhibited TRH-induced MAPK activation in GH4ZR7 or GαoZ7 cells, pretreatment with PTX revealed a partial (50%) rescue of the inhibitory response in the GαoZ7 cells but not in GH4ZR7 cells (Fig. 6A). Apomorphine almost completely inhibited TRH-induced MAPK activation in all clones compared to GH4ZR7 cells (Fig. 6B and 6C), but upon pretreatment with PTX, differences among the clones were observed. In the $G\alpha_i2$-PTX expressing clone (Gi2Z23) PTX pretreatment completely blocked this inhibitory effect, indicating that the $G\alpha_i2$ subunit is not involved in D2S-mediated inhibition of MAPK phosphorylation. Interestingly, PTX pretreatment failed to completely reverse the inhibitory action of apomorphine in both $G\alpha_o$-PTX- and $G\alpha_i3$-PTX-expressing clones, suggesting a role for G_o and G_i3 in D2S-induced inhibition of MAPK activity. The importance of $G\beta\gamma$ subunits in D2S-induced inhibition of MAPK was evaluated using GRK-ct expressing GH4ZR7 clones, GRKZ16 and GRKZ17 (Fig. 7). D2S-mediated inhibition of TRH-stimulated MAPK was identical to that observed in GH4ZR7 cells. To address whether GRK-ct influenced the sensitivity of D2S receptor action, MAPK inhibition was quantitated at a range of apomorphine concentrations (Fig. 8). Apomorphine inhibited TRH-induced MAPK with an EC_{50} of < 1 nM.

Concentration-dependencies of apomorphine were not significantly different in GH4ZR7 cells and both GRK-ct clones, indicating that GRK-ct did not alter the sensitivity of TRH-induced MAPK phosphorylation to D2S receptor activation, suggesting that Gβγ subunits are unlikely to mediate inhibition of MAPK phosphorylation.

Apomorphine decreases phospho-MEK1/2, c-Raf and B-Raf kinase activity.

To further investigate upstream protein kinases involved in D2S inhibition of MAPK phosphorylation, regulation of MEK1/2 phosphorylation was examined (Fig. 9). Apomorphine induced a complete inhibition of TRH-stimulated MEK1/2 phosphorylation with identical concentration dependence to that for inhibition of p42/44 MAPK, indicating that apomorphine inhibits TRH-stimulated MAPK via inhibition of MEK1/2 in rat pituitary cells. Next, actions of the D2 receptor on c-Raf and B-Raf, which phosphorylate MEK1/2, were examined (Fig. 10). Each kinase was separately immunoprecipitated from cell lysates and assayed *in vitro* using a coupled assay involving sequential phosphorylation of recombinant MEK and MAPK. Using this immunoprecipitation/kinase assay, we detected basal activity of both c-Raf and B-Raf after 1 h serum starvation of GH4ZR7 cells. TRH selectively increased c-Raf kinase activity (164±11% of basal), but had no effect on B-Raf kinase activity (104±16% of basal). Apomorphine inhibited TRH-induced c-Raf kinase activity back to the basal level (84±12% of basal), while apomorphine alone inhibited c-Raf activity to 70% of basal activity (appendix 4). Thus D2S receptor mediated a complete inhibition of TRH-induced c-Raf activation. In contrast, B-Raf kinase activity was not significantly induced by TRH treatment in GH4ZR7 cells, but was decreased by apomorphine in the presence

(42±9% of basal) or absence (45%, Appendix 4) of TRH. These results indicate that both c-Raf and B-Raf are involved in D2S action, but that reduction of c-Raf activity is more important for inhibition of TRH action, while inhibition of B-Raf appears to be more important to regulate basal MAPK activity.

DISCUSSION

The dopamine-D2 receptor signals through multiple pathways in lactotrophs to regulate prolactin secretion, synthesis, and cell proliferation (9, 29, 30). Dopamine-D2 agonists, such as bromocryptine, have been used in the clinic for many years to inhibit the growth of prolactin-secreting pituitary adenomas (31, 32). A crucial role for the dopamine-D2 receptor in the anti-proliferative action of dopamine in pituitary cells was identified in homozygous mice deficient in the gene encoding the D2 receptor, which develop pituitary adenomas (33-35). Oppositely, mice that lack the gene encoding the dopamine transporter hypersecrete dopamine, resulting in pituitary hypotrophy due to a paucity of lactotrophs (36). Although of great importance *in vivo*, the detailed signaling pathways mediating the anti-proliferative actions of D2 receptors in pituitary cells remain to be characterized. In contrast to the numerous stimulatory actions in mesenchymal cells, including MAPK activation and enhanced cell proliferation (6, 9), D2S activation causes inhibitory effects in pituitary cells (9, 10, 37). We expressed PTX-insensitive $G\alpha_{i_o}$ protein mutants to evaluate the G protein specificity of D2S-induced inhibition of AC, calcium influx and MAPK activity in GH4ZR7 cells.

Modulation of adenylyl cyclase via distinct G proteins

Inhibition of adenylyl cyclase by receptors that couple to $G_{i/o}$ appears to be a ubiquitous pathway (3, 38) and is mediated by dopamine-D2S receptor activation in a wide variety of cell types (15, 17, 39). Using PTX-insensitive mutants in GH4ZR7 cells $G\alpha_i2$ (and to a lesser extent $G\alpha_i3$) rescued inhibition of forskolin-stimulated cAMP production by D2S receptor activation after PTX treatment, while $G\alpha_o$ was not involved in this pathway. These findings are consistent with previous studies in pituitary and fibroblast cells (6, 7, 18) that implicate $G\alpha_i2$ in regulation of forskolin-stimulated AC activity. Interestingly, selective depletion of $G\alpha_i2$ by stable expression of antisense $G\alpha_i2$ RNA in GH4ZR7 cells had little influence on D2S inhibition of G_s-stimulated AC (17). The different G_i specificity in coupling to G_s- or forskolin-induced AC in these cells could result from different $G\alpha_i$ specificity for different subtypes of AC, or different states of activation (e.g. by G_s or forskolin) of a single AC subtype. Expression of GRK-ct in GH4ZR7 cells blocked D2S-mediated inhibition of calcium influx but did not change D2S-mediated inhibition of cAMP levels, indicating that mobilization of $G\beta\gamma$ subunits is not necessary for the latter signaling pathway (7). Inhibitory regulation of cAMP signaling via $G\alpha_i2$ could play a role in cAMP-dependent MAPK activation, prolactin gene transcription and secretion (40, 41).

G protein specificity for inhibition of calcium influx

In GH4ZR7 cells, D2S receptors mediated inhibition of L-type calcium channels via a PTX-sensitive pathway (17, 19, 42), as observed in lactotrophs (43). $G\alpha_o$-PTX, but not $G\alpha_i2$-PTX or $G\alpha_i3$-PTX, rescued D2S-mediated inhibition of dihydropyridine-sensitive calcium influx indicating that the G_o protein has the prominent role in L-type calcium

channel inhibition. In agreement with these results, antisense depletion of $G\alpha_o$, but not other $G\alpha_i$ subunits, reduced coupling of multiple receptors (including the D2S receptor) to inhibit BayK8644-stimulated calcium influx and prolactin secretion (17, 21, 44). Inhibition of L-type calcium influx was also blocked by expression of GRK-ct in GH4ZR7 cells, indicating a prominent role for $G\beta\gamma$ subunits in this pathway. Although G_o plays a crucial role in coupling to N-type calcium channels, a direct interaction between mobilized $G\beta\gamma$ subunits and the channel α_{1B} subunit actually transduces the receptor signal (45, 46). How G_o regulates L-type channels remains unclear, since both L-type channel α_{1C} and α_{1D} subunits fail to bind $G\beta\gamma$ (47, 48). The L-type channel in GH3 cells may actually be heteromeric including at least one α_{1A} subunit to confer $G\beta\gamma$ sensitivity, since expression studies have identified α_{1A}, α_{1C} and α_{1D} RNA in these cells (48). Activation of L-type calcium channels contributes in part to multiple stimulatory actions of TRH, including TRH-induced sustained calcium entry, MAPK activation, prolactin secretion and gene transcription (25, 28, 44). Hence G_o-mediated inhibition of L-type channel opening could play an important role in dopamine-induced inhibition of TRH action.

D2S-induced inhibition of TRH-stimulated MAPK activation

Our results demonstrate that among PTX-insensitive $G\alpha$ mutants, $G\alpha_i3$-PTX and $G\alpha_o$-PTX could partially rescue D2S inhibition of TRH-induced MAPK phosphorylation, indicating the crucial role of $G\alpha_i3$ and $G\alpha_o$ in this pathway. Although activation of MAPK mediated by G_i-coupled receptors in mesenchymal cells is transmitted via $G\beta\gamma$ subunits (49-51), our results suggest that $G\beta\gamma$ subunits were not involved in D2S-induced MAPK inhibition in GH4ZR7 cells. Although GRK-ct blocked D2-induced inhibition of

BayK8644-induced calcium entry in these cells, we cannot rule out the possibility that Gβγ subunits not blocked by GRK-ct expression are mediating D2S receptor action to inhibit MAPK phosphorylation.

The signaling pathway from $G\alpha_{i_o}$ proteins to inhibition of MAPK phosphorylation is not known yet. In neuronal cells, activation of a $G_{i/o}$-coupled receptor may inhibit MAPK phosphorylation by lowering cellular cAMP level (52, 53). However, TRH has no effect on cAMP formation in these cells, and D2S receptor activation did not affect basal cAMP levels under our conditions. TRH-induced MAPK activation is complex and appears to involve calcium- and PKC-dependent signaling to endocytosis, EGF receptor activation, and initiation of the ras-c-Raf-MAPK pathway (26, 27, 54, 55) (Fig. 11). Consistent with this, in our experiments TRH induced c-Raf kinase and MEK1/2 activity but not B-Raf kinase activity. D2S activation completely blocked TRH-induced c-Raf activation and MEK1/2 phosphorylation, suggesting that TRH-induced signaling to MAPK converges at c-Raf activation, the upstream site of D2 action. However, D2S receptor activation does not inhibit early upstream events such as TRH-induced phosphatidyl inositol turnover or calcium mobilization (56, 57). Consistent with this, TRH-induced Ser259 phosphorylation to desensitize c-Raf (58) was not inhibited by D2 receptor activation (Appendix 3). Hence the most important site of D2S action to inhibit TRH-induced MAPK activation appears to be c-Raf activation, but not earlier Gq-mediated signaling events. D2S-mediated MAPK inhibition could in turn inhibit TRH-induced prolactin gene transcription and prolactin synthesis, which is mediated by a Ras-Raf-MAPK-Ets pathway (28, 41, 59, 60).

Based on our results, the D2S receptor inhibits MAPK phosphorylation in at least two ways (Fig. 11). Apomorphine pretreatment completely blocked TRH-induced c-Raf activation, suggesting that the D2 receptor, via activation of $G\alpha_{i}3$ or $G\alpha_{o}$, may utilize a novel signaling pathway to inhibit TRH-induced MAPK activation via block of c-Raf-dependent MEK1/2 activation. However, it remains to be determined whether this is a direct or indirect action of $G\alpha_{i}3$ and $G\alpha_{o}$. The D2S receptor could act via $G\alpha_{o}$ to inhibit the Rap1-GTP/B-Raf/MEK/MAPK cascade. Activation of $G\alpha_{o}$ releases Rap1-GTPase activating protein (GAP) to inhibit Rap1-induced B-Raf activation (61), providing an appealing mechanism for $G\alpha_{o}$-mediated D2S action. Although basal MAPK phosphorylation not detectable (see also (20)), a basal level of endogenous phospho-MAPK was present in B-Raf immunoprecipitates and was inhibited by apomorphine treatment (data not shown). A B-Raf-dependent mechanism could be important for D2-induced inhibition of basal MAPK activity or stimulation by cAMP or calcium, both of which activate B-Raf kinase activity (52, 62). These findings suggest that one possible pathway for D2S-induced MAPK dephosphorylation in rat pituitary cells is by activation of $G\alpha_{o}$ subunit and inhibition of B-Raf kinase activity that probably involves Rap-GAP.

By mapping Gi/Go signaling pathways using PTX-insensitive or antisense approaches we have defined differences in G protein specificity for particular actions, such as inhibition of cAMP, MAPK or calcium channel activation. Using the antisense approach, we recently found that in contrast to D2S inhibition of BayK8644-stimulated prolactin secretion which required Go primarily, inhibition of TRH-stimulated secretion required Gi2, Gi3, and Go (44). This suggests that recruitment of known Go- or Gi3-induced pathways, as well as Gi2-dependent signaling mediates inhibition of secretion.

Utilization of PTX-insensitive or antisense G protein expression will provide relatively non-perturbing methods to identify G protein-induced signaling networks and identify their functional roles (8).

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MATERIALS AND METHODS

Materials

Apomorphine, dopamine, EGTA, forskolin, 3-isobutyl-1-methyl xanthine (IBMX), pertussis toxin (PTX), puromycin, thyrotropin-releasing hormone (TRH), (+/-)-Bay K8644, vasoactive intestinal peptide(VIP), Sepharose G protein beads, anti-β-actin and anti-Flag antibody were from Sigma (St. Louis, MO); Fura-2 AM was purchased from Molecular Probes (Eugene, OR); [^{125}I] succinyl cAMP (2200Ci/mM) and polyvinylidene difluoride (PVDF) membrane were from New England Nuclear Corp. (Boston, MA); enhanced chemiluminescence (ECL™) detection kits were from Amersham Corp. (Arlington Heights, IL); Sera and media were obtained from Gibco/BRL. Endonucleases and DNA polymerase were purchased from New England Biolabs (NEB; Mississauga, Canada); Anti-$G\alpha_o$ was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-$G\alpha_i1/2$ was obtained from Calbiochem (San Diego, CA); anti-phospho-p42/44 MAPK antibody (T202/Y2040), anti phospho-MEK 1/2 (Ser 217/221) antibody was from New England Biolabs (Boston, MA). Anti B-Raf, anti c-Raf and Raf kinase cascade assay kit was from Upstate biotechnology (Lake Placid, NY). Polyclonal antibody against recombinant bovine GRK2 was kindly provided by Dr. J. L. Benovic (24).

Cell culture and transfection

GH4ZR7 cells and derivative clones were maintained in Ham's F10 medium with 8% fetal Bovine serum (FBS) at 37°C, 5% CO_2. PTX-insensitive $G\alpha_{i/o}$ mutants and His-GRK-ct were constructed previously (7). $G\alpha_i2$-PTX and $G\alpha_i3$-PTX were Flag-tagged at the initiator ATG codon, and subcloned in KpnI/EcoRI-cut pcDNA3 (Invitrogen) to generate Flag-$G\alpha_i2$-PTX and Flag-$G\alpha_i3$-PTX and their sequences were confirmed by DNA sequencing. These constructs were cotransfected individually (20 μg) with pGK-puro (2 μg) into GH4ZR7 cells using calcium phosphate co-precipitation. The transfected cells were cultured in F10 + 8%FBS containing puromycin (20 μg/ml) for 3-4 weeks. Antibiotic-resistant clones were picked (24 clones/transfection) and tested for expression of the corresponding $G\alpha_{i/o}$ proteins by Western blot analysis.

Western blot analysis

Cells ($3x10^5$ cells/well) were harvested and resuspended by pipetting in 50 μl of RIPA-L buffer (10 mM Tris [pH 8], 1.5 mM $MgCl_2$, 5 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1% Nonidet p-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 μg/ml Leupeptin) and incubated on ice for 30 min. The lysate was centrifuged (12,000g, 10 min., 4°C), and the supernatant was recovered and measured for protein content by the bicinchoninic acid protein assay kit (Pierce). Lysate was equally loaded and separated on 12% polyacrylamide gel and transferred onto PVDF membrane. Blots were blocked overnight at 4°C, then incubated at 4°C with primary antibody for 24 h, followed by 45 min incubation with horseradish peroxidase-conjugated secondary antibody, the peroxidase product was developed using the ECL™ protocol. Protein expression was quantified by densiometric analysis of blots.

cAMP measurement

Equal numbers of cells were plated in six-well plates and grown to 70-80% confluence. The cells were incubated at 37°C in1 ml/well of serum-free DMEM/20 mM Hepes, pH 7.0/ 100 μM isobutylmethylxanthine, with or without experimental compounds. After 20 min. the media were recovered and centrifuged at 12000 x g for 2 min. at 4°C. The supernatant was stored at -20°C for further analysis. Samples then analyzed by a specific radioimmunoassay to measure cAMP level. Percent inhibition was calculated as 100-[100(D-C)/(S-C)], where C is cAMP level in nontreated cells, S is stimulated cAMP in forskolin-treated cells, and D is cAMP level in apomorphine/forskolin-treated cells. These values were normalized to control GH4ZR7 cells (=100%).

Measurement of $[Ca^{2+}]_i$

Cells were grown to 80% confluence in 15-cm plates and harvested with HBBS+EDTA. The cells were resuspended in 2 ml of HBBS+Ca^{2+} with 2.5 μM Fura-2 AM, and incubated at 37°C for 30 min. with gentle shaking (100 rpm) (7). Cells were washed twice and resuspended in 2 ml of HBBS+Ca^{2+} and subjected to fluorometric measurement of $[Ca^{2+}]_i$ as described (7). Experimental compounds were added directly to cuvettes at times indicated in the figures. Because of fluorescent interference of the Fura-2 signal by apomorphine autofluorescence, dopamine was used in these experiments.

Measurement of phospho-MAPK and -MEK1/2

Equal number of cells (3×10^5 cell/well) were plated in six-well plates. At 80% confluence, the cells were placed in serum-free Ham's F10 medium (1 hr, 37°C). Cells were treated with the indicated drugs at 37°C and after the indicated time the plates transferred on ice and washed 2 times with cold PBS. Cells were lysed in 50 μl of 5×

sodium dodecyl sulphate (SDS) loading buffer (500 mM Tris pH 6.8, 2% SDS, 40 µl/ml 2-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol), stored on ice, sonicated for 10 sec and centrifuged at 12,000 x g for 5 min. at 4°C. The supernatant (30 µl) was heated (100°C, 2 min.) and rapidly cooled on ice. Samples were centrifuged 30 sec. and were separated by SDS-polyacrylamide gel electrophoresis (PAGE), blotted on PVDF membrane, and subjected to Western blot analysis. Phosphorylation was detected using (1:1000) anti-phospho-p42/44 MAPK or -phospho-MEK1/2 antibody. The corresponding band for p42 MAPK and p44 MAPK (collectively referred to as p42/44 MAPK) was digitally quantified using UN-SCAN-IT program (Silk Scientific Inc., Orem, Utah). The results were normalized to the control.

Immunoprecipitation/kinase assay

The ability of c-Raf and B-Raf to activate MEK was measured by an immune complex coupled assay in which recombinant GST-MEK1 activates and phosphorylates GST-p42 MAPK. Equal number of cells in 6-well plates were serum starved for an hour and then treated by indicated drugs and were lysed in buffer containing 1% Nonidet P-40, 50 mM Tris-Cl , pH=7.5, 150 mM NaCl plus protease and phosphatase inhibitors. Lysates with equal amount of protein were incubated with antibodies against c-Raf or B-Raf for 2h at 4°C while rotating. Immune complex was collected with protein G-sepharose for 1h at 4°C centrifuged and washed 3 times. The pellet was used for the following kinase assay. For each reaction 20 µl of assay dilution buffer I (ADBI) (20mM MOPS, pH=7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT), and 10 µl of Mg/ATP cocktail (75 mM $MgCl_2$ and 500µM ATP in ADBI) were added to dephosphorylated GST-MEK1 and GST-MAPK2 plus immunoprecipitate or active B-Raf as a positive control. After 30 min shaking at 30°C, the reaction was

terminated by adding SDS-loading dye, boiled for 2 min., and loaded on SDS-PAGE. Specific phospho-GST-MAPK2 bands were detected with anti-phosphoMAPK to assay kinase activity.

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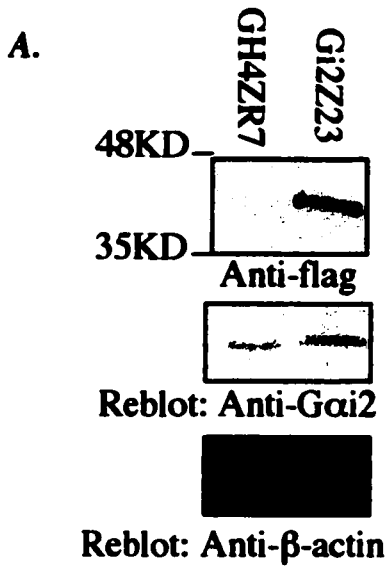
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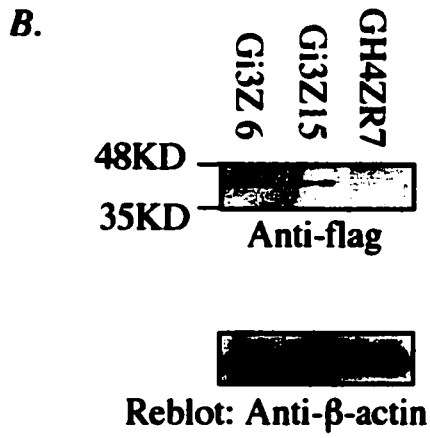
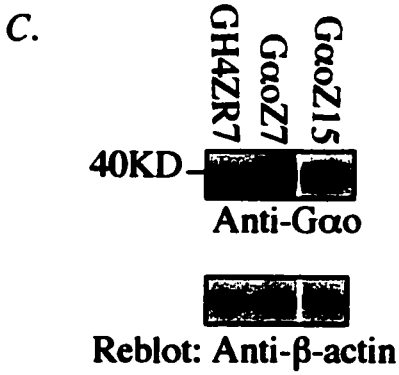
GH4ZR7
Gi2Z23
48KD
35KD
Anti-flag
Reblot: Anti-Gαi2
Reblot: Anti-β-actin


B.
Gi3Z 6
Gi3Z15
GH4ZR7
48KD
35KD
Anti-flag
Reblot: Anti-β-actin


C.
GH4ZR7
GαoZ7
GαoZ15
40KD
Anti-Gαo
Reblot: Anti-β-actin

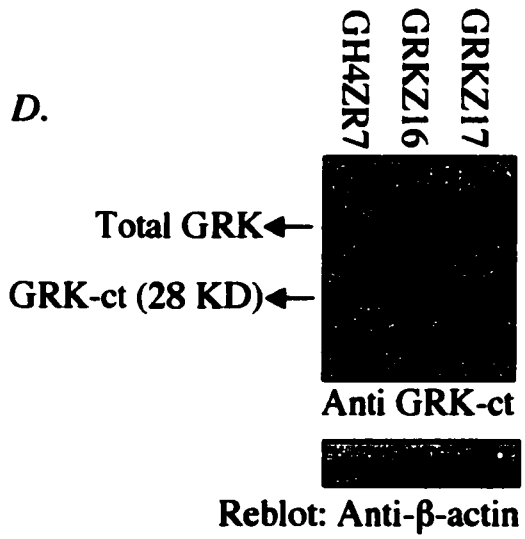

D.
GH4ZR7
GRKZ16
GRKZ17
Total GRK
GRK-ct (28 KD)
Anti GRK-ct
Reblot: Anti-β-actin

Fig 1. Protein level of stably-transfected PTX-insensitive $G\alpha_{i-o}$ mutants and GRK-ct.

Equal amount of cell extracts (100 μg/lane) were subjected to Western blot analysis as described in Materials and Methods: GH4ZR7 cells (wild type) were compared to: a, GH4ZR7 cells expressing Flag-$G\alpha_i2$-PTX (Gi2Z23); B, Flag-$G\alpha_i3$-PTX (Gi3Z6 and Gi3Z15); C, $G\alpha_o$-PTX (GαoZ7 and GαoZ15); D, GRK-ct (GRKZ16 and GRKZ17). The blots were probed with anti-Flag (A and B), anti-$G\alpha_i2$ (A), anti-$G\alpha_o$ (C), anti-β-actin (B and C), and anti-GRK-ct (D).

Figure 2.

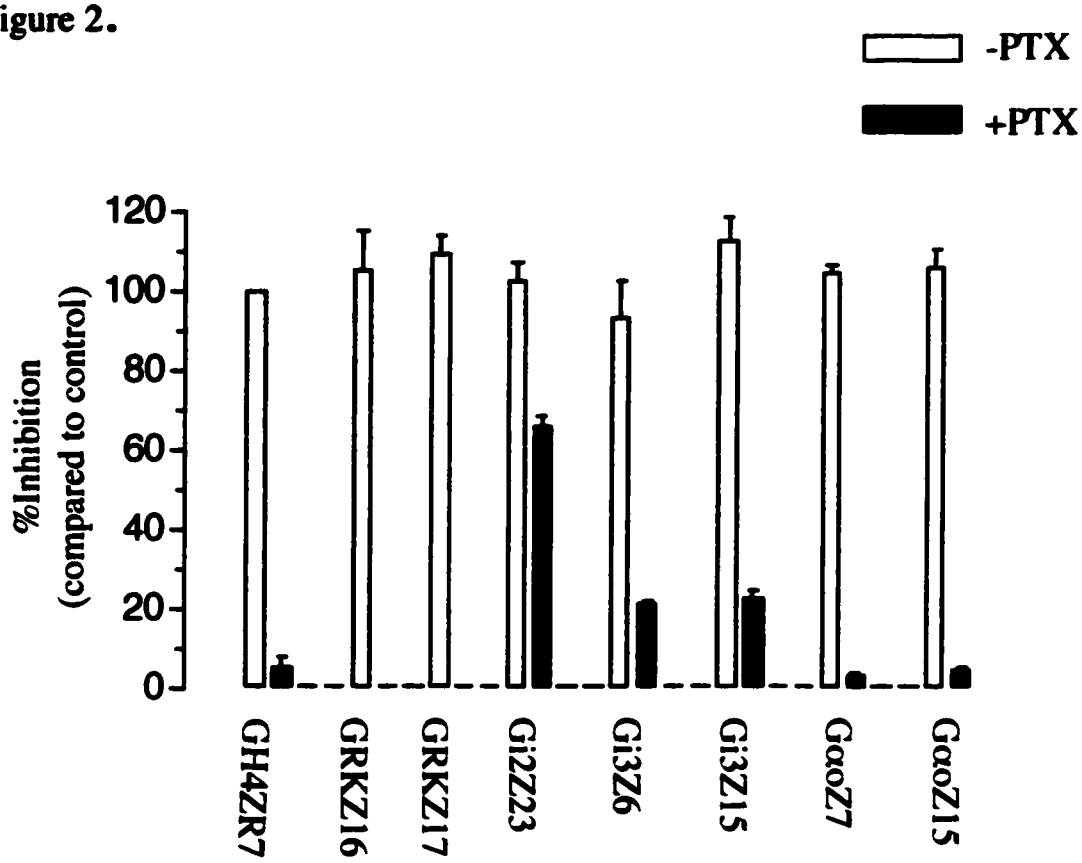

-PTX
+PTX
%Inhibition
(compared to control)
120
100
80
60
40
20
0
GH4ZR7
GRKZ16
GRKZ17
Gi2Z23
Gi3Z6
Gi3Z15
GαoZ7
GαoZ15

Fig. 2. D2S-induced inhibition of forskolin-stimulated cAMP requires $G\alpha_{i}2$.

Cells were incubated with no drug, forskolin (1 μM), apomorphine (1 μM), or both, with or without pretreatment with PTX (20 ng/ml, 12 hours) as indicated. Percent inhibition of apomorphine action was calculated as described in Materials and Methods and normalized to the value for GH4ZR7 (100%). The data are expressed as mean ± SEM of three independent experiments. In all clones, basal and forskolin-stimulated cAMP levels were not significantly different from corresponding levels in GH4ZR7 cells. GH4ZR7 clones are labeled as shown in Fig. 1.

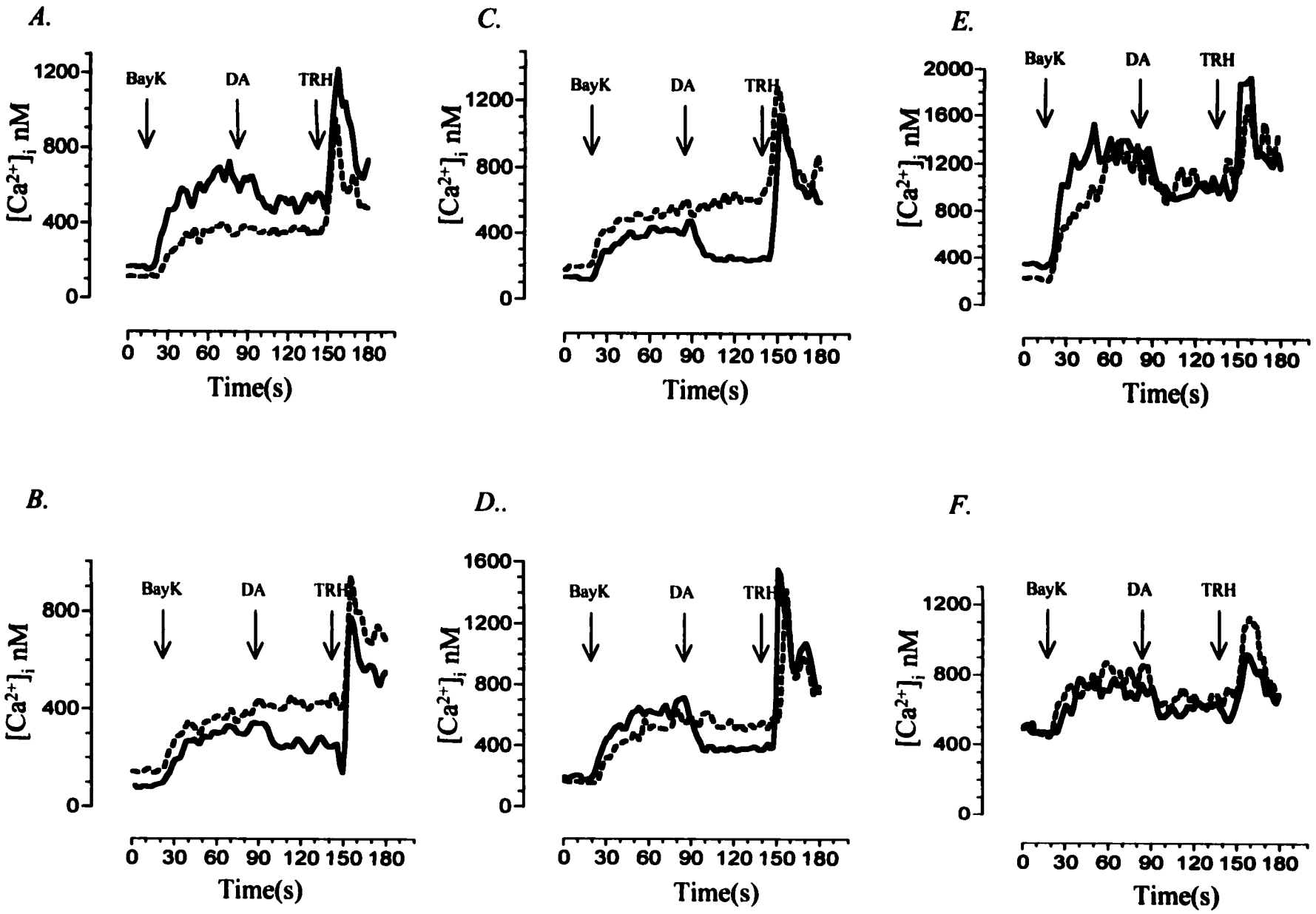

A.
C.
E.
B.
D..
F.
BayK
DA
TRH
[Ca2+]i nM
Time(s)
0
400
800
1200
1600
2000
0 30 60 90 120 150 180

Figure 3.

Fig. 3. $G\alpha_o$ subunits mediate inhibition of dihydropyridine-induced $[Ca^{2+}]_i$.

A, $[Ca^{2+}]_i$ was measured in GH4ZR7 cells and GH4ZR7 cells expressing: B, $G\alpha_i2$-PTX (Gi2Z23); C, D, $G\alpha_i3$-PTX (Gi3Z6 and Gi3Z15); and E, F, $G\alpha_o$-PTX (GαoZ7 and GαoZ15). The cells were not treated (solid line) or treated (dashed line) with PTX (20 ng/ml, 12 hour), and the change in $[Ca^{2+}]_i$ level in response to BayK8644 (BayK, 100 nM), dopamine (10 μM) or TRH (100 nM) was measured (Materials and Methods). Arrows show the time of addition of drugs. Similar results were obtained in at least three independent assays for each clone.

Figure 4.

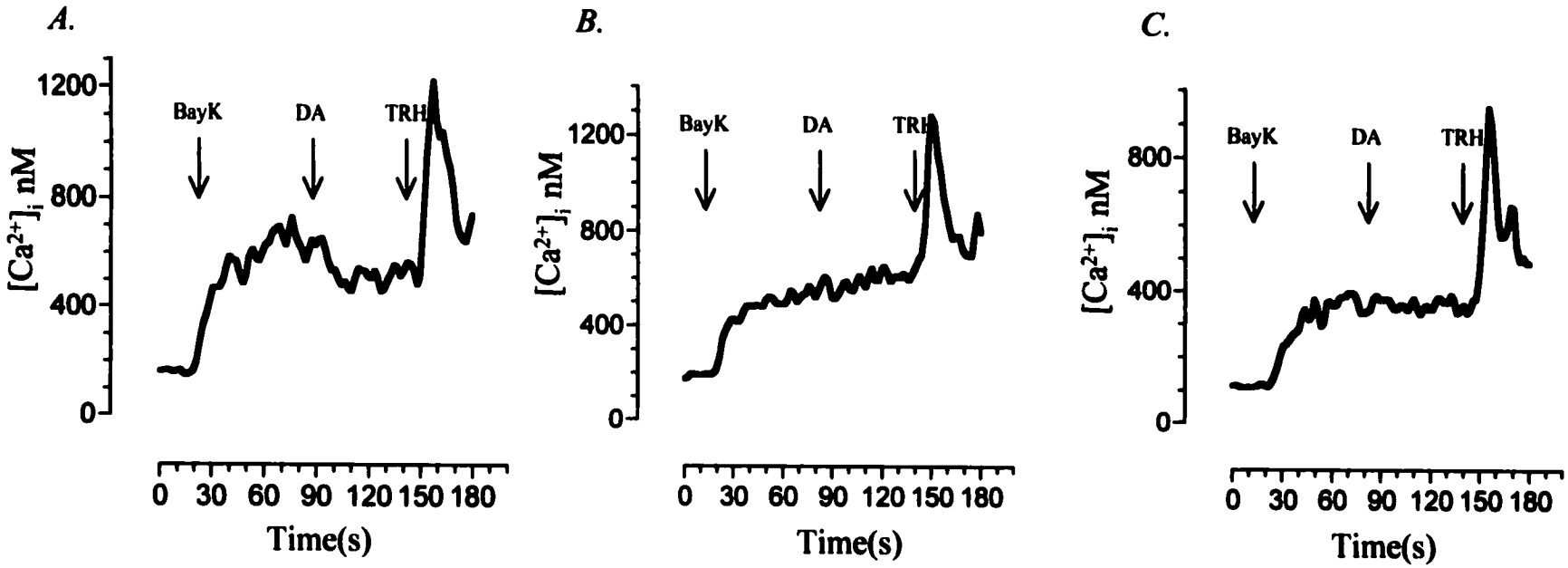

A.
B.
C.
BayK
DA
TRH
[Ca2+]i nM
1200
800
400
0
0 30 60 90 120 150 180
Time(s)

Fig. 4. Expression of GRK-ct blocks D2S-mediated inhibition of calcium entry.

A, Change in $[Ca^{2+}]_i$ was measured in GH4ZR7 cells (wild type) and GH4ZR7 cells expressing GRK-ct protein (B, GRKZ16 and C, GRKZ17). Arrows indicate the addition of drugs as explained in Fig. 3. These results were reproduced in three independent assays.

Figure 5.

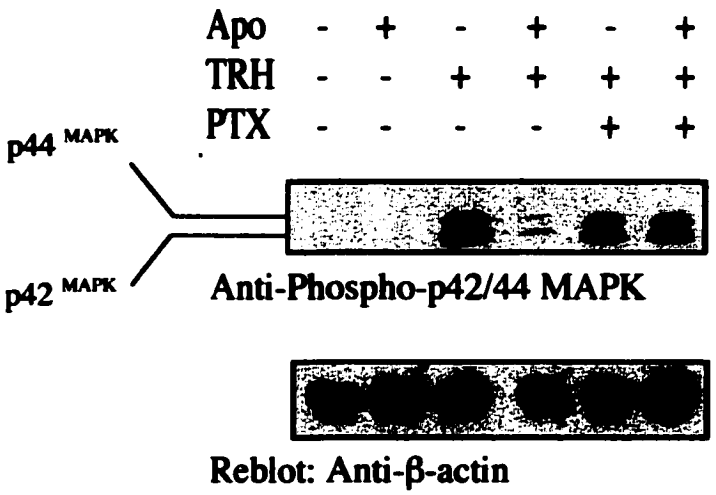

Apo - + - + - +
TRH - - + + + +
PTX - - - - + +
p44 MAPK
p42 MAPK
Anti-Phospho-p42/44 MAPK
Reblot: Anti-β-actin

Fig. 5. TRH-induced MAPK activation is blocked by apomorphine via $G_{i/o}$ proteins. GH4ZR7 cells were treated or not with PTX (20 ng/ml, 12 hours) and incubated for 1 hour in serum-free medium. For assay, cells were incubated with no drug (control), or pretreated with Apomorphine (1 μM) for 15 minutes, then TRH (1 μM) was added to wells for 7 minutes followed by cell lysis (Materials and Methods). Western blot analysis of lysates was done using specific antibody against phospho-p42/44 MAPK. Membranes were reprobed with β-actin antibody as a loading control.

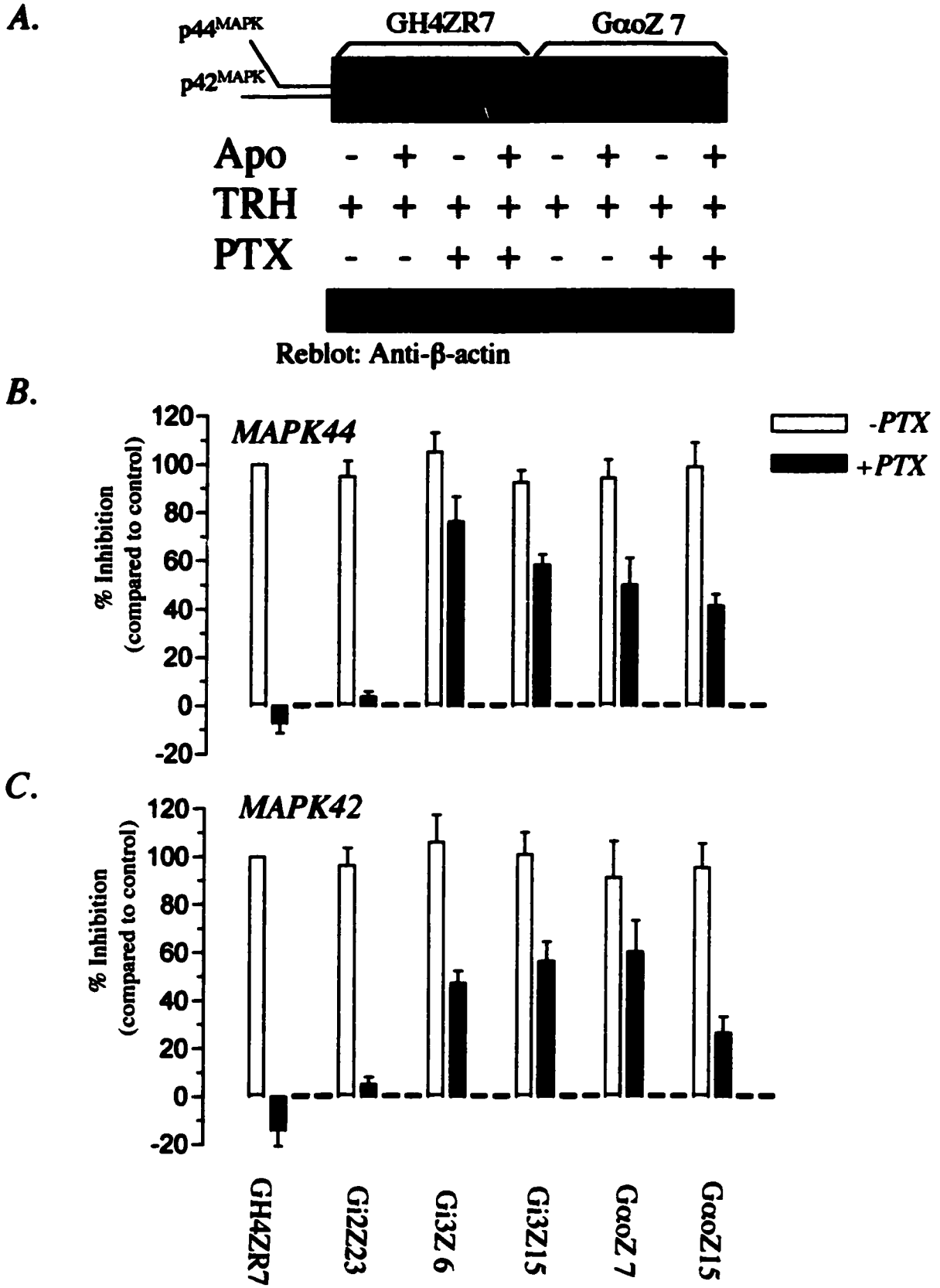

A.
p44MAPK
p42MAPK
GH4ZR7
GαoZ 7
Apo - + - + - + - +
TRH + + + + + + + +
PTX - - + + - - + +
Reblot: Anti-β-actin
B.
MAPK44
-PTX
+PTX
% Inhibition (compared to control)
120
100
80
60
40
20
0
-20
C.
MAPK42
% Inhibition (compared to control)
GH4ZR7
Gi2Z23
Gi3Z 6
Gi3Z15
GαoZ 7
GαoZ15

Figure 6.

Fig. 6. $G\alpha_o$ and $G\alpha_i3$ mediate D2S inhibition of TRH-activated MAPK.

A, Western blot showing an example apomorphine-induced MAPK inhibition in GαoZ7 cells compared to original GH4ZR7 cells. Cells were pretreated with or without PTX (20 ng/ml for 12 hour). Experimental compounds were added as indicated and the level of phosphorylated MAPK was measured. B, C, D2S-induced MAPK inhibition was calculated as percent inhibition of TRH (1 μM)-induced MAPK activity produced by apomorphine (1 μM) compared to control, based on densitometric analysis of phospho-p44 (B) and phospho-p42 (C) MAPK bands. Data are expressed as mean ± SEM of at least three independent experiments.

Figure 7.

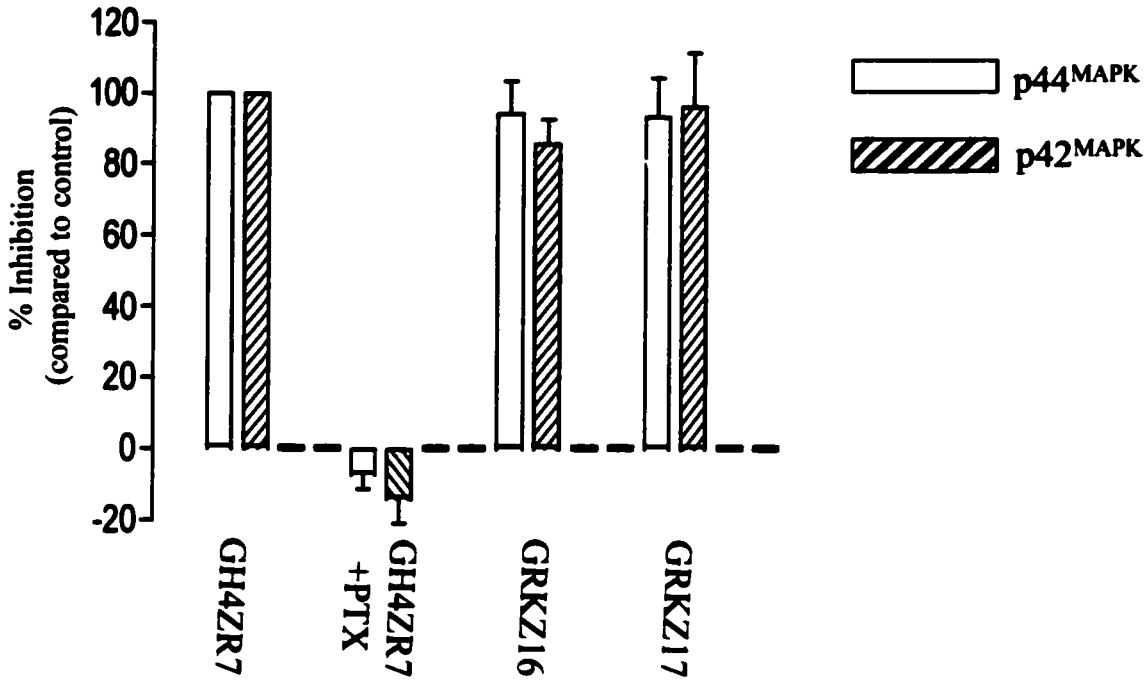
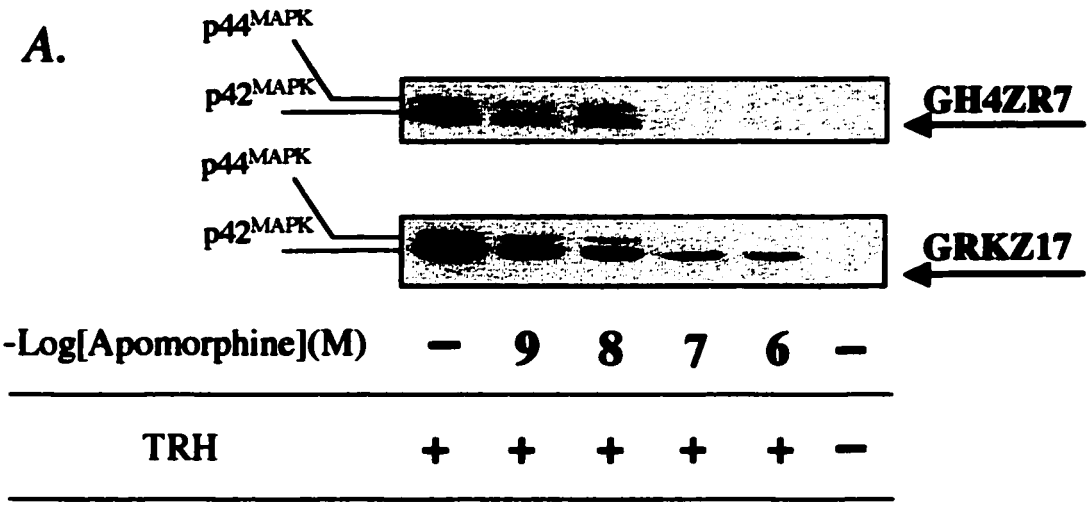

120
100
80
60
40
20
0
-20
% Inhibition
(compared to control)
GH4ZR7
+PTX
GH4ZR7
GRKZ16
GRKZ17
p44MAPK
p42MAPK

Fig. 7. D2S-induced MAPK inhibition in GH4ZR7 cells expressing GRK-ct protein. MAPK phosphorylation was measured in GRKZ16 and GRKZ17 cells, after addition of TRH and apomorphine as in Fig. 5. Densitometric analysis data are expressed as mean ± range (n=2) of the percent inhibition by apomorphine of TRH-stimulated phospho-MAPK (p44 or p42, as indicated) compared to control GH4ZR7 cells (=100%).

Figure 8.


A.
p44MAPK
p42MAPK
GH4ZR7
p44MAPK
p42MAPK
GRKZ17
-Log[Apomorphine](M) – 9 8 7 6 –
TRH + + + + + –

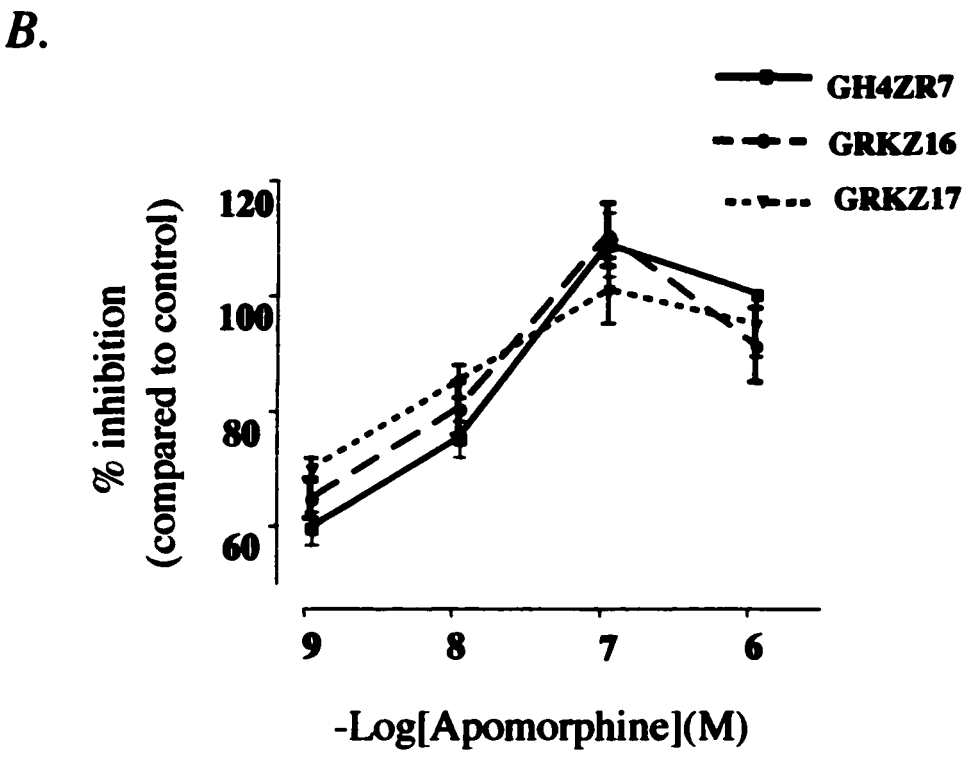
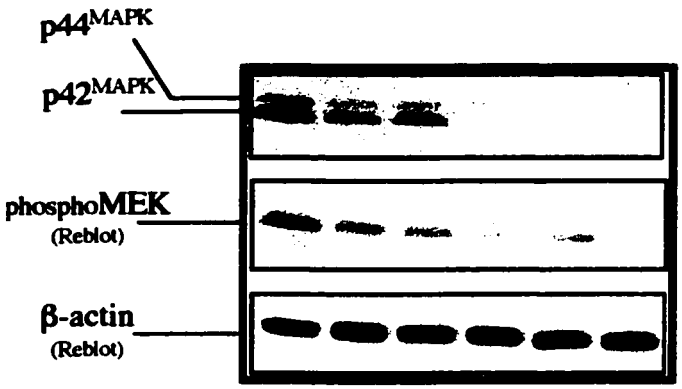

B.
GH4ZR7
GRKZ16
GRKZ17
% inhibition (compared to control)
120
100
80
60
9
8
7
6
-Log[Apomorphine](M)

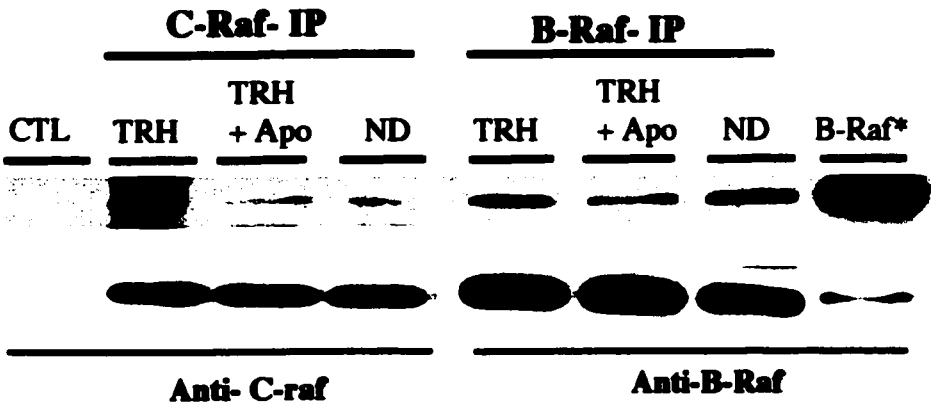
Fig. 8. Concentration-dependence of apomorphine inhibition of TRH-induced MAPK phosphorylation is similar in GH4ZR7 cells and GRK-ct expressing clones. A, western blots illustrating a sample of apomorphine (10^{-9} to 10^{-6} M) inhibition of TRH-induced MAPK activation in GH4ZR7 and GRKZ17 clones. B, D2S-induced MAPK inhibition was calculated as percent inhibition of TRH (1 μM)-induced MAPK activity produced by different concentrations of apomorphine compared to control, and plotted as a concentration-dependence curve for GH4ZR7 cells (wild type) and GRKZ16 and GRKZ17 clones. Each value represents the mean ±SD of three independent experiments.



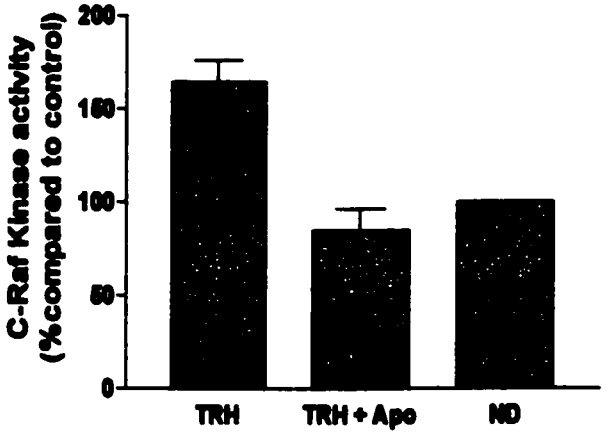
-Log[Apomorphine](M)	–	9	8	7	6	–
TRH	+	+	+	+	+	–

Fig. 9. Dopamine D2 stimulation by apomorphine inhibits TRH-induced phosphorylation of MEK1/2 in GH4ZR7 cells. Western blot showing the effect of different concentration of apomorphine on GH4ZR7 cells on MEK1/2 phosphorylation. Cells were pretreated with the indicated concentration of apomorphine for 15 min. and then subjected to TRH stimulation for 7 minutes at 37° C. Each figure is representative of three independent sets of experiments.

A.


C-Raf- IP
B-Raf- IP
CTL
TRH
TRH + Apo
ND
TRH
TRH + Apo
ND
B-Raf*
Anti- C-raf
Anti-B-Raf

B.


C-Raf Kinase activity (%compared to control)
200
150
100
50
0
TRH
TRH + Apo
ND

C.

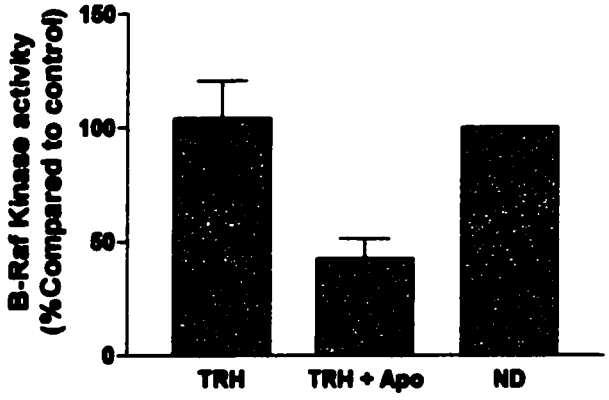

B-Raf Kinase activity (%Compared to control)
150
100
50
0
TRH
TRH + Apo
ND

Fig. 10. D2S stimulation decreases c-Raf and B-Raf kinase activity.

Cells were treated with TRH (1 μM) and/or apomorphine (Apo, 1 μM) or untreated (Basal) as explained in Figure 5. Equal amounts of lysate protein were immunoprecipated using anti c-Raf or anti-B-Raf. Kinase activity in the precipitate was measured by kinase cascade assay (see Methods). Buffer alone (CTL) or constitutively-active B-Raf protein (B-Raf*) was used in the assay as negative or positive controls, respectively. **Above**, kinase activity was assayed by phosphorylation of GST-ERK2 detected by Western blot analysis. Total c-Raf and B-Raf in the immunoprecipitate was determined by reprobing with anti-c-Raf or anti-B-Raf antibody. **Below**, c-Raf or B-Raf kinase activity was calculated based on densitometric analysis of phospho-ERK2 bands compared to basal level. Data are shown as mean ± SEM of three independent experiments, *$p<0.05$ vs. Basal.

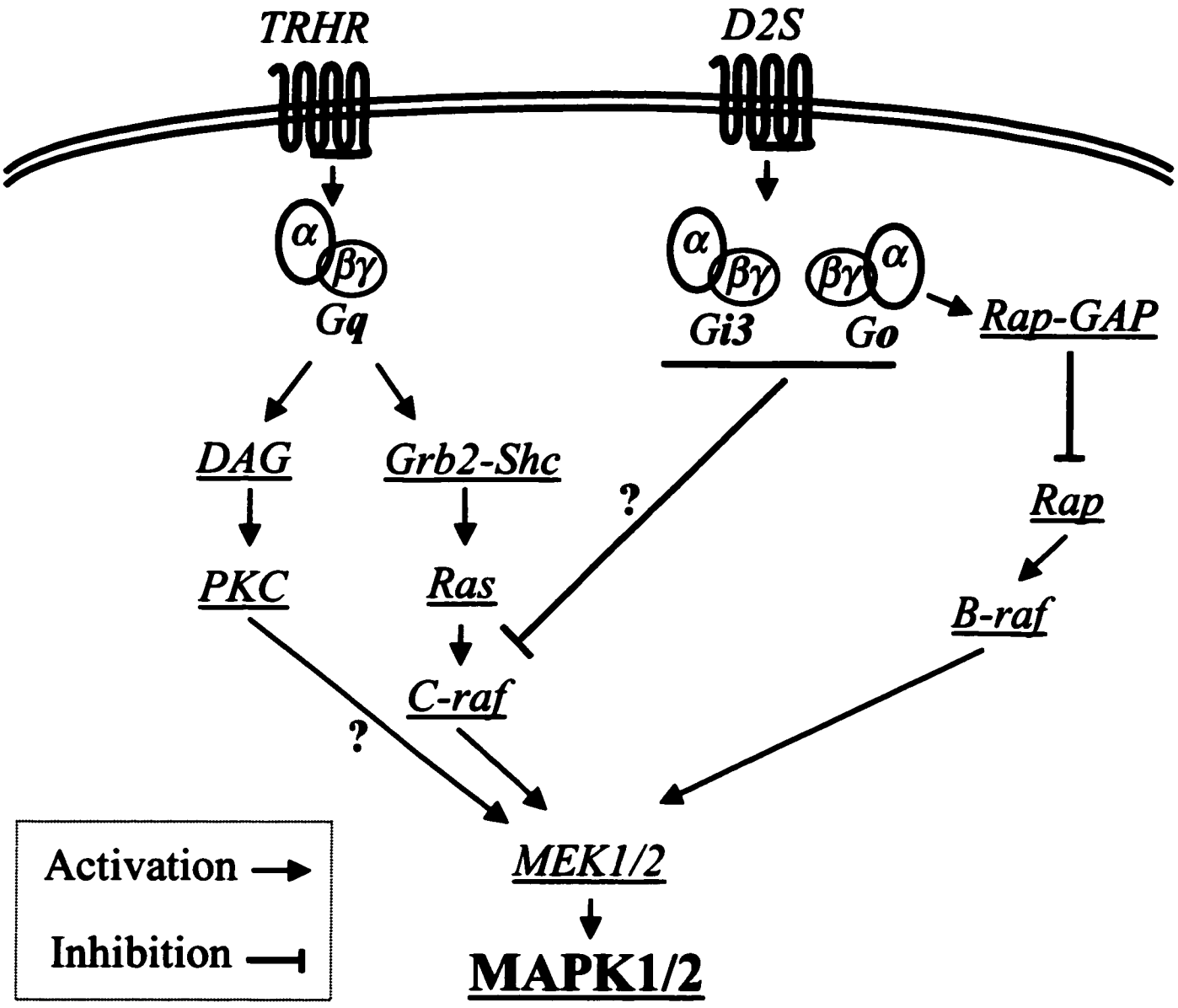

TRHR
D2S
α
βγ
Gq
Gi3
Go
Rap-GAP
DAG
Grb2-Shc
?
Rap
PKC
Ras
B-raf
C-raf
?
Activation
Inhibition
MEK1/2
MAPK1/2

Fig. 11. Pathways of D2S-induced inhibition of MAPK in GH4ZR7 cells.

A summary of D2S receptor actions on basal and TRH-induced MAPK is presented. TRH (via Gq) stimulates MAPK via Shc/GRB2-dependent and PKC-dependent pathways. Our data indicate that both $G\alpha_i3$ and $G\alpha_o$ mediate D2S inhibition of TRH-induced MAPK via inhibition of c-Raf, leading to decreased phosphorylation of MEK1/2 and MAPK. The D2S may utilize $G\alpha_o$-induced activation of rapGAP to inhibit rap and decrease B-Raf activity, as reported for other systems.

CHAPTER III

Discussion

Since the discovery of GPCRs, the specificity of signaling pathways and the cellular effectors for this family of the receptors has expanded dramatically. GPCRs are involved in many physiological and pathological systems. Considering the diversity in the functionality of these receptors, the specificity and selectivity in each system is an inevitable component of GPCR performance. GPCRs can regulate their specificity in four levels:

- receptor level, which depending to receptor structure, specificity and sensitivity to agonists and antagonists are dictated and coupling to distinct G protein partners is directed.
- G protein level, which determines coupling specificity to downstream effectors as the primary signal transduction component of GPCR function.
- Effector level, where G protein coupling to immediate effectors(e.g. ion channels or AC) provides second messenger or intermediate effectors, initiates a cascade of events. At this level, not only different effectors, but also different structural isoforms of effectors direct specificity of signaling.
- Cross-regulation, when other receptors or regulatory mechanisms affect the signal transduction of GPCR. This cross talk between different pathways increases the complexity of each signaling pathway.

The outcome of GPCR signal transduction is formulated by the signaling specificity at the above levels and the interaction of the contributing pathways, regulators and proteins involved in the signaling.

The dopamine D2 receptor (a GPCR) regulates many signaling pathways. Bromocriptine, a dopamine D2 agonist, has been used for a long time to inhibit growth and prolactin-secretion of pituitary adenomas (Colao et al., 1995); (Kanasaki et al., 2000). Recently, gene knockout studies in mice have illustrated the role of D2 receptor in regulation of pituitary development as well as PRL secretion. In mice deficient in dopamine D2 receptors, there was a hyperproliferation of lactotrophs leading to pituitary adenomas (Saiardi et al., 1997); (Kelly et al., 1997); (Asa et al., 1999). Conversely, augmentation of dopamine release in mice lacking the dopamine transporter, which mediates reuptake of dopamine and termination of its action (please refer to introduction), has an apposite pituitary phenotype (Bosse et al., 1997). The mice had hypotrophic pituitaries, due to a lack of somatotrophs and lactotrophs, illustrating the important role of D2 receptors in negative regulation of pituitary cell growth. In contrast to the numerous stimulatory actions in mesanchymal cells, including MAPK activation and increase in cell proliferation (reviewed in introduction), it is shown that D2S receptors have inhibitory effects in pituitary cells. Regarding the known importance of D2S receptors in pituitary in vivo, the detailed signaling pathways of this receptor in pituitary are not completely known. In this thesis, I have tested the actions of the dopamine D2S receptor on specific signaling pathways such as adenylyl cyclase, Ca^{2+} mobilization and MAPK regulation in rat pituitary adenoma cells. I have also looked at signaling specificity and selectivity at G protein level for these pathways.

Signaling specificity of dopamine D2S receptor to regulate adenylyl cyclase:

Coupling of Gi/o proteins to inhibit AC has been described in variety of cell systems for most (if not all) of the receptors in this family as well as dopamine D2 receptors (Clapham and Neer, 1997); (Bourne, 1997b); (Izenwasser and Cote, 1995). The inhibition of AC by Gi/o coupled receptors is considered to be a ubiquitous pathway in all cell systems tested. However, the level of AC inhibition by the receptor could be affected by the Gi/o protein subtypes involved in the inhibition and by the AC isoforms expressed in the cell system. In both Ltk- cells and Balb/c-3T3 cells expressing D2S receptor, activation of D2S receptors inhibits forskolin- and PGE1-stimulated cAMP production (Ghahremani et al., 1999). We observed the same results in GH4ZR7 cells, which is consistent with others (Senogles, 1994a). Ghahremani et al has shown that in Ltk- and Balb/c-3T3 cells, D2S-induced inhibition of forskolin-stimulated AC is mediated by Gαi2 and Gαi3 (Ghahremani et al., 1999); (Ghahremani et al., 2000). To explore further the G protein subunit specificity of this signaling event, we have used a series of PTX-insensitive Gαi/o subunits. Our results indicate that in GH4ZR7 cells, D2S inhibition of forskolin-stimulated AC is mediated mainly by Gαi2 and to a lesser extent by Gαi3. Gαo had no role in this pathway. By using antisense Gαi2 RNA in GH4ZR7 cells, Liu et al have shown that Gαi2 had little influence on inhibition of Gs-stimulated AC (Liu et al., 1994b). The reason for the distinct G protein specificity of these states of AC is not completely clear. It could be that different sensitivity of AC subtypes to forskolin or Gs also indicates the coupling sensitivity to Gi/o subtypes. It is shown that Gαs activates ACII more efficiently than ACI, ACV and ACVI while forskolin preferentially stimulates ACI over ACII, ACV or ACVI (Sutkowski et al.,

1994). Liu et al have shown that the rank order of RNA expression of ACs in GH4C1 cells was II= VI > III>> (I, IV and V) (Liu et al., 1999b). These results suggest that the level of specificity is a dynamic event among the players involved in this signal transduction and is dictated by activation state of AC and the cell content of AC subtypes.

The other part of G protein trimeric complex, i.e. Gβγ is known to influence AC activation. In GH4ZR7 cells, using GRK-ct, to block Gβγ, has no effect on the AC activity. This is consistent with the known biochemical activity of purified Gαi to inhibit AC subtypes *in vitro* (Gilman, 1987).

Dopamine D2S modulation of calcium mobilization:

Dopamine inhibited activation of L-type calcium channels in GH4ZR7 cells and lactotroph cells (Vallar et al., 1990); (Seabrook et al., 1994b); (Lledo et al., 1990). Using different PTX-insensitive mutants of Gα subunits, we have shown that L-type calcium channel inhibition could be rescued by Gαo-PTX, and not Gαi2-PTX or Gαi3-PTX. By using GRK-ct, we also shown that βγ subunit is involved in inhibition of L-type calcium channels in GH4ZR7 cells. Meanwhile, it was observed that depletion of Gαo but not other Gαi subunits, reduced inhibitory coupling of D2S receptors to L-type calcium channels (Liu et al., 1994b); (Liu et al., 1999b). Taken together, our result indicates that it is the Gβγ subunit of Go protein, which is involved in inhibition of L-type calcium channels influx. It is already shown that βγ subunit can directly interact with α_{1B} subunit of N-type calcium channels (Herlitze et al., 1996); (Zamponi and Snutch, 1998), but the mechanism for interaction of this subunit with L-type channels is not clear yet. Knowing the importance of calcium mobilization in different actions of pituitary cells as MAPK activation, PRL secretion and gene transcription, one can realize that Go-mediated

inhibition of L-type calcium channels could play an important role in dopamine-induced inhibition of TRH action.

D2S modulation of MAPK activation:

Studying the role of GPCR on cell proliferation has indicated that multiple signaling pathways are involved in this process. Although cAMP inhibition or increase in IP3/DAG levels may indirectly participate in these events, there is growing evidence implicating other signaling pathways in the regulation of proliferation by heterotrimeric G proteins. Extracellular signal regulated kinase (ERK)- mitogen activated protein kinase (MAPK)- cascade is an important mechanism to transduce mitogenic signals from the cell membrane to the nucleus. In mesenchymal cells like Balb/c-3T3 cells, COS, CHO and C6-glioma cells, D2S activation augments MAPK phosphorylation. There are many studies regarding the pathways implicated in MAPK activation but there are not so many studies regarding inactivation of MAPKinases. Using western blot analysis to measure phosphorylated MAPK in GH4ZR7 cells, we could not detect any phosphorylated MAPK in basal level in these cells, as was observed in GH3 cells previously (Ohmichi et al., 1994a); (Ohmichi et al., 1994b). Ohmichi et al have shown that in GH3 cells TRH activates MAP kinase through a PKC-dependent pathway as well as a second pathway possibly involving tyrosine phosphorylation (Ohmichi et al., 1994b). D2S stimulation produced a rapid decrease in TRH-induced MAPK phosphorylation. This is consistent with what was already reported that dopamine, a physiologic prolactin inhibitory factor, inhibits TRH-induced MAPK phosphorylation in primary cultures of anterior pituitary cells. This inhibition goes through Gi/o proteins because it could be completely reversed

with PTX pretreatment. It is obvious that Gz or other G proteins are not involved in these signaling pathways, since all the D2S-induced inhibition produced in our experiments were completely reversed by PTX treatment. MAPK activation is involved in prolactin synthesis and cell differentiation in rat pituitary cells (Yonehara et al., 2001). It is also demonstrated that 5HT1 agonists inactivate ERK by dephosphorylation and in this way inhibit gene transcription (reviewed in (Albert and Tiberi, 2001). Furthermore, inhibitory forms of p42 MAPK inhibit Ras and Raf activation of the rat prolactin promoter in GH4 pituitary cells (Conrad et al., 1994). So it is possible that D2S stimulation by inhibiting MAPK phosphorylation, through Gi/o proteins, inhibit gene transcription and prolactin synthesis in rat pituitary cells.

Our result demonstrated that among PTX-insensitive Gα mutants, Gαo and Gαi3 could partially recruit D2S inhibition of TRH-induced MAPK phoshorylation, indicating the crucial role of Gαi3 and Gαo in this pathway.

cAMP is a potent mitogen in some cell lines, so AC inhibition could have inhibitory effect in this pathway. At present, no cAMP-dependent effector that mediate inhibitory cross-talk with MAPKs is known. However, in our experiments it was Gi2 that mainly rescued D2S-induced decrease in AC, but for inhibition of TRH-stimulated MAPK phosphorylation, Gi3 and Go were the main subunits. These findings support the idea that D2S inhibits MAPK phosphorylation in a cAMP-independent manner.

The signaling pathway from Gi/o proteins to dephosphorylation of MAPK is not known yet. There are several known pathways that connect GPCR activation to MAPK phosphorylation., among them:

- cAMP dependent Rap1-B-raf signaling pathway, that can activate MAPK in a PKA -dependent or –independent way (de Rooij et al., 1998); (Beebe, 1994).

- PLC/PKC dependent pathway, that can signal through RasGAP -Ras system or Shc –Grb –SoS -Ras system. In any case activation of Ras by GTP binding activates MAPK (Kolch et al., 1993); (Marais et al., 1998).

- Direct interaction of G protein subunits with Rap-GAP, which modulates ERK activity by inhibiting Rap1 activation, inactivating Rap1 activity upon Gi/Go activation, which leads to ERK inhibition (Jordan et al., 1999); (Mochizuki et al., 1999).

- GPCRs can modulate MAPK pathway through transactivation of other receptors as receptor tyrosine kinases (reviewed in (Pierce et al., 2001).

Meanwhile, the relationship between dopaminergic stimulation and decrease in phospho-ERK1/2 is unclear. In a recent study, Kievit et al have shown that overexpression of GTPase activating protein Rap1/GAP reduced basal and stimulated levels of phospho-ERK2 (Kievit et al., 2001). Phosphorylation of C-raf at Ser259 is required for interaction with the scaffolding protein 14-3-3 required for C-raf-mediated activation of MEK1/2 but also inhibits raf activity and thus provides an indirect read-out of C-raf activation (Dhillon et al., 2002); (Jaumot and Hancock, 2001); (Muslin et al., 1996). By measuring phospho-C-raf and phospho-MEK level by western blotting, our results indicate that D2-induced inhibition of TRH-stimulated MAPK involves inhibition of MEK1/2. These observations suggest that the D2 receptor may use a Rap1/B-raf-dependent pathway to inhibit MEK and MAPK, but does not appear to inhibit ras-C-raf activation.

In our immunoprecipitation/kinase assay, we showed that TRH could increase c-Raf kinase and MEK1/2 activity but not B-Raf kinase activity. D2S stimulation completely blocked TRH-induced c-Raf kinase activity indicating that D2S signals through c-Raf to inhibit TRH induced MAPK activity. Meanwhile, D2S inhibits B-Raf to decrease basal level of MAPK42/44 activity. We suggest that D2S through Gαo activates Rap-GAP to inhibit Rap1, one of the main activators of B-raf. The detailed molecules involved in these pathways remained to be clarified.

Another possible mechanism for D2 receptor inhibition of MAPK phosphorylation is MAPK phosphatases (MKPs) activation by the receptor. 5HT1 agonists can inactivate MAPK through calcium-dependent Gi/o-mediated induction of MKPs (Durham and Russo, 1998). This pathway was maximally activated after four hours of receptor stimulation. Our observation that D2 stimulation inhibits ERK1/2 within minutes suggests that more rapid mechanisms be involved. It is also shown by Zhang et al (Zhang et al., 2001) and Ryser et al (Ryser et al., 2001) that in pituitary cell lines and primary culture cells MKPs are induced by hormones that activate PKC and calcium channels (like TRH), contrary to the known signaling pathways of D2 receptors in these cells. Moreover, it is demonstrated that agents that stimulate MAPK signaling, rather than inhibit, increased MKP expression (Brondello et al., 1997); (Cook et al., 1997). So, it seems unlikely that dopamine D2 receptors increase expression of MKPs. Finally, it is also possible that D2 receptors, via activation of Gαi3 and Gαo, use a novel signaling pathway to inhibit TRH-induced MAPK activation.

With our GRK-ct clones and PTX-mutants we showed that Gαi3 and Gαo are involved in this pathway but it seems unlikely that Gβγ subunits have a major

contribution to MAPK inhibition. Interestingly, TRH-induced MAPK activation was also observed in GRK-ct clones, suggesting that Gβγ signaling may not be necessary for TRH-induced activation of MAPK. This is consistent with results observed for other Gq-coupled receptors.

This indicates that G αo/Gαi3 couples to MEK1/2 and MAPK42/44 through c-Raf and B-raf to inhibit TRH-induced and basal MAPK activity respectively, although the intervening steps remain to be identified.

Conclusion:

In contrast to the classical roles of GPCRs as a minute-to-minute regulators of intermediary metabolism, it is increasingly being apparent that heptahelical receptors play vital roles in the longer term regulation of cellular physiology as hypertrophy, apoptosis, cell growth and proliferation. This thesis reveals that different subtypes of Gi/o proteins play a specific signaling role in regulating growth-related pathways by dopamine D2S receptors. It should be remembered that great caution has to be taken when interpreting results obtained in a recombinant setup employing coexpression/overexpression strategies. These observations have to be validated by appropriate studies on signaling components in native environments before real conclusions pertaining to a physiological relevance of experimental findings can be reached.

Furthermore, the results point out to some downstream effectors of dopamine D2 receptor signaling. The physiologic significance of these novel signaling mechanisms remains to be illuminated. Understanding of relevant signaling pathways used by

dopamine system may ultimately reveal novel pharmacological targets and the design of strategies to selectively regulate distinct stimuli in vivo. It is known that the role of MAPK proteins in proliferation is being the result of the balance among different members of MAPK family including ERK 1/2, which we investigated in this thesis, Jun N-terminal kinase (JNK), p38 MAPKinase and ERK5. Therefore, examining the effect of Gi/o-coupled D2S receptor on ERK1/2 only uncovers part of the balance of MAPKinases and the role of other family members should be addressed further.

In this thesis, we have begun to identify novel pathways that mediate actions of D2S receptors. The next step will be to identify proteins that mediate these actions in nervous system where dopamine D2 receptors mediate important actions on control of movement and reward. To further study that, one can follow two approaches: First, to identify the downstream and/or upstream components by using antisense to lower protein expression, or inhibit the coupling by domain negative proteins or inhibitory drugs targeted to known specific player in that pathway. Second, to identify interacting proteins by implying protein-protein interaction systems such as yeast two hybrid. Investigating the role of these pathways in cell growth and proliferation or gene expression will provide insight into not only the downstream events, but also the respective cross talks and regulatory links among pathways. Ultimately these results can contribute to the original knowledge of G protein signaling and regulation of growth and proliferation.

CHAPTER IV

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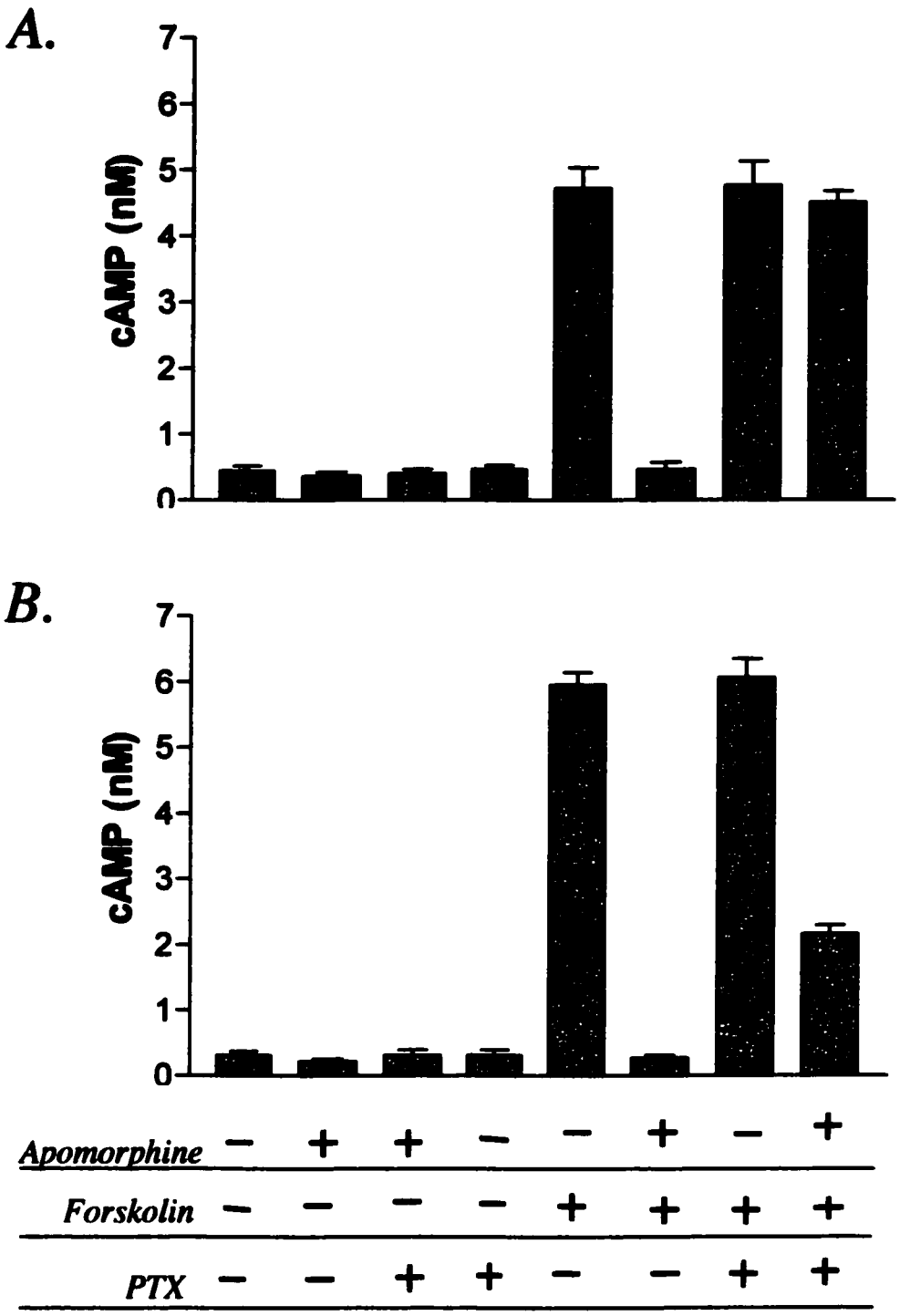
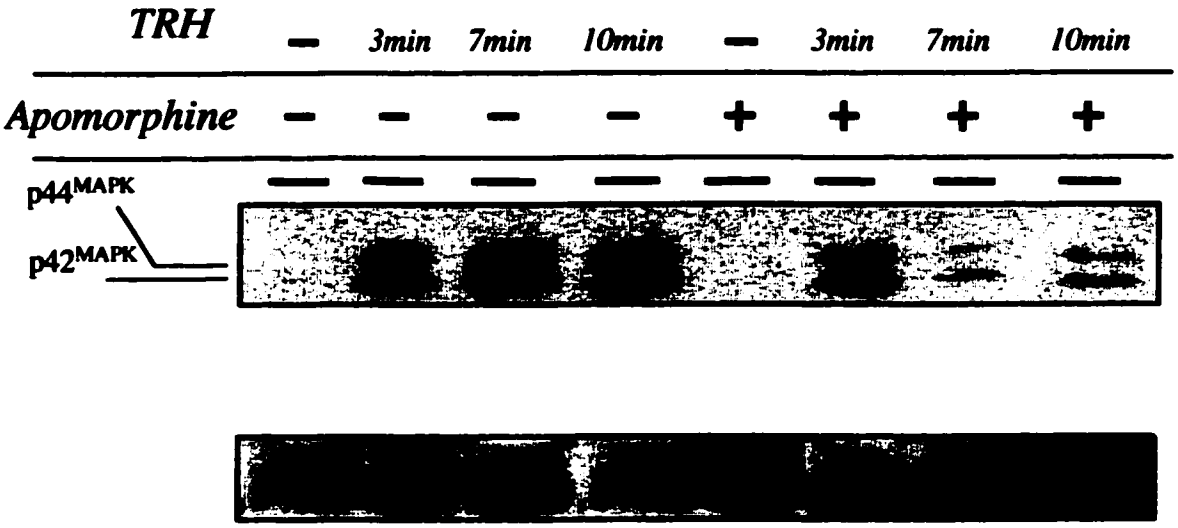
Appendices


A.
cAMP (nM)
0
1
2
3
4
5
6
7
B.
cAMP (nM)
0
1
2
3
4
5
6
7
Apomorphine − + + − − + − +
Forskolin − − − − + + + +
PTX − − + + − − + +

Appendix 1. The effect of apomorphine, forskolin and PTX (separately or in combination) on cAMP level in GH4ZR7 and Gi2Z23 cells.

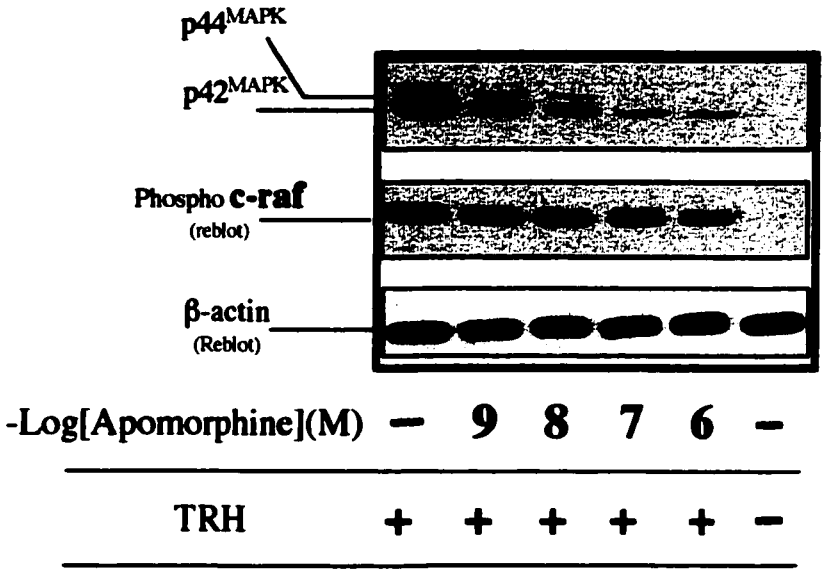
Cells were incubated with forskolin (1μM), apomorphine (1μM), with or without pretreatment with PTX (20 ng/ml, 12h) as indicated. The level of cAMP was measured as explained in Materials and Methods. Graphs are sample results of a set of experiments from 6 separate wells of A, GH4ZR7 cells and B, Gi2Z23 cells.



Reblot: Anti-β-actin

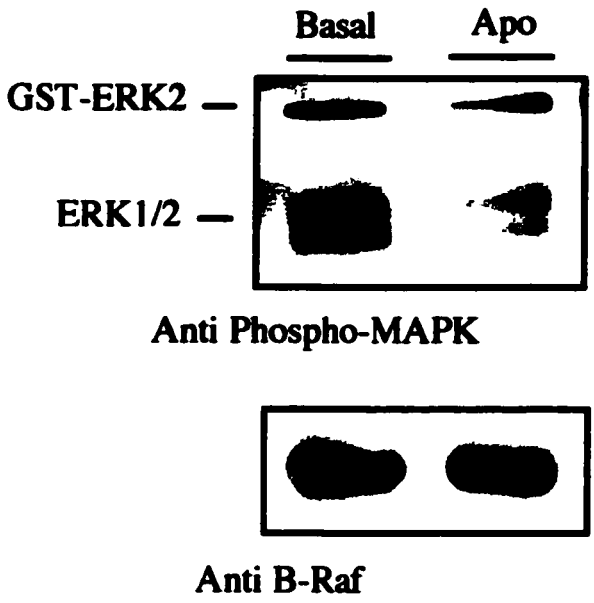
Appendix 2. Time-course inhibition of TRH-induced MAPK phosphorylation by apomorphine.

GH4ZR7 cells were serum starved for 1 hour before assay. For assay, cells were incubated with no drug (control), or pretreated with Apomorphine (1 μM) for 15 minutes, then TRH (1 μM) was added to wells for indicated time, followed by cell lysis (Materials and Methods). Western blot analysis of lysates was done using specific antibody against phospho-p42/44 MAPK. Membranes were reprobed with β-actin antibody as a loading control.

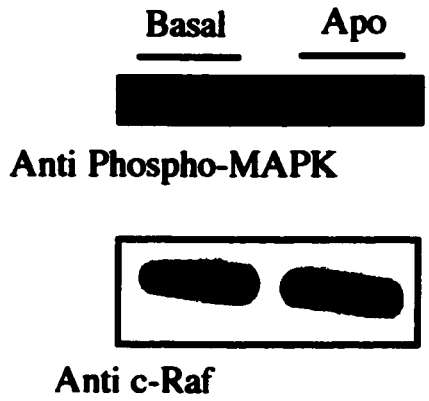

p44MAPK
p42MAPK
Phospho c-raf
(reblot)
β-actin
(Reblot)
-Log[Apomorphine](M) – 9 8 7 6 –
TRH + + + + + –

Appendix 3. Dopamine D2 stimulation by apomorphine does not inhibit TRH-induced (Ser 259)phosphorylation of c-raf in GH4ZR7 cells. Western blot showing the effect of different concentration of apomorphine on GH4ZR7 cells on c-raf phosphorylation. Cells were pretreated with the indicated concentration of apomorphine for 15 min. and then subjected to TRH stimulation for 7 minutes at 37° C.

A.


Basal
Apo
GST-ERK2
ERK1/2
Anti Phospho-MAPK
Anti B-Raf

B.


Basal
Apo
Anti Phospho-MAPK
Anti c-Raf

Appendix 4. Apomorphine inhibits basal level of B-Raf and c-Raf kinase activity. Immunoprecipitation/Kinase assay showing the effect of apomorphine on B-raf (A) and c-raf (B) kinase activityon in GH4ZR7 cells . Endogenous MAPK42/44 were also co-immunoprecipitated with B-Raf and were inhibited by apomorphine treatment. Cells were pretreated with 1μM of apomorphine for 15 min, Immunoprecipitated with c-Raf or B-raf and then subjected to kinase assay.