KUOPION YLIOPISTON JULKAISUJA G. - A.I. VIRTANEN -INSTITUUTTI 28
KUOPIO UNIVERSITY PUBLICATIONS G.
A.I. VIRTANEN INSTITUTE FOR MOLECULAR SCIENCES 28

JOHANNA MAGGA

G protein mediated calcium signaling in the regulation of synaptic transmission

Doctoral dissertation

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ISBN 951-781-387-2
ISBN 951-27-0092-1 (PDF)
ISSN 1458-7335
Kopijyvä
Kuopio 2005
Finland

Magga, Johanna. G protein mediated calcium signaling in the regulation of synaptic transmission. Kuopio University Publications G. - A.I. Virtanen Institute for Molecular Sciences 28. 2005. 78 p.
ISBN 951-781-387-2
ISBN 951-27-0092-1 (PDF)
ISSN 1458-7335

ABSTRACT

Neuronal signaling takes place mainly in the close neuronal contact sites known as synapses. Neurotransmitters packed within vesicles are secreted due to depolarization of the presynaptic terminal, and ultimately, the influx of Ca^{2+} via voltage-gated calcium channels. Secretion is mediated by protein-protein interactions between synaptic vesicles and the plasma membrane, driving the fusion of the membranes, resulting in subsequent neurotransmitter release. Upon activation of G protein-coupled receptors, both N-type and P/Q-type channels undergo a pronounced voltage-dependent inhibition. Gβγ, as well as synaptic proteins (e.g. syntaxin and CSP) are known to couple to voltage-gated calcium channels directly. In this study, the contribution of synaptic proteins syntaxin 1A and CSP to G protein-mediated inhibition of N-type calcium channels was investigated. Syntaxin 1A and CSP were shown to have specific functions in the G protein modulation of N-type channels. In addition, subcellular location of these constituents and the effect of localization on the formation of protein complexes were investigated. Caveolin, the scaffolding protein forming the plasma membrane signaling domains, was shown to interact with synaptic proteins, namely the core complex protein SNAP-25, in a dynamic way. The study focused also on G protein binding properties of a peptidergic receptor involved in divergent calcium signaling pathways. Multiple coupling of the orexin 1 receptor to phosphoinositide activation and adenylate cyclase stimulatory/inhibitory G proteins was shown.
Overall, the data indicate that synaptic proteins promote tonic inhibition of N-type Ca^{2+} channel activity, and thus, are involved in the fine tuning of neuronal transmission. The study presents novel G protein-mediated calcium signaling pathways modulating the presynaptic inhibition or signaling cascades that take part in the regulation of synaptic transmission.

National Library of Medicine Classification: QV 126, QV 276, WL 102.8
Medical Subject Headings: neurotransmitters; synapses; synaptic vesicles; exocytosis; synaptic transmission; vesicular transport proteins; nerve tissue proteins; calcium channels; GTP-binding proteins; caveolae; hippocampus; receptors, G-protein-coupled; receptors, neuropeptide; calcium signaling; signal transduction

TIIVISTELMÄ

G proteiinivälitteinen kalsiumviestitys hermosolujen synaptisen viestinsiirron säätelyssä

Hermosolujen väliseen viestinsiirtoon kuuluu lukuisia vaiheita, joita solutasolla säädellään tarkasti kalsiumin välityksellä. Kalsiumviestitykseen osallistuvien mekanismien selvittäminen on olennaista hermosolujen yksityiskohtaisen toiminnan ymmärtämisessä. Monet keskushermoston sairaudet aiheutuvat solujen häiriintyneestä kalsiumsignaloinnista. Tämä johtuu reseptorien, ionikanavien tai niitä säätelevien proteiinien poikkeavuudesta. Väitöskirjatyössä tutkittiin kalsiumviestitystä ja siihen osallistuvia proteiineja hermosolussa välittäjäaineen vapauttaja- ja vastaanottajapuolella sekä pre- että postsynaptisessa hermosolussa. Kalsium itsessään toimii solujen toisiolähettinä, ja aktivoi solutyypistä ja solun olosuhteista riippuen solun liikkuvuutta, eritystä, kasvua tai kuolemaa. Hermovälittäjäaineet säätelevät hermosolujen toimintaa pääasiassa nopeiden ionikanavareseptoreiden välityksellä viestintäherkkyyttä lisäämällä tai vähentämällä. Lisäksi monet hermovälittäjäaineet, mukaanlukien neurohormonit sekä proteiinirakenteiset neuropeptidit käyttävät G proteiinivälitteistä signalointia hermosoluviestityksen muokkaamisessa ja mukauttamisessa. Väitöskirjatyössä pyrittiin kuvaamaan uusia välittäjäaineen vapautumisen säätelyyn osallistuvia proteiineja sekä luonnehtimaan näiden proteiinien ja tunnettujen viestintäproteiinien välisiä vuorovaikutuksia.

Tutkimuksessa selvitettiin siis välittäjäaineen vapautumista sääteleviä molekulaarisia mekanismeja: miten presynaptiset kalsiumriippuvaiset proteiinit sekä G-proteiinikytkentäiset reseptorit säätelevät välittäjäaineiden vapautumista ja miten tätä viestiä viedään edelleen G-proteiinikytkentäisten reseptorien ja kalsiumin välityksellä postsynaptisessa hermosolussa. Tätä tutkittiin sekä aivokudoksessa, muistitoimintoihin osallistuvassa hippokampuksessa, että soluviljelymalleissa pääasiassa selvittämällä proteiinien sitoutumisia toisiinsa sekä kuvantamalla kalsiumvasteita elävissä soluissa reseptoreja aktivoimalla. Tutkimuksessa keskityttiin selvittämään synaptisen vesikkelin vapautumiseen liittyviä tekijöitä, jotka osallistuvat G proteiinivälitteiseen jänniteherkkien kalsiumkanavien estoon. Aihe on keskeinen koko kemiallisen neurotransmission kannalta ja se tunnetaan kokonaisuudessaan vielä varsin puutteellisesti. Tutkimuksessa saatiin myös molekyylitason tietoa oreksiinipeptidin kalsiumvälitteisestä signaloinnista. Tämä on kiinnostavaa siksi, että oreksiinin vaikutuksia solussa ja elimistössä ei vielä tarkasti tunneta ja että oreksiiniviestitys tapahtuu tavanomaisesta G proteiinisignaloinnista poikkeavien mekanismien välityksellä.

Tutkimuksesta saatavaa tietoa hermoston säätelyjärjestelmistä voidaan käyttää hyväksi kehiteltäessä uusia tai uudentyyppisiä lääkeaineita hermoston sairauksien hoitoon. Kolmasosa nykyisin myynnissä olevista lääkkeistä kohdistuu G proteiinikytkentäisiin reseptoreihin. Aivoista on löydetty yli kolme sataa tämän reseptoriperheen jäsentä, joiden toimintaa ei täysin tunneta. Tämä tutkimus tuo uutta tietoa erityisesti jänniteherkkien kalsiumkanavien inhibitiosta, jota voidaan hyödyntää kehiteltäessä keskushermostoon vaikuttavia lääkeaineita.

ACKNOWLEDGEMENTS

This study was mainly carried out in the University of Calgary during the years 1999-2000. The thesis project was completed in the A.I. Virtanen Institute, University of Kuopio during the years 2002-2004.

I am sincerely grateful to Professor Wayne R. Giles, the Head of the Department of Physiology and Biophysics, for giving me the opportunity to work in the University of Calgary. I am very thankful to Assistant Professor Jan Braun for her precise and patient supervision during the very early years of my research project and also for the advice and co-operation during the later years. It has been a privilege to be part of her Neuroscience research group. I thank Professor Karl Åkerman for the opportunity to work in his Cell Biology research group. I also thank him for familiarizing me further with calcium signaling.

I am very grateful to co-authors and co-workers for the fruitful collaboration.

I am thankful to Professor Eero Castrén, University of Helsinki, and Professor Kid Törnquist, Åbo Akademi University, for careful review of the manuscript and for the constructive comments to improve my thesis. I express my thanks to Dr. Tessa Campbell, University of Calgary, for kindly revising the language.

I am thankful to Professor Pekka T. Männistö, the former Head of the Department of Pharmacology and Toxicology, University of Kuopio, for letting my undergraduate and graduate studies go smoothly despite the unconventional order. I also thank him for the encouragement to continue in the field of research. I thank the personnel in the Department of Pharmacology and Toxicology and in the Department of Physiology who have helped me with the studies.

I thank all the co-workers and friends in the University of Calgary who made the stay in Calgary enjoyable. Special thanks go to my colleagues in the lab and to the Cardiovascular research group lead by Professor Giles.

I thank all the researchers in the Cell Biology and Molecular Signaling research groups for their help and advice. I also thank the personnel in A.I. Virtanen Institute who have assisted me in various ways. My special thanks go to Veera Pevgonen and doctors-to-be Hanna Peltonen, Miia Antikainen, Virve Kärkkäinen and Lauri Louhivuori for cheering up the atmosphere. I also appreciate the multicultural environment provided by the group. I thank my colleagues and friends in and out the Institute who have helped me with the project.

My warmest thanks go to my family and family in-law for their support and care. Finally, I thank my companion Jarkko for supporting me and my studies over the past decade and keeping the time high-spirited as ever. The research projects were financially supported by University of Calgary Funds, the Canadian Psychiatric Research Foundation and the Academy of Finland. The thesis was also supported by the Research and Science Foundation of Farmos.

Kuopio, November 2004

Johanna Magga

ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase enzyme
cAMP	cyclic adenosine monophosphate
CAPS	calcium activated protein for secretion
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CO	carbon monoxide
CRAC	calcium release activated channel
CSP	cysteine string protein
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
ER	endoplasmic reticulum
G protein	guanine nucleotide binding protein
G$\alpha\beta\gamma$	guanine nucleotide binding protein α,β,γ subunits
GABA	gamma amino butyric acid
GAP	GTPase activating protein
GIRK	G protein activated inwardly rectifying potassium channel
GPCR	G protein coupled receptor
GDP	guanosine diphosphate
GRK	G protein coupled receptor kinase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase enzyme
HEK293	human embryo kidney cell line 293
IP_3	inositol 1,4,5-triphosphate
IP_3R	inositol 1,4,5-triphosphate receptor
H_2O_2	hydrogen peroxide
LGCC	ligand gated calcium channel
LTD	long-term depression
LTP	long-term potentiation
MAP	mitogen-activated protein kinase
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
n-Sec1	neuronal Sec1
NSF	N-ethylmaleimide sensitive factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cyclic AMP-dependent protein kinase A
PKC	protein kinase C

PLC	phosphoinositide phospholipase C
RACC	receptor activated calcium channel
RGS	regulator of G protein signaling
RYR	ryanodine receptor
SDS	sodium dodecyl sulphate
SERCA	sarco-endoplasmic reticulum calcium ATPase pump
SNAP	soluble NSF attachment protein
SNARE	soluble NSF attachment protein receptor
SOC	store-operated channel
SNAP-25	synaptosome associated protein
synprint	synaptic protein interaction site on calcium channels
TRP	transient receptor potential channels
TRPC	transient receptor potential canonical
TRPM	transient receptor potential melastatin
VAMP	vesicle associated membrane protein
VGCC	voltage-gated calcium channels

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles referred to by their corresponding Roman numerals:

I Jarvis SE, Magga JM, Beedle AM, Braun JE, Zamponi GW. (2000) G protein modulation of N-type calcium channels is facilitated by physical interactions between syntaxin 1A and Gβγ. J Biol Chem 275:6388-6394

II Magga JM, Jarvis SE, Arnot MI, Zamponi GW, Braun JE. (2000) Cysteine string protein regulates G protein modulation of N-type calcium channels. Neuron 28:195-204

III Magga JM, Kay JG, Davy A, Poulton NP, Robbins SM, Braun JE. (2002) ATP dependence of the SNARE/caveolin 1 interaction in the hippocampus. Biochem Biophys Res Commun 291:1232-1238

IV Magga JM, Bart G, Oker-Blom C, Åkerman KE, Näsman J. Physical and functional interactions of the orexin receptor type 1 with G proteins. Manuscript (in preparation)

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1. INTRODUCTION

Neurological disorders are among the major causes of severe disabilities, the occurrence of which increases with the increase in life expectancy. The cures for neurological disabilities, such as stroke, epilepsy, various degenerative disorders and psychiatric illnesses, are widely sought after, and enormously needed. To be able to intervene in the disorders of the neuronal system, we need to understand the cellular and molecular mechanisms mediating the complex and varied functions of the human brain. We have come a long way since the discovery of vesicular neurotransmitter release, the building block of the neuronal signaling. It is known to be strictly regulated by calcium, the ion, which plays a dominant role in various kinds of cellular signaling and thus, also acts as a second messenger. Lately, the view of the regulation of neurotransmission has broadened out. It is this phenomenon on which this thesis is focused.

Neuronal signals are transmitted from neuron to neuron at specialized sites of contact known as synapses. The cells are electrically isolated from one another, the presynaptic cell being separated from the postsynaptic cell by a narrow synaptic cleft. A change in electrical potential in the presynaptic neuron triggers it to release a small signaling molecule known as a neurotransmitter, which is stored in synaptic vesicles and is released by exocytosis. The neurotransmitter rapidly diffuses across the synaptic cleft and, by binding to transmitter-gated ion channels or receptors in the postsynaptic neuron, provokes an electrical change or metabotropic action in the postsynaptic cell. A typical neuron receives thousands of excitatory and inhibitory inputs, which combine by spatial and temporal summation to produce a grand postsynaptic potential in the cell body. The magnitude of this is translated into the rate of firing action potentials. This encoding is achieved by a special set of gated ion channels that are present at high density at the base of the axon, adjacent to the cell body. For example, in a motor neuron cell in the spinal cord, thousands of nerve terminals synapse on the cell body and dendrites. The nerve terminals deliver signals from other parts of the organism to control the firing of action potentials along the single axon of a particular nerve cell.

The life cycle of the synaptic vesicle begins with the synthesis of synaptic vesicle components in the cell body. The selective targeting of these components to the nerve terminal is mediated by axonal transport. The entire synaptic vesicle cycle takes place locally in nerve terminals. It consists of vesicle exocytosis with neurotransmitter release, endocytosis of empty vesicles and neurotransmitter uptake, and regeneration of fresh vesicles. The vesicle cycle allows synapses to function at long distances from the cell body, a requirement for fast exocytosis and recycling. The synaptic vesicles are triggered to release their contents within a fraction of a millisecond when an action potential arrives at a nerve terminal. The synaptic vesicle life cycle, particularly the fast calcium regulated exocytic and an efficient endocytic process, distinguishes the release of neurotransmitter from other regulated secretory pathways in several ways that are critically important for synaptic function. However, no matter how elaborate the secretory machinery of our highly developed nervous system is, it has not deviated far from the simplest secretory systems like those found in yeast.

2. REVIEW OF THE LITERATURE

2.1. G protein coupled receptors

2.1.1. The role of G protein coupled receptors in the central nervous system

G protein coupled receptors (GPCRs) form a large heterogeneous family of signaling proteins that take part in physiological processes throughout the body, ranging from mammals to plants to protozoa. They convert a variety of divergent signals from exogenous as well as endogenous sources into cellular messages. Sensory signals from extracellular sources, like light and taste, exert their functions via light-sensing rhodopsin or chemosensory GPCRs, respectively. These make approximately half the amount of the total number of human GPCRs, which is currently estimated to be 750 (Vassilatis et al., 2003). The other half consists of GPCRs for endogenous ligands, such as hormones, neurotransmitters, chemokines and local mediators.

Chemosensory (exogenous) GPCRs are not well conserved among different species, and the genes are primarily expressed in the sensory organs. Contrary to this, GPCRs for endogenous ligands are well conserved among the species (e.g. mouse and human) and the genes are widely expressed throughout the body. From the 367 human endogenous GPCRs, 93 % are expressed in the brain (Vassilatis et al., 2003). The large number of GPCRs makes it challenging to clarify the role of multiple GPCRs in physiological and pathological processes. Nevertheless, it also offers the possibility of developing more specific methods to treat malfunctions. Today, 30% of drugs available have an effect on GPCRs. These drugs target only 10% of the endogenous GPCRs, leaving a huge potential for drug development.

Each neuron in the central nervous system (CNS) receives thousands of excitatory and inhibitory synaptic inputs. In the CNS, neuronal signaling mainly relies on fast amino acid transmitters, mostly on the excitatory transmitter glutamate and the inhibitory transmitter gamma amino butyric acid (GABA). In addition, endogenous ligands for GPCRs form another important group of transmitters taking part in brain physiology (Table 1). The discovery that a neuron releases the same transmitter substance at each of its synaptic terminals dates back to 70 years. With the growing evidence that neurons may contain more than one transmitter substance (Hokfelt et al., 1980), the hypothesis has been modified to indicate that the neuron will secrete the same set of transmitters from all its terminals.

At some point, the different pathways, whether excitatory or inhibitory, ionotropic or metabotropic, converge to take part in regulating the same cellular signaling circuitry. Different transmitters may even regulate each other's release from the presynaptic terminal by stimulation of a presynaptic heteroreceptor. In the CNS, neurons are outnumbered by nonneuronal supportive glial cells, which are involved in neuronal signal propagation by taking part in axonal conduction and synaptic transmission. Glial cells assist neurons in many physiological functions such as maintaining the ion balance, controlling ion fluxes, and handling the excessive excreted neurotransmitters (Fields and Stevens-Graham, 2002). Recently, astrocytes, a type of glial cell, have been found to regulate synaptic transmission, both by influencing presynaptic neurotransmitter release and by stimulating postsynaptic

neurons (Newman, 2003). Signal propagation in the multineuronal circuits is by no means straightforward or easily predictable.

As seen in Table 1, GPCRs mediate signals originating from various types of transmitters. The simplest neurotransmitter consists of a single amino acid while the most complex mediators consist of specific peptides. Peptides make a special set of transmitters: they are synthesized by gene expression, and no active reuptake mechanism for peptides has been found. They mainly act in concert with coexisting transmitters (e.g. glutamate, GABA or noradrenaline). However, peptide and classical transmitters are not stored within the same compartments: for example, in a catecholaminergic neuron, amines are found preferentially in small (<70 nm) dense-core vesicles, while peptides are contained exclusively in large (>70 nm) vesicles, most commonly together with amines (Boehm and Kubista, 2002).

2.1.2. G protein-mediated signal transduction

Ionotropic receptors are ligand-gated ion channels, in which the binding of a transmitter induces the conformational change of an ion channel from a closed state to an open one. GPCRs are more complicated in their action: the receptor is accompanied by a heterotrimeric guanine nucleotide binding protein (G protein). When transmitter binds to the receptor, it causes activation of a G protein that binds to a specific ion channel or an effector, which induces the formation of intracellular second messengers. Compared to ion channel receptors, GPCRs convert their message from the transmitter to an intracellular signal relatively slowly, within seconds or minutes. For an ionotropic receptor, signal conversion only takes milliseconds.

GPCRs are seven transmembrane proteins that bind to heterotrimeric G proteins consisting of a guanine nucleotide binding α subunit (Gα) and a tightly bound dimer of β and γ subunits (Gβγ). When in the inactive state, the receptor is associated with a Gα subunit occupied by guanosine diphosphate (GDP). When converted to the active state, the receptor facilitates the binding of guanosine triphosphate (GTP) to the Gα subunit and thus, activation of both Gα and Gβγ subunits and signal transduction cascades mediated by these proteins. Termination of signal transmission results from hydrolyzation of GTP to GDP by an intrinsic Gα subunit enzyme GTPase. This causes the reassociation of Gα and Gβγ subunits and is also thought to be the limiting factor for Gβγ action. The conformation of free Gβγ is identical to Gβγ in the heterotrimer, suggesting that Gα inhibits Gβγ interactions with its effectors through the Gα binding site on Gβγ (Ford et al., 1998). Because Gα can inhibit the actions of Gβγ, the Gα binding residues are candidates for effector activation determinants (Hamm, 1998).

Several different G protein subunits have been identified: 17 Gα, 6 Gβ and 14 Gγ subunits (Gainetdinov et al., 2004; Hermans, 2003). G proteins are divided into four families, Gsα, Giα/Goα, Gqα/G11α and G12α/G13α, according to their Gα subunits that activate different signal transduction pathways (Neves et al., 2002). The Gsα and Gqα/G11α families have well defined effector pathways: activation of adenylyl cyclase (AC) and phospholipase Cβ (PLC) pathways, respectively. The Giα and Goα families are more diverse, as the signal flows through both the Gα and Gβγ subunits. The Gα subunit of Giα inhibits adenylyl cyclase. The Gβγ unit has various effects, such as coupling to phospholipases, ion channels,

Table 1. Neurotransmitter pharmacology in the central nervous system. The lines shown in bold text refer to ionotropic receptors. The rest of the receptors exert their function via G proteins.

Transmitter type	Transmitter	Receptor subtype	Effector	Presynaptic receptor
Amino acid	glutamate aspartate	**NMDA ($NR_{1,2A\text{-}D,3}$)**	**↑Na^+,K^+,Ca^{2+} conductance**	
		AMPA Glu (1-4)	**↑Na^+,K^+ conductance**	
		Kainate Glu (5-7)	**↑Na^+,K^+ conductance**	
		mGlu (1,5)	↑IP_3, ↓K^+ conductance	
		mGlu (2,3,4,6,7)	↓cAMP	$mGlu_4$, $mGlu_6$
	GABA	**$GABA_A$ (isoforms consist of α,β,γ,δ,σ)**	**↑Cl^- conductance, ↓cAMP**	
		$GABA_B$	↑K^+,Ca^{2+} conductance	
	glycine	**Consist of α/β subunits**	↑Cl^- conductance	
Amine	acetyl-choline	**Nicotinic (α2-4,β2-4)**	**↑Na^+,K^+,Ca^{2+} conductance**	
		Muscarinic (M1-5)	M1,M3, M5: ↑IP_3, ↓K^+ conduct. M2,M4: ↓cAMP, ↑K^+ conduct.	M3 M2
	dopamine	D (1-5)	D1,D5: ↑cAMP D2:↓cAMP,↑K^+,↓Ca^{2+}conduct.	D2, D3
	noradrenaline	$\alpha_{1A\text{-}D}$	↑IP_3, ↓K^+ conductance	
		$\alpha_{2A\text{-}C}$	↓cAMP, ↑K^+, ↓Ca^{2+} conduct.	α_{2A}
		$\beta_{1\text{-}3}$	↑cAMP	β_2
	serotonin	$5\text{-}HT_{1A\text{-}F}$	↓cAMP, ↑K^+ conductance	$5\text{-}HT_{1A,1B,1D}$
		$5\text{-}HT_{2A\text{-}C}$	↑IP_3, ↓K^+ conductance	
		$5\text{-}HT_3$	**↑Na^+,K^+ conductance**	
		$5\text{-}HT_{4\text{-}7}$	$5\text{-}HT_{4,6,7}$: ↑cAMP	
	histamine	H_1	↑IP_3, ↓K^+ conductance	
		H_2	↑cAMP	
		H_3		H_3
Peptide	opioids:			
	endorphins	μ	↓ cAMP, ↑K^+ conductance	
	enkephalins	δ	↓ cAMP, ↑K^+ conductance	
	dynorphins	κ	↓Ca^{2+} conductance	
	substance P	Tachykinins: NK1, NK2, NK3	↑IP_3, ↓K^+ conductance	
	neuropeptide Y	Y_1, Y_2, Y_3,	↓ cAMP, (↑IP_3), ↓ Ca^{2+},↑K^+ conductance	
	cholecystokinin	CCK_A, CCK_B	↑IP_3, ↓K^+ conductance	
	somatostatin	SS1 SS2 SS3, SS4	↓ cAMP	
	orexins	OX1	↑Ca^{2+},Na^+ conductance ↑IP_3	
		OX2	↑Ca^{2+},Na^+ conductance, ↓ cAMP, ↑cAMP	
Purine	adenosine	P (A_1, A_{2A}, A_{2B}, A_3)	↓ cAMP, ↓ Ca^{2+},↑K^+ conduct.	
	ATP	P_{2X}	↑Ca^{2+},K^+,Na^+ conduct.	
	ATP	P_{2Y}	↑IP_3, ↓K^+ conductance	

adenylyl cyclases and various kinases and accessory proteins (Clapham and Neer, 1997). Once activated, Gα and Gβγ contribute to the modulation of either the same or unrelated effectors in a synergistic or an antagonistic way. Gβγ effects often require higher concentrations of agonist than those mediated by Gα (Hermans, 2003), probably reflecting the lower potency of Gβγ in activating the effectors. However, for some receptors, the action of Gβγ may be the dominant signaling route (Stehno-Bittel et al., 1995). Concentration of Giα/Goα proteins in the cell considerably exceeds those of other families, especially in brain, where Goα may amount to 1-2 % of membrane protein (Gudermann et al., 1997). Functions of the G12α/G13α family and receptors preferentially coupling to this family are not yet fully established.

The different G protein families route signals to several distinct intracellular signaling pathways. However, these pathways interact with one another to form a network that regulates components of the cellular machinery controlling of cellular processes such as transcription, motility, contractility and secretion (Neves et al., 2002). These cellular processes in turn regulate systemic functions such as embryonic development, organismal homeostasis and learning and memory. The members of the same GPCR family can activate different G proteins, as is the case with α_2 and β adrenergic receptors, which activate Giα and Gsα subunits, respectively. Also, a single receptor can activate more than one type of G protein and therefore modulate multiple intracellular signals (Hamm, 1998). On the other hand, multiple receptors can converge on a single G protein. The different G protein mediated pathways may also modulate each other at the effector level. For example, there is an interaction between two G protein coupled signal transduction pathways by cAMP and PLCβ effector crosstalk (Liu and Simon, 1996).

Action of GPCRs is highly regulated in various levels and the recent data has proven G protein signaling to be even more versatile than it previously was thought to be. First, the classification of GPCRs strictly as specific G protein activating subtypes can no longer be considered accurate, as there is growing evidence for multiple coupling and even for switching of coupling to different G proteins. Second, specific GPCRs have been shown to perform dimerization or oligomerization with the same subtype of receptors and also with different GPCRs as well. Third, GPCRs may be regulated and even coupled physically with other types of receptors and effectors. In some cases, GPCRs may transfer signals in a G protein-independent way. These issues will be discussed further in the following section.

2.1.3. Versatility of G protein coupled signaling

G protein mediated signal transduction is a complex signaling network with diverging and converging transduction steps at each coupling interface. There is increasing evidence that many GPCRs have the ability to couple to multiple G proteins. This has been documented for closely related G proteins, such as Gqα and G11α in muscarinic receptors (Mullaney et al., 1993), or Giα and Goα in metabotropic glutamate receptors (Kammermeier et al., 2003). This has also been shown to happen with distinct types of G proteins and a variety of different receptors, such as in dopamine (Kimura et al., 1995), serotonin (Cussac et al., 2002) and peptidergic receptors, such as neurotensin (Gailly et al., 2000) and orexin (Zhu et al., 2003). In some situations, the coupling to multiple G proteins is related to anatomical differences, such as different brain regions (Jin et al., 2001) or different cell types (Selkirk et

al., 2001). This may suggest that coupling occurs in different neuronal populations in the brain and vice versa. The multiple coupling may also reflect the level of receptor expression (Cordeaux et al., 2000). In addition, the switching of coupling may follow the phase of the cell cycle (Abel et al., 2000), the developmental phase, or changes in the energy status (Karteris and Randeva, 2003). In some cases, receptor regulation (e.g. by phosphorylation) may switch the coupling to a different G protein (Vazquez-Prado et al., 2003). Phosphorylation of adrenergic β_2 receptors by protein kinase A (PKA) switches the coupling of the receptor from Gsα to Giα (Daaka et al., 1997).

Differential coupling may also be the result of alternative splicing. Though most mammalian GPCRs (>90 %) have no introns, splice variation has since been documented in many GPCR types, where differences in ligand binding, signaling efficiency and desensitization have been reported (Minneman, 2001). Alternative splicing may lead to distinct and possibly antagonistic functions: splice variants of the dopamine D2 receptor (D2 short and long forms) act at postsynaptic sites or serve presynaptic autoreceptor functions, respectively (Usiello et al., 2000). Some GPCRs spontaneously assume an active conformation without agonist binding, and differences in such constitutive activity have been found for several splice variants. Constitutively active GPCRs have been linked to many diseases, such as hyperthyroidism and hypocalcemia (Parnot et al., 2002). In addition, many clinically important medicines, such as the antipsychotic agent olanzapine or the antihypertensive agent losartan, have been demonstrated to act as inverse agonists, i.e. to negatively antagonize the function of the receptor by converting it from the active state to an inactive one (Milligan, 2003).

Recent studies have shown that GPCRs can assemble as homo- and hetero-oligomeric complexes. This can result in altered receptor ligand binding, signaling or intracellular trafficking. Since many individual GPCRs can exist as homodimers, the GPCR dimerization may be regarded as a requisite for function (Milligan, 2004). GPCRs have been demonstrated to have a natural tendency to form hetero-oligomers when co-transfected into cells (Salim et al., 2002), as indicated by serotonin $5HT_{1A}$ receptors coupling to multiple receptors. Many studies performed with dopamine receptors show oligomerization to be characteristic of the dopaminergic neurotransmitter system. Antagonistic adenosine/dopamine interactions in the CNS may partly be caused by an intramembrane interaction between subtypes of adenosine and dopamine receptors, specifically between A_1 and D1 and between A_{2A} and D2 (Gines et al., 2000). Also, D2 and the somatostatin receptor SSTR5 interact physically to create a novel receptor with enhanced functional activity (Rocheville et al., 2000).

Structurally and functionally distinct neurotransmitter receptors have been demonstrated to couple as well. For example, dopamine receptor D5 may couple physically with the $GABA_A$ ligand gated channel leading to mutually inhibitory interactions between the systems (Liu et al., 2000). This also shows that a GPCR can dynamically regulate synaptic strength independently of classically defined second messenger systems. In addition, GPCRs have been shown to transduce proliferative signals through the activation of receptors with intrinsic tyrosine kinase activity (Vazquez-Prado et al., 2003). Tyrosine kinase receptors can also negatively modulate the activity of GPCRs. Furthermore, GPCRs couple widely to signaling proteins in a heterotrimeric G protein-independent way. These GPCR interacting proteins, such as various ion channels, Ca^{2+}-binding proteins, Na^+/H^+ exchanger related proteins, non-receptor tyrosine kinases, nitric oxide synthases, and small G proteins, enable

neurotransmitters to directly contribute to cell survival, proliferation, and differentiation and to regulate the trophic responses (Heuss and Gerber, 2000). Also, a variety of GPCR interacting proteins serve as accessory/chaperone molecules to modulate ligand recognition, signaling specificity and receptor trafficking (Brady and Limbird, 2002).

An important feature of G protein signaling systems is that they are not constant but exhibit a memory of prior activation or signaling tone. High activation of the GPCR leads to a reduced ability to be stimulated, i.e. desensitization, while low activation leads to an increased ability to be stimulated, i.e. sensitization. Thus, regulation of GPCRs is modulated with accessory proteins, such as the regulators of G protein signaling (RGS) that act as GTPase activating proteins (GAPs) to promote the inactivation of GTP-bound Gα. The activated state of GPCRs also serves as a substrate for protein phosphorylation by a family of GPCR protein kinases (GRKs). Phosphorylation of the receptor leads to binding of an arrestin protein that further leads to binding of various other factors as well as displacement from the cell membrane (Gainetdinov et al., 2004). Furthermore, activity of GRKs can be regulated with Ca^{2+} sensing proteins, such as calmodulin and neurocalcin (Sallese et al., 2000). Prolonged activation by the agonist leads to down-regulation of the receptor.

2.2. Neuronal Ca^{2+} signaling

2.2.1. Receptor operated Ca^{2+} channel maintenance of intracellular calcium balance

Calcium is the ultimate molecule to trigger physiological functions in all the cell types. It is one of the most versatile and universal signaling agents. Since many different signaling pathways are facilitated with the same molecule, it is the spatial and temporal aspect of the Ca^{2+} signaling that matters, and the amplitude, to a lesser extent (Berridge et al., 1998). Within a cell, the signaling molecules are clustered in signaling domains, which, when activated, lead to mediation of a specific set of messages. Therefore, different signaling domains can be regulated with local Ca^{2+} spikes. Ca^{2+} signals may propagate within the cell to form a global Ca^{2+} wave or may act intercellularly. As prolonged increases in intracellular signaling lead to cellular death, cells prefer to use brief, low-amplitude signals. When information has to be relayed over longer time periods, cells use repetitive signals (e.g. Ca^{2+} oscillations). Both the local and global signals can oscillate. Ca^{2+} sensing accessory proteins serve as counters translating the repetition of signals to different messages. In neurons particularly, the combination of local and global signals has been adapted to regulate a range of processes.

A wide variety of Ca^{2+} channels present in neurons make the picture of neuronal Ca^{2+} signaling intricate. Cytosolic Ca^{2+} may originate from two different sources, extracellular or intracellular, following activation of plasma membrane Ca^{2+} channels or endoplasmic reticulum (ER) channels, respectively. The plasma membrane and the ER form a binary membrane system that functions to regulate a variety of neuronal processes including excitability, neurotransmitter release, synaptic plasticity and gene transcription. In addition, the balance between the ER and the mitochondrial Ca^{2+} concentration is crucial for the well-being of the cell. Overload of the mitochondrial Ca^{2+} store leads to abnormal mitochondrial metabolism that may activate the programmed cell death pathways (Berridge et al., 1998).

Also, the Golgi has been found to have Ca^{2+} uptake and release activity that may modulate the receptor-evoked Ca^{2+} signal (Pinton et al., 1998). Therefore, there are many mechanisms to remove Ca^{2+} from the cytosol: the high affinity Ca^{2+} pumps of the ER and plasma membrane, the low affinity high capacity Na^+/Ca^{2+} exchanger in the plasma membrane, and the mitochondrial Ca^{2+} uniporter (Usachev et al., 2001).

Neurons have an elaborate ER that extends throughout the cell and appears to be a continuous membrane system (Werry et al., 2003). However, it consists of spatially distinct Ca^{2+} compartments that can be individually loaded and unloaded (Blaustein and Golovina, 2001). Beginning in the soma, the ER extends up into dendrites and down the axon. Ca^{2+} release occurs by activation of the inositol 1,4,5-trisphosphate receptors (IP_3R) or ryanodine receptors (RYR), expressed throughout the ER or mainly in the soma, respectively (Berridge, 1998). The phosphoinositide system is particularly well developed in the brain. Activation of PLC stimulates the hydrolysis of the membrane bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to form the second messengers, diacylglycerol (DAG) and IP_3. The binding of IP_3 increases the sensitivity of IP_3R to Ca^{2+}, which has a biphasic action. Low concentrations of Ca^{2+} (100-300 nM) are stimulatory but above 300 nM Ca^{2+} becomes inhibitory and switches the channel off (Berridge et al., 2000; Berridge et al., 2003). Release of Ca^{2+} is sensitive to a variety of factors, including Ca^{2+} itself. An increase in the resting cytosolic Ca^{2+} level as well as an increase in the luminal Ca^{2+} increases the sensitivity of IP_3R to IP_3. Ca^{2+}-induced Ca^{2+} release may also occur, which is responsible for amplifying Ca^{2+} signals coming from the outside and for setting up Ca^{2+} waves. This phenomenon is mainly related to RYRs and may display the function of RYRs as amplifiers of the Ca^{2+} signals originating from the IP_3 pathway (Berridge, 1998). RYRs are also activated by cyclic ADP ribose, mainly in a Ca^{2+} co-operative way.

The amount of stored Ca^{2+} in the ER lumen is highly variable. The store may be only partially full under resting conditions. Since the ER is capable of fast sequestration of cytosolic Ca^{2+} through its sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) pumps, it represents a major buffering system as a sink for Ca^{2+} signals. Therefore, the ER can function as a memory and an integrator of the oncoming signals. This way, the localized Ca^{2+} transients that are rapidly buffered may be gathered up to transmit regenerative global signals (e.g. for gene transcription) (Berridge, 1998). Whereas the plasma membrane memory depends on the phosphorylation of the signaling molecules, the ER uses the accumulation of luminal Ca^{2+} as a memory of the previous events. In addition, the release of Ca^{2+} from the ER modulates neuronal excitability by altering the membrane potential by inducing the Ca^{2+}-activated potassium channels.

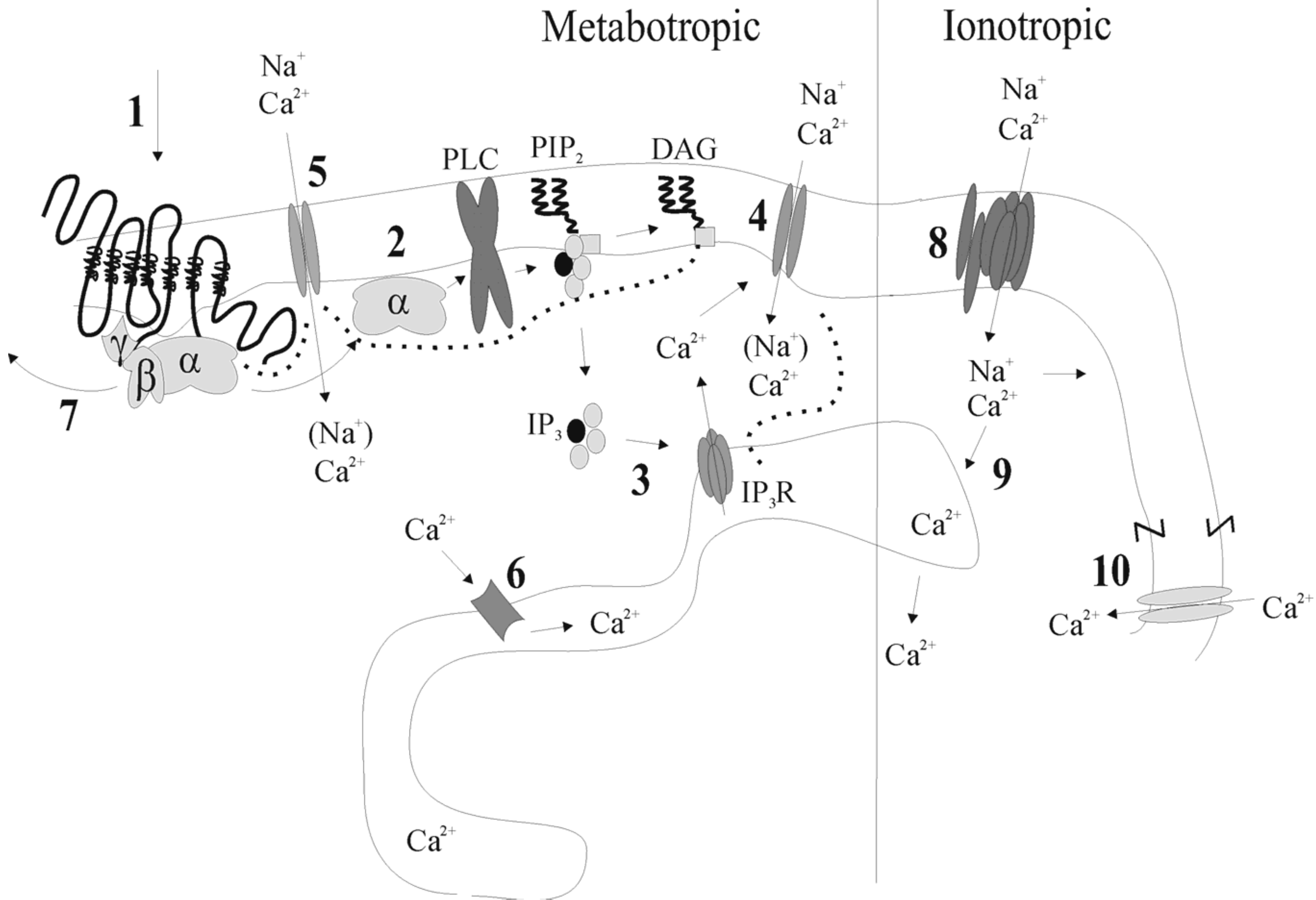
There is a variety of Ca^{2+} channels in the plasma membrane that take part in maintaining the balance of intracellular Ca^{2+} stores and to enhance the signaling evoked by Ca^{2+} release. These receptor-activated channels include: 1) channels activated by intracellular messengers (e.g. cGMP, cAMP, IP_3 or arachidonic acid) 2) store-operated channels (SOC), the activation of which require the release of calcium from the ER and 3) calcium channels activated by direct interaction between the channel protein and a trimeric G protein (Barritt, 1999). A variety of coupling mechanisms have been proposed to SOC activation (Putney, Jr. et al., 2001). ER store depletion may cause the release of a diffusible messenger that activates plasma membrane SOC. Depletion of stores may also activate physical interactions rather than a diffusible messenger: depletion of stores may cause an insertion of channels into the

membrane by vesicle fusion or, cause a conformational change that leads to coupling between the ER and the channels in plasma membrane by a direct protein-protein interaction. Also, the change in the calcium concentration itself may activate store-operated channels by a calcium sensing mechanism. There is a family of receptor-activated nonselective Ca^{2+} channels called transient receptor potential channels (TRP) that is involved in a wide variety of activation mechanisms and functional couplings in neurons. They mainly act as cellular sensors that translate fluctuations in the extracellular milieu into changes in membrane excitability and second messenger signals, particularly Ca^{2+} (Clapham, 2003). They have also been adapted to respond to stimuli within the cell. TRP channels are activated by GPCRs and tyrosine kinases as well.

As TRP channels work as sensors for both physical and chemical stimuli, they seem to be much more than SOCs (or CRACs, to a lesser extent) mediating the capacitative Ca^{2+} entry. The intracellular candidates for the modulation mechanisms are many: IP_3, PIP_2, PLC, DAG, DAG derived fatty acids such as arachidonic acid, PKC, and naturally, Ca^{2+} and Ca^{2+} binding proteins (Barritt, 1999; Obukhov et al., 1998; Hardie, 2003; Montell et al., 2002). The search for a receptor-activated messenger that directly binds and specifically activates a TRP channels still continues. Also, the physiological role of these channels is not known. Disturbed Ca^{2+} signaling originating from the abnormal function of specific sets of TRP channels has been encountered in many severe diseases such as muscular dystrophy (Vandebrouck et al., 2002), hypomagnesia (Schlingmann et al., 2002), and polycystic kidney disease (Cantiello, 2004; Koulen et al., 2002).

2.2.2. Ca^{2+} channel activation and modulation of exocytosis

There is increasing evidence that exocytosis might be regulated by the release of Ca^{2+} from intracellular stores (Berridge, 1998). Since TRP channels are open for many seconds, allowing Ca^{2+} to accumulate in the cytoplasm or to bind to Ca^{2+} sensors, TRP channels are also hypothesized to regulate exocytosis (Obukhov and Nowycky, 2002; Munsch et al., 2003). Together with voltage-gated Ca^{2+} channels (VGCC) and store-operated channels, these receptor-operated channels form an additional pathway for Ca^{2+} entry to support secretory and possibly other Ca^{2+} dependent processes in neuroendocrine cells. A similar mechanism may be involved in stimulating or modulating exocytosis in other neuronal systems in physiological or pathophysiological conditions (Obukhov and Nowycky, 2002). As TRPs are nonselective cationic channels, the conductance of Na^+ may lead to depolarization and the opening of VGCCs, and thus, exocytosis (Barritt, 1999). Na^+ may also alter the uptake of Ca^{2+} by mitochondria and activate the plasma membrane Na^+/Ca^{2+} exchanger. Some studies suggest a reverse mode Na^+/Ca^{2+} exchange for the Ca^{2+} entry mechanism (Rosker et al., 2004). Mitochondria may contribute to synaptic potentiation by releasing Ca^{2+} through its Na^+/Ca^{2+} exchangers upon cell stimulation (Yang et al., 2003). TRP channels have been linked to glutamatergic transmission in many studies. Metabotropic glutamate receptors have been demonstrated to activate postsynaptic conductance mediated by TRPC1 channels (Gee et al., 2003; Kim et al., 2003). Also, TRPM8, when activated by menthol, has been shown to contribute to Ca^{2+} entry and the release of Ca^{2+} from intracellular stores in the sensory neurons. This demonstrates that specific TRP channels might be able to facilitate spontaneous glutamate release as well as to potentiate evoked glutamate release presynaptically (Tsuzuki et al., 2004).



Metabotropic
Ionotropic
1
2
3
4
5
6
7
8
9
10
PLC
PIP_2
DAG
IP_3
IP_3R
α
β
γ
Na^+
Ca^{2+}
(Na^+)

Figure 1. Cellular Ca^{2+} signaling. *Metabotropic signaling mediated by a Gq coupled receptor.* 1. Ligand (neurotransmitter) binds to a GPCR, activating the Gqα and Gβγ subunits. 2. Gqα activates PLC that hydrolyzes PIP_2, giving rise to second messengers, membrane bound DAG and soluble IP_3. 3. IP_3 exerts its function by binding to IP_3R located in the surface of the ER, activating Ca^{2+} channels in the ER and causing the release of Ca^{2+} from the intracellular stores. 4. IP_3 pathway-activated Ca^{2+} or store-derived diffusible messenger, activate store-operated Ca^{2+} channels in the plasma membrane, causing the influx of Ca^{2+}, to keep the Ca^{2+} balance inside the cell. SOCs also probably have other functions in the cell. 5. DAG-derived or activated second messengers, such as AA, other fatty acids, PKC, the G protein subunits themselves, or other G protein linked messengers, activate the receptor-operated Ca^{2+} channels in the plasma membrane, causing the influx of Ca^{2+}, or in the case of a nonselective channel, the influx of both Ca^{2+} and Na^+. Na^+ may cause depolarization of the cell, leading to propagation of the signal further from the site of signaling. 6. Excessive Ca^{2+} is pumped out of the cytosol, to the ER, or to the outside of cells (plasma membrane Ca^{2+} pumps or exchangers are not shown). 7. Gβγ may display multiple functions, coupling directly with enzymes or ion channels, or by acting synergistically or antagonistically with Gqα pathways. *The stimulatory ionotropic signaling*. 8. The ligand (neurotransmitter) binds to an ionotropic receptor, activating and thus, opening the ion channel, causing the influx of Ca^{2+}/Na^+, leading directly to Ca^{2+} actions or depolarization induced Ca^{2+} influx and Ca^{2+} activated pathways. 9. Ca^{2+} itself may enhance the signaling by releasing more Ca^{2+} from the stores and thus, initiating a more global signal. 10. Depolarization propagates along the membrane, finally reaching its targets in soma or nerve terminals, leading to synaptic vesicle exocytosis, in assistance with VGCCs. In neuronal signaling, the ionotropic and metabotropic pathways overlap in a co-operative or contradictory way.

Regulated exocytosis is ultimately activated by intracellular Ca^{2+} increases mediated through voltage-gated Ca^{2+} channels (VGCC) at the presynaptic terminal. N-type and P/Q-type VGCCs play the predominant role in neuronal transmitter release. At some nerve terminals, only N-type channels contribute to transmitter release and in others, only P/Q-type channels contribute, but in many terminals both sub-types are active. The α_1 pore-forming subunit of the channel is a key determinant of the properties of the channel subtypes. The P/Q-type channels contain α_{1A} subunits, whereas N-type channels contain α_{1B} subunits. A robust form of synaptic inhibition is produced by the down-regulation of Ca^{2+} channel function by a G protein-mediated mechanism. For N-type channels, G protein-mediated modulation is stronger than for P/Q-type channels. Therefore, the terminals with a greater complement of P/Q-type channels will be blocked less than those dominated by N-type channels. N-type channels play a major role in developing synapses, while P/Q-type channels become more prominent during maturation. Hence, the immature, N-type occupied terminals are more likely to be inhibited by diffuse modulators. This may provide a protective mechanism by which the immature synapses are shut down under conditions of neuronal hyperexcitability (Reid et al., 2003). The non-uniform distribution of VGCCs has also other beneficial roles for regulated synaptic transmission as they are both present in mature synapses as well.

Different Ca^{2+} entry pathways (voltage gated (VGCC), ligand gated (LGCC) and receptor activated (RACC)) may have evolved to allow the generation of Ca^{2+} signals with different intensities, durations and spatial locations and to accommodate the different intracellular sites of the enzymes and proteins targeted for Ca^{2+} action. VGCCs deliver large quantities of Ca^{2+} to specific restricted locations in the subplasmalemmal space. Restriction is achieved by cytoplasmic Ca^{2+} buffers and by the rapid inactivation of open VGCCs. The opening of LGCCs, such as ionotropic glutamate receptors, leads to inflow of large amounts of Na^+ and

Ca^{2+}. Na^+ causes depolarization of the membrane and the opening of VGCCs, consequently, which admit further amounts of Ca^{2+} into the intracellular space. LGCCs cause a rapid and large increase in Ca^{2+}, which together with VGCCs induce cell responses such as exocytosis. The roles of RACCs are more diverse, as there are a variety of different channels and mechanisms of activation and function. GPCRs are often attached to multimolecular complexes by scaffolding proteins, adding an extra level of complexity (Clapham, 2003).

2.3. Transmitter release controlled by presynaptic G protein-mediated receptors

2.3.1. Presynaptic receptor control of transmitter release

It has long been known that impulse propagation in a neuron, and thus, transmitter release, is the summary of excitatory and inhibitory inputs of a given neuron. The firing is either an on or off phenomenon. However, only about one third of the action potentials invading the nerve terminal are able to cause significant vesicle exocytosis (Goda and Sudhof, 1997). Thus, voltage dependent vesicle release is not strictly an event determined by depolarization and activation of VGCCs. Many studies have proven that this event can be regulated with presynaptic receptors, both ionotropic and metabotropic, in a membrane excitability altering or VGCC modulating way (Boehm and Kubista, 2002). The action may be stimulatory or inhibitory, which does not necessarily correspond to the role the neurotransmitter usually plays in the postsynaptic signaling. For example, GABA has been shown to exhibit both stimulatory and inhibitory effects on transmitter release depending on the intracellular ionic balance, Cl^- particularly (Boehm and Kubista, 2002).

For many receptors, there is a specific subtype of the receptor that takes part in presynaptic signaling. Therefore, some of these presynaptic receptors may pharmacologically be distinguished from the postsynaptic receptors. The corresponding antagonists may inhibit presynaptic inhibition of transmitter release and thus, act as transmitter release enhancing drugs. Therefore, the antagonist can stimulate neurotransmission and, in this regard, the agonist can inhibit the release of a transmitter and thus, lead to inhibition of synaptic transmission. Presynaptic receptors may be activated with transmitters released from the very same nerve ending or from a different axon terminal. Presynaptic receptors sensitive to the transmitter it releases on its own can also be referred as autoreceptors (Raiteri, 2001). The homologous receptors do not, however, always mediate the regulatory effect on transmitter release as they may have other functions as well (Kalsner, 2001).

Presynaptic ionotropic receptors may alter release by many mechanisms (Boehm and Kubista, 2002). First, they can interrupt the action potential invading the axon terminal by manipulating the conductance of cation or anion channels. Second, they can shorten the action potentials by augmentation of repolarizing K^+ currents. Third, they can prolong the action potential duration through mechanisms opposite to aforementioned ways, which will augment Ca^{2+} entry. Finally, the presynaptic receptors can directly facilitate or inhibit transmembrane Ca^{2+} entry, leading to triggering or diminishing Ca^{2+} dependent exocytosis, respectively. In addition to presynaptic ionotropic receptors, most types of G proteins have been shown to be potentially involved in the presynaptic regulation of VGCC and K^+ channels. The following paragraph will focus on metabotropic presynaptic signaling that takes part in the regulation of vesicle exocytosis.

Various protein kinases, such as cAMP dependent kinase (PKA), PKC, Ca^{2+} calmodulin dependent kinase, mitogen-activated protein kinase (MAP), cyclin-dependent kinase, and PI3K have been shown to activate neurotransmitter release positively (Takahashi et al., 2003). Metabotropic facilitatory presynaptic signaling may originate from the stimulation of cAMP-dependent cascades or IP_3 or DAG-dependent mechanisms. Presynaptic GPCRs that facilitate transmitter release are thus linked to Gsα or Gqα proteins. The action of cAMP is likely mediated by activation of cAMP dependent protein kinases that may augment the Ca^{2+} currents or act downstream of Ca^{2+} entry at the level of exocytotic machinery. Also, DAG mediated modulation is probably due to activation of PKC that facilitates transmitter release via a direct effect on exocytotic machinery. To a lesser extent, the Gqα activated pathways may lead to facilitation by IP_3 mediated liberation of Ca^{2+} from intracellular stores or by the direct interaction of PKC with VGCCs. G protein activation may lead to an inhibition of VGCCs, and thus cause an inhibitory effect on transmitter release via the liberation of Gβγ subunits. This effect can be observed with both stimulatory and inhibitory G proteins but is more prominent with inhibitory G proteins, Giα and Goα (Boehm and Kubista, 2002).

Some GPCRs that are able to couple to multiple pathways may reduce transmitter release by inhibition of VGCCs and stimulate release through generation of intracellular messengers, such as cAMP or PKC. Also, receptors linked to Giα/Goα control not only the gating of VGCCs but also the synthesis of cAMP in an inhibitory manner. In addition, PKC may also originate by Gβγ mediated activation of PLC, which demonstrates that activation of inhibitory GPCRs may support the facilitatory action of Gqα coupled receptors (Boehm and Kubista, 2002). Also, it has been shown that PKC prevents the inhibitory effect of Gβγ (Zamponi et al., 1997) and, thereby, may counteract the reduction of transmitter release caused by inhibitory presynaptic receptors (Cox et al., 2000). The complex interactions of GPCRs may happen at the effector (see above) as well as receptor level. Receptor interactions can occur by ionotropic-ionotropic, ionotropic-metabotropic or metabotropic-metabotropic methods. The activation or inhibition of a single GPCR, for example, can cause the same response or a contradictory one to another receptor without the specific signal for the latter receptor. This works as a spare mechanism since activation of two such receptors at the same time does not cause more stimulation or inhibition than the activation of one receptor only (Boehm and Kubista, 2002).

As seen above, the different presynaptic receptors work in a synergistic or inhibitory way to modulate transmitter release. This way, one presynaptic receptor may stimulate, facilitate, or reduce transmitter release in addition to interacting with the functions of other presynaptic receptors. As a whole, presynaptic modulation can be regarded as a safety procedure, allowing efficient transmission during neuronal activity and yet serving as a mechanism to prevent overstimulation (Boehm and Kubista, 2002). Transmitters that act on both ionotropic and metabotropic receptors, such as glutamate and GABA, play an especially interesting role in the field of regulation of transmitter release. For example, whereas ionotropic $GABA_A$ receptor activated presynaptic Cl^- conductance lasts for 1-2 ms, $GABA_B$ receptor mediated responses are slower, often persisting for several hundred milliseconds (MacDermott et al., 1999). Thus, the different patterns of presynaptic stimulation appear to differentially recruit presynaptic $GABA_A$ and $GABA_B$ receptors.

The location of the receptor is the crucial determinant concerning the regulatory role of presynaptic receptor activation. With metabotropic glutamate receptors, for example, there

are many different presynaptic receptors located in the synaptic area or further in the perisynaptic membrane. During normal synaptic activity, glutamate concentrations do not reach sufficient levels for diffusion from the release site to activate mGlu2 receptors located away from the active zone. However, under high-frequency stimulation, activation of these receptors might prevent pathologically high levels of glutamate from accumulating in the synaptic cleft by inhibiting further glutamate release (Cartmell and Schoepp, 2000). This kind of transmitter spillover has been documented in dopaminergic and GABAergic systems as well (Schmitz et al., 2003).

2.3.2. The inhibitory role of Gβγ in voltage-gated presynaptic Ca^{2+} signaling

The G protein Gβγ complex, which was originally thought to be an inactive membrane-anchoring protein of Gα, has turned out to be a versatile molecule with divergent roles. GPCR activation was first found to reduce action potential duration (Dunlap and Fischbach, 1978), and subsequently, this effect was found to result from inhibition of VGCCs (Dunlap and Fischbach, 1981). The inhibition was demonstrated to be very localized and to happen more likely via receptor channel coupling than via diffusible second messengers (Forscher et al., 1986). Later, the inhibition of VGCCs was found to be mediated by the Gβγ subunit (Herlitze et al., 1996; Ikeda, 1996) by a direct interaction of the Gβγ subunit with VGCCs (De Waard et al., 1997).

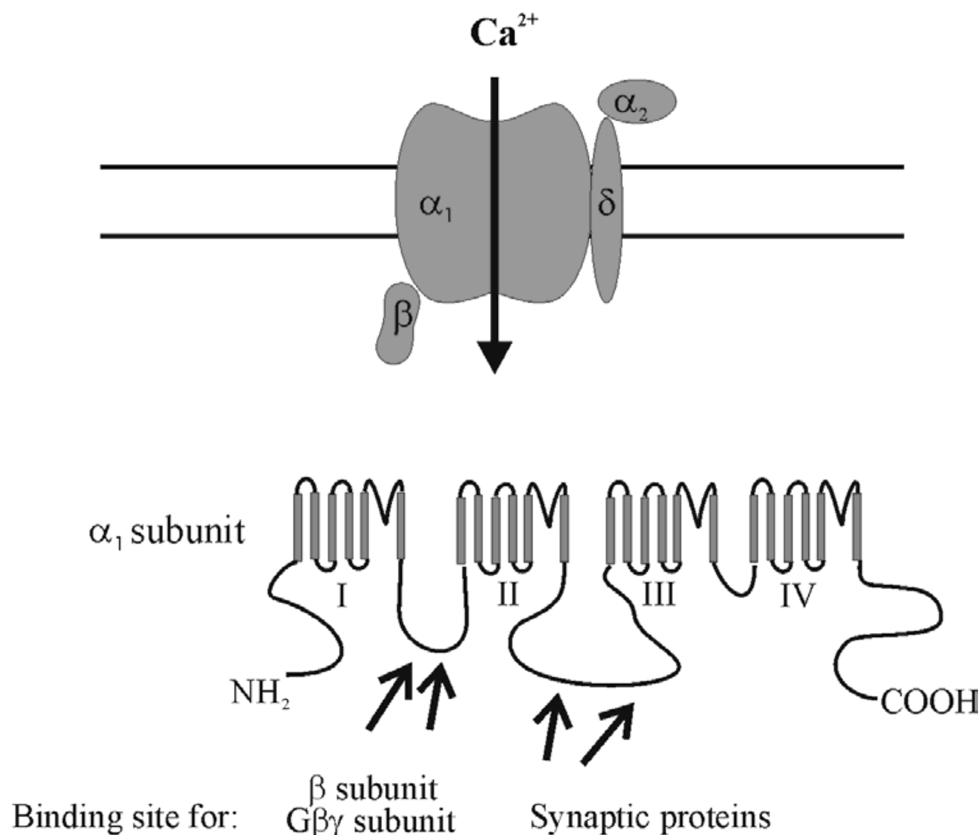


Figure 2. The structure of a voltage gated Ca^{2+} channel.

Neuronal Ca^{2+} channels are heterotrimeric complexes composed of a pore-forming α_1 subunit associated with β and $\alpha_2\delta$ subunits (Bourinet et al., 1999). The binding site of $G\beta\gamma$ is localized to the α_1 pore-forming region of VGCC, more specifically to the cytoplasmic linker connecting the first and second transmembrane repeats (I-II linker) of the α_1 subunit. This site is also known as a mediator for VGCC β subunit stimulation (Pragnell et al., 1994) and PKC up-regulation (Zamponi et al., 1997). The VGCC β subunit, if expressed exogenously, has been shown to increase maximum conductance of N or P/Q-type VGCCs about 10-fold (Dolphin, 2003). The binding of $G\beta\gamma$ to the same site may act in a competitive way, having the opposite effect on the channel function. The direct linkage of $G\beta\gamma$ to VGCCs mainly applies to the Ca_V2 (see Table 2) family of Ca^{2+} channels.

Table 2. Classification of voltage gated Ca^{2+} channels.

	type	tissue	α1 subunit	Ca_V	blocker
High Voltage Activated	P/Q	neuronal	A	2.1	ω-agatoxin
	N	neuronal	B	2.2	ω-conotoxin
	R	neuronal	E	2.3	
	L	sceletal muscle	S	1.1	dihydro-pyridines
		cardiac/ smooth muscle	C	1.2	
		neuronal/secretory cells, heart	D	1.3	
		retina, sensory neurons	F	1.4	
Low Voltage Activated	T	brain	G	3.1	
		heart	H	3.2	
		brain	I	3.3	

$G\beta\gamma$ binding to VGCCs makes the channel reluctant to open (Bean, 1989) by changing its voltage dependency. Thus, stronger depolarizations are needed for channels to open. It is suggested that $G\beta\gamma$ binding slows the voltage sensor movement and thus inhibits the subsequent transduction of this movement to channel opening. $G\beta\gamma$ could also work as a voltage sensor trap (Dolphin, 2003). $G\beta\gamma$ modulation of VGCCs is voltage dependent itself. Large depolarizations shift the channels from reluctant to open to willing to open channels. The termination of $G\beta\gamma$ mediated inhibition by depolarization is a result of rapid dissociation of $G\beta\gamma$ from the channel at depolarizing potentials. Removal of inhibition can also be induced with a depolarizing prepulse (Dolphin, 2003). The $G\beta\gamma$ does not exhibit strict specificity for its different subunits by the VGCC inhibition. However, there is some difference concerning the $G\beta$ subunit being antagonized by PKC (Cooper et al., 2000) or inhibiting the channel (Wolfe et al., 2003).

As was noted before, the $G\beta\gamma$ inhibition of VGCCs in brain mainly originates from the G_o family of G proteins. No convincing data has been shown for $G\alpha$ to play a role in VGCC inhibition. However, the activation of $Gq\alpha$ family does not produce typical voltage dependent inhibition mediated by $G\beta\gamma$ but instead produces a voltage independent inhibition (Kammermeier et al., 2000). Indeed, the voltage dependent inhibition by $G\beta\gamma$ can be reversed

by co-activation with Gqα (Zamponi et al., 1997). Gqα activated PKC phosphorylation of the G$\beta\gamma$ interaction site counteracts G$\beta\gamma$ binding to the I-II linker and thus counters the inhibitory modulation. Inhibition of G$\beta\gamma$ is not just localized to the I-II linker. The N terminus of the α_1 pore forming subunit is essential in this regard as well (Page et al., 1998; Canti et al., 1999). Interactions between presynaptic VGCCs and exocytotic machinery proteins regulating neurotransmission will be discussed further in the next chapter.

2.4. Molecular mechanisms of transmitter release

2.4.1. Ca^{2+}-induced vesicular release in regulated exocytosis

Release of transmitters may be vesicular or non-vesicular and occur constitutively or in a stimulation-dependent manner (Boehm and Kubista, 2002). Classical Ca^{2+} dependent vesicular release is the major interneuronal method of communication, which is suitable for fast and discretely localized processes. Nonsynaptic transmission, also known as volume transmission, may, however, provide an important mechanism for synaptic communication. Some transmitters, such as catecholamines and acetylcholine, can be released from both synaptic and nonsynaptic sites (Vizi et al., 2004). The neuropeptides are mainly released from nonsynaptic sites. Several brain pathologies have been linked to alterations in volume transmission (Zoli et al., 1999). For example, during ischemia, release due to axonal stimulation is inhibited, but there is an extensive release of transmitter due to reverse operation of the transporter (Vizi et al., 2004).

Dendrites are capable of propagating retrograde signals to modulate synaptic transmission (Ludwig and Pittman, 2003). The regulation of dendritic transmitter release is complex and only partially dependent on axonal release. Dendrites of many neural populations transmit information back to their synaptic inputs through release of neuroactive substances, such as NO, CO or arachidonic acid (AA). It has also been shown that classical transmitters as well as neuropeptides take part in retrograde signaling. Neuropeptides released from dendrites act at high local concentrations and have long half-lives because of shorter acting active degradation products. As a consequence, diffusion of neuropeptides through the brain extracellular fluid might also allow them to act on receptors on neurons at a distance from the sites of their release (Ludwig and Pittman, 2003). This type of neuropeptide signaling is especially involved in developmental and morphological plasticity as well as autoregulation of the presynaptic activity itself.

Basically, two classes of vesicles have been identified, small clear-core vesicles (SV) and large dense-core vesicles (LV). They contain mainly classical and peptidergic transmitters, respectively. The fusion time constants have been found to exhibit enormous diversity among different vesicles and cells (Ninomiya et al., 1997). The time course of increases in Ca^{2+} concentrations regulates the exocytosis of each type of secretory vesicle. In the preparation for fast exocytotic release, synaptic vesicles dock at the active zone and are primed to be Ca^{2+} responsive. The speed with which Ca^{2+} triggers the release (under 400 microseconds) suggests that Ca^{2+} binding to the Ca^{2+} sensor only induces the fusion pore opening and does not initiate a complex reaction cascade (Sudhof, 2004). Docking does not, however, guarantee the short time courses of vesicle fusion (Kasai, 1999).

Ca^{2+} influx triggers two components of release that might be mechanistically distinct. A fast, synchronous phasic component is induced rapidly (in 50 microseconds) after the Ca^{2+} transient develops whereas a slower asynchronous component continues for a longer time period (over 1 second) as an increase in the rate of spontaneous release after the action potential. Even at rest, synapses have a finite probability of release that can cause spontaneous events of exocytosis (Sudhof, 2004).

2.4.2. Exocytotic machinery regulation of vesicle fusion

More than a thousand proteins function at the presynaptic nerve terminal, hundreds of which are thought to participate in exocytosis. Synaptic vesicles themselves contain two classes of proteins, transporters involved in neurotransmitter uptake and trafficking proteins that participate in synaptic vesicle exo- and endocytosis. The number of proteins constitutively occupying the vesicles is estimated to be around 50, but there are a number of extra proteins only transiently binding the vesicles. These were first studied in the yeast *Saccharomyces cerevisiae* (Novick et al., 1980) and were later discovered to be homologous to mammalian secretory apparatuses. Almost every step in membrane trafficking is carried out by a distinct set of SNARE (SNAP, soluble N-ethylmaleimide sensitive factor (NSF) attachment protein, receptor) pairs that are also conserved from yeast to humans (Bonifacino and Glick, 2004; Pelham, 1999; Finger and Novick, 1998). This basic machinery underlies all membrane trafficking, including the divergent secretory pathways of hormonal and digestive release (Barg, 2003).

During exocytosis, the synaptic vesicle protein called synaptobrevin (also referred to as VAMP, vesicle associated membrane protein) and presynaptic plasma membrane proteins SNAP-25 (synaptosome associated protein of relative molecular mass 25000) and syntaxin 1A assemble into a complex called the core complex. Complex formation is energetically favorable, and the complex is thermally and chemically very stable, even in the presence of SDS (sodium dodecyl sulphate) up to 80 degrees Celsius (Kubista et al., 2004). In vivo, the SNARE complex can only be dissociated with ATPase NSF that is thought to occur as a post fusion step. The stability of the complex has led to a proposal that the energy gained from the complex formation may be harnessed to drive the membrane fusion reaction. Experiments with neurotoxins revealed that the core complex is essential for exocytosis. Botulinum and tetanus toxins enter nerve terminals and irreversibly inhibit synaptic vesicle exocytosis by protease activity towards SNARE proteins. SNAREs are highly susceptible to toxin attack in monomeric form, but they are protected to various degrees in macromolecular complexes. Poisoning of nerve terminals with botulinum or tetanus toxins almost completely blocks exocytosis without interfering with vesicle docking. In fact, neurotoxins increase the amount of docked vesicles, probably as a result of blocked fusion. This demonstrates a surplus of docking sites in synaptic vesicles (Murthy and De Camilli, 2003).

Tetanus and botulinum neurotoxins are derived from Clostridium bacteria. Tetanus toxin acts mainly at CNS synapses by blocking the inhibitory interneurons of the spinal cord while botulinum toxins (type A, B, C, D, E, F and G) act peripherally in cholinergic neurons (Montecucco and Schiavo, 1995),(Rossetto et al., 2004). They underlie the opposite clinical symptoms but affect the same target, exocytosis. Tetanus toxin and botulinum toxins B, D, F and G cleave VAMP, botulinum toxin C cleaves syntaxin and SNAP-25, and botulinum toxin

A and E cleave SNAP-25. Botulinum toxins have value in clinical use to treat syndromes caused by hyperfunction of cholinergic terminals. They are especially useful in treating diseases with excessive muscle contraction, such as dystonia (Rossetto et al., 2004).

SNAREs have two major functions in exocytosis. First, they promote fusion itself by localizing to opposing membranes and assembling into a protein complex to bring the two membranes into close position. Second, they help ensure the specificity of membrane fusion. SNARE knockout studies have shown core complex formation to be essential to fast Ca^{2+} triggered exocytosis (Sorensen et al., 2003; Schoch et al., 2001; Washbourne et al., 2002). However, the SNARE complex formation rather catalyzes the subsequent fusion reaction than actually executes it. By reconstitution approach, the SNARE complex and the Ca^{2+} sensor, synaptotagmin, has been shown to compose the minimal protein complement for Ca^{2+} triggered exocytosis (Tucker et al., 2004).

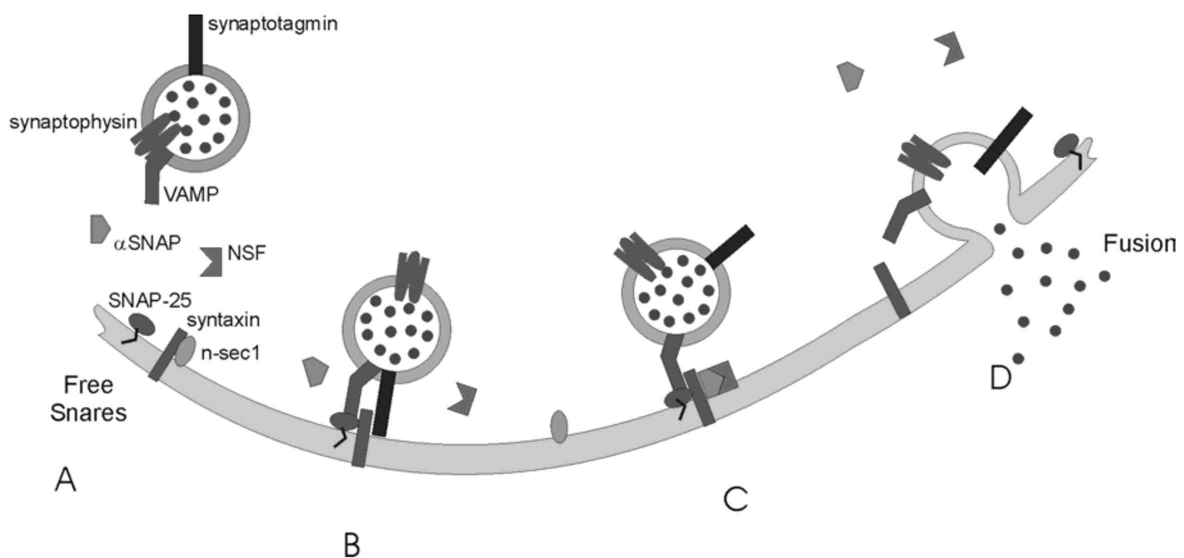


Figure 3. Formation of core complex in synaptic vesicle exocytosis
A) Synaptobrevin is bound to synaptophysin on the synaptic vesicle, and syntaxin is bound to n-sec1 (neuronal sec1) on the acceptor membrane prior to docking. B) The vesicle proteins synaptobrevin and synaptotagmin and the plasma membrane proteins SNAP-25 and syntaxin associate to form the core complex. C) As the α-SNAP adds to the complex, synaptotagmin is displaced. NSF binds to the complex, and D) ATP hydrolysis dissociates the interactions. Following ATP hydrolysis, several steps are thought to precede membrane fusion. Ca^{2+} acts as the final trigger for exocytosis.

The process of vesicle targeting requires molecular motors and actin or microtubule-based cytoskeletons to bring a vesicle from one part of the cell to another. An intact actin matrix is required for normal membrane trafficking, suggesting that the cytoskeleton mediates vesicle transport through the cytoplasm. A role for actin is supported by the findings that the unconventional myosin, myosin V, associates with synaptic vesicles via synaptobrevin and that pharmacological inhibition of either myosin ATPase activity or myosin light chain kinase reduces neurotransmitter secretion. Other cytoskeleton elements, like microtubules, are also involved in the vesicle movement (Bajjalieh, 1999). Recently, a family of presynaptic cell-adhesion proteins, α-neurexins, has been shown to contribute to vesicle exocytosis by

functionally coupling Ca^{2+} channels to the presynaptic machinery. Interestingly, α-neurexins link the pre- and postsynaptic compartments by binding extracellularly to postsynaptic cell adhesion molecules and intracellularly to presynaptic PDZ domain proteins (Missler et al., 2003).

2.4.3. Additional regulators in the precision of exocytosis

The synaptic core complex is accompanied by a variety of proteins regulating vesicle exocytosis (Gerst, 2003). The mammalian homologue of the yeast protein Sec1, nSec1, is the prototypic plasma membrane SNARE protector. NSec1 binds directly and tightly to syntaxin 1A and this binding blocks the association of vesicle and plasma membrane SNAREs. NSec1 binds to a protein complex that may act as a target specifier for vesicle release, and recruit SNAREs to this site through nSec1 (Pfeffer, 1999). Snapin is a protein exclusively located on synaptic vesicle membranes that associates with the core complex through direct interaction with SNAP-25, modulating sequential interactions between the SNAREs and synaptotagmin, a Ca^{2+} sensor. Recombinant snapin injected into presynaptic neurons reversibly inhibited neurotransmitter release at synapses in culture. Neurons also express the unique actin-binding protein synapsin, which links synaptic vesicles to the cytoskeleton in a phosphorylation-dependent manner. Disruption of synapsin expression decreases the number of synaptic vesicles distal to the active zone and results in synaptic fatigue (Bajjalieh, 1999).

Syntaphilin, a membrane-associated protein, competes with SNAP-25 for binding to syntaxin and therefore inhibits SNARE complex formation at nerve terminals. Syntaphilin may function as a molecular clamp that controls the availability of free syntaxin for the assembly of the SNARE complex and thereby regulates synaptic vesicle exocytosis. SNARE complex formation is also regulated with cytoplasmic syntaxin interacting proteins, Munc-18 and tomosyn. In vitro binding studies show that Munc-18 interacts with syntaxin, preventing its binding to SNAP-25 or synaptobrevin and thereby preventing formation of the SNARE complex, while tomosyn promotes SNARE complex assembly. It has been demonstrated that the interaction of Munc-18 with syntaxin is essential for fusion of docked vesicles (Mochida, 2000).

Since the core complex is very stable, assembled complex has to be dissociated enzymatically by a chaperone. This chaperone, protein called NSF, is recruited to the membrane by adaptor proteins called SNAPs (soluble NSF attachment protein, no relation to SNAP-25). NSF, which uses the energy of ATP hydrolysis to disassemble the core complex, serves to recycle the components of the complex so that they can participate in a new round of fusion (Rizo and Sudhof, 1998). Rab3 is a synaptic vesicle protein that belongs to a rab family of low molecular weight GTP-binding proteins. At nerve terminals, rab3A, the most abundant rab protein in the brain, and its binding proteins, rabphilin3A and Rim, are involved in exocytosis via hydrolysis of GTP. Based on studies made with knockout mice lacking rab3A, this small protein appears to be a constitutive negative regulator of fusion that acts at a late step of fusion, coincidental with synaptotagmin. The regulatory role of rab3A is essential for long-term potentiation in the hippocampus. In rab3A knockout mice, long-term potentiation is not normally produced, presumably because the synapses are already in an activated state (Fernandez-Chacon and Sudhof, 1999). Rab proteins are suggested to have a role in Ca^{2+}-dependent regulation of exocytosis.

Table 3. SNAREs and SNARE-associated proteins involved in exocytosis

Classification	Proteins	Localization	Function
SNAREs (SNARE core complex)	synaptobrevin	synaptic vesicle	fusion machinery
	syntaxin	plasma membrane	fusion machinery
	SNAP-25	plasma membrane	fusion machinery
SNARE core complex interacting proteins	NSF (ATPase)	cytoplasm	SNAREs disassembly
	α-SNAP	cytoplasm	SNAREs disassembly
	snapin	synaptic vesicle	modulation of SNARE-synaptotagmin interaction
	N-and P/Q-type Ca^{2+} channels	plasma membrane	synchronous neurotransmitter release, transmission of voltage signal to SNARE complexes
Syntaxin-interacting proteins	tomosyn	cytoplasm and plasma membrane	stimulation of SNARE assembly
	Munc-18	plasma membrane	inhibition of SNAREs assembly, essential for synaptic vesicle fusion
	syntaphilin	plasma membrane	inhibition of SNAREs assembly
	n-Sec1	cytoplasm ?	regulation of SNARE complex assembly
GTP-binding proteins	rab3A (GTPase)	synaptic vesicle	limiting fusion machinery, generation of LTP, recruitment of synaptic vesicles to active zone
	rabphilin	synaptic vesicle	modulation of synaptic vesicle fusion and synaptic vesicle trafficking
	rim	active zone	promotion of synaptic vesicle fusion
Ca^{2+} binding proteins	Synapto-tagmins	synaptic vesicle	trigger of synaptic vesicle fusion
	CSP	synaptic vesicle	coupling of Ca^{2+} channels to exocytosis
Proteins involved in synaptic plasticity	synapsin	synaptic vesicle	maintaining a stable pool of synaptic vesicles that can be rapidly recruited during synaptic plasticity
	synaptophysins	synaptic vesicle	essential functions in synaptic plasticity
	synaptogyrin	synaptic vesicle	essential functions in synaptic plasticity

CSP (cysteine string protein) is a synaptic vesicle membrane protein, which contains a J-domain. The J-domain of CSP shares homology with the universally conserved DnaJ family, a group of proteins that act as co-chaperones with Hsc70 and its homologs. Hsc70 is an abundant neural protein with ATPase activities (Braun et al., 1996). CSP associated with the secretory vesicle cycle is suggested to regulate Hsc70 function. CSP does not significantly increase the activity of NSF protein, another ATPase required for transport vesicle function. CSP interacts with voltage-sensitive N-type Ca^{2+} channels. The precise role of CSP in exocytosis is unknown, but it has been suggested that it operates as a molecular chaperone, assisting in the folding and conformational change of proteins participating in membrane trafficking (Braun et al., 1996).

Acidic phospholipids may play important roles in membrane transport. They may act as fusogens: an increase in the acidic phospholipid content of synthetic liposomes increases their fusion ability. Alternatively, acidic phospholipids may regulate the interaction of cytoskeleton proteins with vesicles. Phosphatidylinositol (PI) and its higher phosphorylated forms, PIP and PIP_2, regulate the activity of many actin-associated proteins. Therefore, changes in the concentrations of these lipids in either the vesicle or acceptor membrane are predicted to alter the surrounding actin cytoskeleton (Bajjalieh and Scheller, 1995). CAPS (Ca^{2+}-activated protein for secretion) is a lipid-binding protein that binds PIP_2 at micromolar Ca^{2+} concentrations (Bajjalieh, 1999). CAPS binds actin in a Ca^{2+}-dependent fashion and therefore could function as a regulator of cytoskeleton assembly or disassembly or to regulate actin-mediated vesicle movement (Bajjalieh and Scheller, 1995).

2.4.4. Ca^{2+} binding protein and Ca^{2+} channel interaction with the synaptic core complex

Synaptotagmins are suggested to work as Ca^{2+} sensors to trigger the vesicle fusion (Meldolesi and Chieregatti, 2004). Synaptotagmins are a family of Ca^{2+}-binding proteins specific to higher eukaryotes. The absence of synaptotagmins in yeast suggests that they are not part of the basic membrane trafficking machinery. Alterations in synaptotagmin expression have effects on the regulation of exocytosis, but do not prevent it. Therefore, synaptotagmin 1 is essential for fast Ca^{2+}-triggered exocytosis, but not directly involved in the fusion reaction itself. Because loss of synaptotagmin alters the ability of Ca^{2+} to trigger fast exocytosis, the primary action of synaptotagmin as a regulator is hypothesized to result from Ca^{2+}-sensitive interactions. Synaptotagmin interactions with different proteins could supply both negative and positive control to exocytosis, thus inhibiting the progression of fusion at low Ca^{2+} concentrations and promoting fusion at high Ca^{2+} levels (Bajjalieh, 1999).

Different from Ca^{2+}-triggered exocytosis, hypertonic sucrose stimulates exocytosis of all docked synaptic vesicles, but Ca^{2+} selectively picks out only one vesicle and leaves others intact. These facts indicate that the Ca^{2+}-regulated step in the fusion reaction is strongly inhibited and can be activated only occasionally (Fernandez-Chacon and Sudhof, 1999). The relation of Ca^{2+} action to the assembly of the core complex remains unknown: the core complex either has already been assembled before Ca^{2+} acts but is not sufficient for fusion, or Ca^{2+} unleashes a rapid assembly reaction that drives fusion (Fernandez-Chacon and Sudhof, 1999). The SNARE complex directly interacts with N-type and P/Q-type Ca^{2+} channels that

provide Ca^{2+} to trigger exocytosis in the peripheral and central nervous systems (Stanley, 1997; Zamponi and Snutch, 1998b).

Docked synaptic vesicles are suggested to form a low-affinity complex with the Ca^{2+} channel through binding to syntaxin and SNAP-25 at resting Ca^{2+} flow. Ca^{2+} influx greatly increases the affinity of this complex, so the binding energy of Ca^{2+} to the docked complex may contribute to the energetic driving force for the early priming steps of exocytosis. Finally, as the free Ca^{2+} reaches the threshold for release, the binding affinity to the Ca^{2+} channel is reduced, and syntaxin and SNAP-25 dissociate from the presynaptic Ca^{2+} channel to allow membrane fusion to occur. This is probably regulated with Ca^{2+} binding to synaptotagmin or vesicle phospholipids (Sheng et al., 1996).

2.4.5. Caveolin-induced co-localization of molecules into signaling microdomains

As noted before, the different receptor mediated signaling pathways, despite utilizing many identical intracellular components, generate specific cellular responses. One way to achieve this is to organize components needed for a specific pathway into signaling microdomains, the activation of which leads to fast and spatially defined signaling. An important feature leading to the signaling complex hypothesis was the finding of plasma membrane cave-like ultrastructural invaginations half a century ago. These formations, referred as caveolae, have since been getting a massive amount of attraction and shown to play a crucial role in regulation of signal transduction events. Present in almost every cell type, certain cells, such as adipocytes, epithelial cells and muscle cells, have an extraordinary abundance of caveolae. Other cells, such as some types of neurons in CNS and lymphocytes are devoid of caveolae (Razani et al., 2002). Caveolae, 50-100 nm in their size, consist of an oligomerized 22 kDa protein called caveolin (Rothberg et al., 1992; Glenney, Jr., 1992; Glenney, Jr. and Soppet, 1992). Caveolin-1 and caveolin-2 are most abundantly expressed in adipocytes, endothelial cells and fibroblasts, whereas the expression of caveolin-3 is muscle-specific. Because of detergent resistance and buoyancy of caveolae, these microdomains can be separated from all other cellular constituents (Razani et al., 2002).

Caveolae are considered as specialized lipid rafts. Caveolae are morphologically distinct from lipid rafts because of structural protein caveolin that gives caveolae their flasklike appearance. Despite the similarities in their overall structure, these microdomains are not equivalent in their function. Several proteins have been shown to preferentially localize to either caveolae or lipid rafts (Liu et al., 1997), including GPCRs and G proteins (Oh and Schnitzer, 2001; Ostrom and Insel, 2004). Caveolae have also been found in CNS, in glial cells, such as astrocytes and oligodendrocytes (Cameron et al., 1997). The role of caveolae in neurons is still controversial. In hippocampal neurons, glutamate has been shown to regulate caveolin expression indicating the role of caveolae in signal transduction events in neurons (Bu et al., 2003). Alteration in the function of lipid rafts and caveolae have been linked to many diseases (Cohen et al., 2004; Simons and Ehehalt, 2002). Caveolin 1 expression is increased in e.g. Alzheimer's disease (Gaudreault et al., 2004). Increases in cellular cholesterol levels, linked to pathophysiology of the disease, is associated to up-regulation of caveolin.

Caveolae and thus, caveolins, are abundant in terminally differentiated cells. The number of caveolae and the amount of caveolin decreases dramatically in immortalized cultured cells, indicating that caveolae are important in inhibiting certain signaling pathways that regulate cellular proliferation. Therefore, caveolin-1 is a common target among activated oncogenes. Caveolin-1 expression is remarkably reduced during cell transformation by a specific set of activated oncogenes (Okamoto et al., 1998). In caveolin-1 knockout mice, the absence of caveolae impaired NO and Ca^{2+} signaling pathways, leading to severe vascular and pulmonary defects, as well as uncontrolled cell proliferation (Drab et al., 2001). Caveolin mutations have been encountered mainly in muscular disorders and tumorigenesis (Razani et al., 2002). Few signaling proteins are exclusively localized to caveolae, but a remarkable exception includes several Ca^{2+} regulatory proteins. Because some cells and even caveolin-1 knockout animals manage to survive without caveolae, there might be other noncaveolae pathways to compensate for caveolae when they are absent (Parton, 2001).

Regarding caveolae, interest has mainly targeted on caveolin-1. It is a non-conventional membrane protein, since its N and C terminus reside intracellularly. It exists primarily as a higher ordered oligomeric complex of 14 to 16 monomers, making the size of the homo-oligomer 350-400 kDa. Overexpression of caveolin-1 in cells lacking the endogenous caveolins results in production of caveolae (Razani et al., 2002; van Deurs et al., 2003). Caveolin-1 has high affinity binding with cholesterol and a certain threshold level of cellular cholesterol needs to be exceeded for caveolae formation to occur. Not surprisingly, depletion of cellular cholesterol causes a reduction in the number of caveolae. Caveolae do not just function as the structural formation of the plasma membrane where the signaling proteins localize. Caveolins might also act as scaffolding proteins by directly interacting with and modulating the activity of signaling molecules localized in caveolae (Razani et al., 2002; van Deurs et al., 2003). Caveolae should be considered as multifunctional organelles with a physiological role that varies depending on the cell type and needs.

Caveolae are dynamic structures. Plasma membrane caveolae are anchored by the actin cytoskeleton, but they may be internalized under special conditions. Interference with the actin cytoskeleton, may result in the internalization of Ca^{2+} complexes (Lockwich et al., 2001), which shows localization of the complexes to be dependent on the status of the actin cytoskeleton. Thus, there is growing evidence indicating that caveolae also exist intracellularly (Pelkmans et al., 2001; Pelkmans et al., 2002). Caveolin associated vesicles are involved in trafficking between caveolin associated compartments (van Deurs et al., 2003). Interactions with caveolin-1 may also occur in intracellular compartments, especially along the exocytic pathway. For example, the AT1 receptor is located in caveolae and immunoprecipitates with caveolin-1, but is not located in plasma membrane caveolae (Wyse et al., 2003). Caveolae may serve as specialized transport and sorting devices in intracellular transmembrane traffic. Caveolar membranes connect with endocytic and exocytic organelles, and unlike lipid rafts and clathrin coated vesicles, they preserve their compartmentalization, retaining a unique lipid and protein composition. Therefore, the caveolar vesicles retain their identity also in intracellular compartments. Instead of disassembly of the complex, the release or sequestration of cargo in caveolar vesicles are controlled by local cues. Ligands not destined for a particular compartment stay trapped or get loaded only when arrived in a compartment that provides the right trigger for activation (Pelkmans et al., 2004).

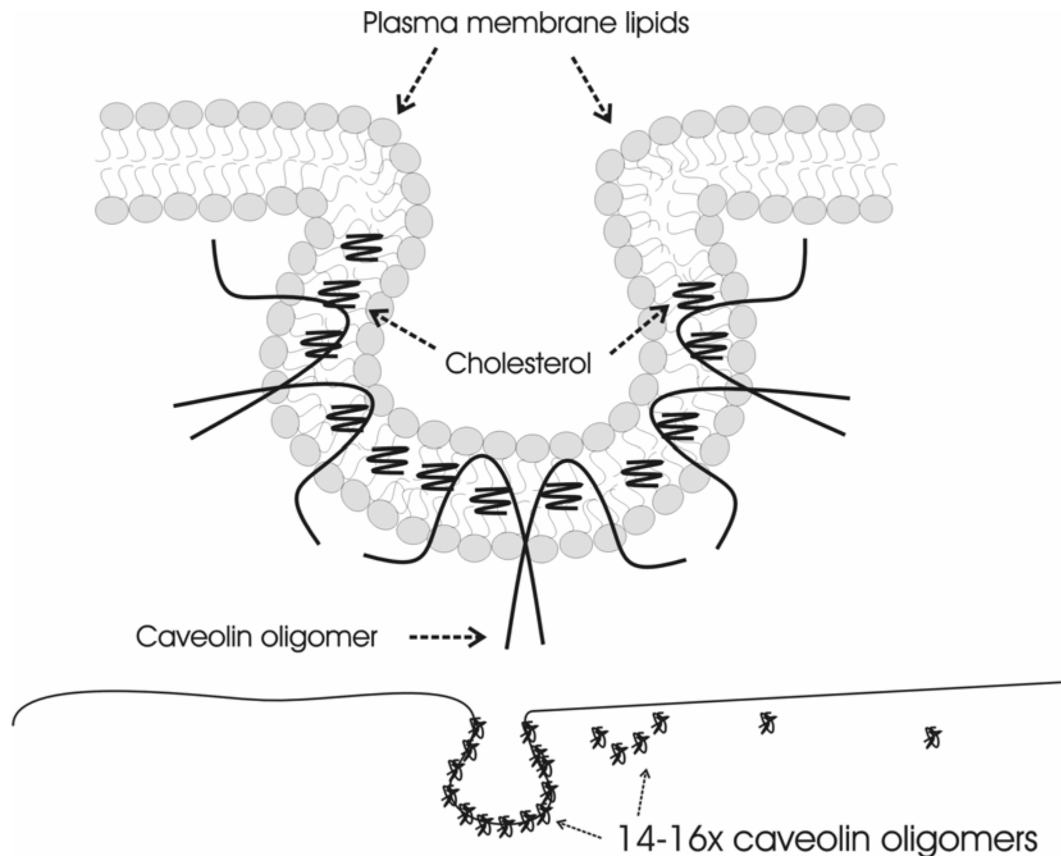


Figure 4. Caveolin proteins form the caveolae structure. Caveolae enriched in cholesterol and sphingolipids are formed by oligomerization of caveolin protein, which represents a cytoplasmically oriented C- and N-terminus. Oligomerized 14-16 caveolin-containing complexes are preformed before attachment to the membrane.

Different G proteins may also be differentially targeted to lipid rafts and caveolae, and thus, coupling is efficient only when the receptor meets the G protein (Chini and Parenti, 2004). For example, in systems where Gqα interacts with caveolin-1 while Giα does not, the Giα mediated inhibitory pathway plays a role only in the signaling events generated when the receptor is excluded from lipid rafts (Rimoldi et al., 2003). Quite similarly, signaling microdomains define the specificity of receptor mediated IP_3 pathways in neurons. Bradykinin 2 (B2) receptors and muscarinic M1 receptors both activate PLC and produce IP_3, but only IP_3 formed by B2 has the ability to activate IP_3R. This exclusive coupling results from the fact that B2 and IP_3 receptors form signaling microdomains, while M1 receptors, in contrast, are randomly distributed and not directly linked to IP_3 receptors (Delmas et al., 2002). Localization in caveolae and caveolin interactions have been shown to modulate many GPCR function (Bhatnagar et al., 2004; Ostrom and Insel., 2004; Toews at al., 2003,).

There is a long list of proteins identified in caveolae (Razani et al., 2002). A variety of GPCRs, G proteins, growth factor receptors, and tyrosine kinases have been shown to localize in caveolae and even interact with caveolins. Also, enzymes like adenylyl cyclases, phospholipases, eNOS, nNOS, non-receptor kinases, PKA, PKC, ion pumps, ion channels (especially Ca^{2+} channels) and synaptic proteins have been found in caveolae. The clustering

of VGCCs and SNARE proteins in lipid microdomains in presynaptic terminals plays an especially important role in organizing the membrane sites for synaptic vesicle exocytosis (Taverna et al., 2004). The precise assembly and function of synaptic protein complexes, and the fine-tuning of synaptic transmission by regulation with Ca^{2+} channel interactions, are not completely understood.

3. AIMS OF THE STUDY

The aim of the present study was to investigate the role of synaptic protein modulation of G protein mediated Ca^{2+} signaling and how these interactions regulate the synaptic transmission. This was achieved by investigating presynaptic signaling protein complexes and by studying postsynaptic signaling of a specific G protein coupled receptor. More specifically, this study was undertaken to:

1. Investigate the role of the synaptic core complex protein, syntaxin 1A, in the regulation of G protein mediated inhibition of N type voltage gated Ca^{2+} channels.

2. Investigate the role of the synaptic protein, CSP in synaptic transmission: whether it regulates the transmission by modulating N type voltage gated Ca^{2+} channels and G protein subunits.

3. Investigate the role of caveolin-1 in the regulation of synaptic vesicle exocytosis

4. Investigate the G protein pathways of a peptidergic G protein coupled orexin 1 receptor

4. MATERIALS AND METHODS

4.1. Molecular biology

4.1.1. DNA constructs for transfections

DNA encoding for rat syntaxin 1A, the entire length of CSP, the carboxyl terminus of βARK (corresponding to residues 495-689), Ca^{2+} channel α_{1B} II-III linker (corresponding to residues of 718-963), the entire α_{1C} II-III linker (corresponding to residues 754-901) and Ca^{2+} channel subunits α_{1B}, α_2-δ and β_{1b}, were amplified by PCR from rat brain cDNA. DNA encoding for bovine $G\beta_1$ and $G\gamma_2$ were amplified by PCR from bovine brain cDNA. Sequences were verified and subcloned into pMT2-SX for expression. The pMT2 constructs were used for transient transfection of HEK cells for electrophysiological experiments.

The human OX_1R cDNA in pcDNA3 (Invitrogen) was received from Dr. M. Detheux (Euroscreen s.a., Bruxelles, Belgium). For expression of the epitope-tagged receptor, the open reading frame of OX1R was amplified by PCR. A PCR fragment of correct size was inserted into pGEM-T Easy (Promega) to make pGEM-OX_1R. To generate a fusion protein the 99 bp MbiI-XhoI 3' end fragment was cut out from pGEM-OX_1R and ligated together with the 1.1 kbp EcoRI-MbiI 5' end fragment of the original OX_1R cDNA into pIRES-hrGFP-1a (Stratagene). DNA sequencing confirmed that the OX_1R was in frame with the triple flag sequence.

Generation of chimeric G proteins: The cDNAs for G11α, Giα1, GsαL (long-form), and Goα1 were of bovine origin. The cDNA for Gqα was of human origin. To generate chimeric G proteins, a fragment of the GsαL cDNA in pBluescript, including cDNA encoding the carboxyl terminus domain of Gsα, was further subcloned into pBluescript. The whole coding sequences of the different constructs were transferred to pFastBac1. For mammalian cell transfection, a fragment from pEGFP-N1 (Clontech), including the cytomegalovirus (CMV) promoter and the gene for EGFP, was subcloned in pFastBac1, removing the polyhedrin promoter. This vector construct was further used to subclone Gsα, Giα and the chimeric Gsα/Gx constructs behind the CMV promoter. Recombinant baculoviruses for all constructs in the pFastBac1 were obtained using the Bac-to-Bac expression system (Invitrogen).

4.1.2. DNA constructs for fusion proteins

DNA encoding for rat caveolin 1α, 1β, 2 and 3, were amplified by PCR from either rat brain or muscle (caveolin 3) cDNA, subcloned into pGEX-KG (Guan and Dixon, 1991), verified and expressed in E. Coli AB1899. Similarly, a GST fusion protein encoding the full length CSP, the amino terminus of CSP (amino acids 1-82), the cytoplasmic portion of syntaxin 1A, VAMP2 and SNAP-25, were constructed in the vector pGEX-KG and expressed in the AB1899 strain of *E. coli.* DNA encoding residues 718-963 from the I-II linker of the α_{1B} Ca^{2+} channel subunit synprint region were amplified using PCR, subcloned into pTrcHisC (Invitrogen), and expressed in *E. coli* TOP1O.

4.2. Cell culture transfections

4.2.1. Stable transfection of HEK cells

The cDNA for Flag peptide (3XFLAG) -tagged human orexin receptor 1 was cloned into the pIRES-hrGFP-1a vector containing resistance to hygromycinB. We used pIRES-hrGFP-1a vector containing an IRES (internal ribosomal entry sequence) followed by the coding sequence of a green fluorescent protein to be able to monitor the expression of the protein. The vector was transfected into HEK293 cells with Fugene6 (Roche). Antibiotics (0.1 mg/ml of hygromycin B, Invitrogen) were added to the medium 48 hours after transfection. The cells were then cultured for 3 weeks to obtain stable clones.

4.2.2. Transient transfection of HEK cells

Human embryonic kidney tsa-201 cells were grown in standard DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin. The cells were grown to 85% confluence, split with trypsin-EDTA, and plated on glass coverslips at 10% confluence 12 h before transfection. Immediately prior to transfection, the medium was changed, and a standard Ca^{2+} phosphate protocol was used to transfect the cells with cDNAs encoding for Ca^{2+} channel subunits α_{1B}, α_2-δ and β_{1b}, green fluorescent protein (EGFP-Clontech, CA), and, as appropriate, cDNA constructs encoding CSP, βARK, syntaxin 1A, and/or Gβγ. After 12 h, the cells were washed with fresh DMEM and allowed to recover for another 12 h. The expression of transfected proteins was confirmed by Western blot analysis. Subsequently, the cells were incubated at 28°C in 5% CO, for 1-3 days prior to recording. For experiments examining protein levels of syntaxin 1A and Gβγ, the cells were plated directly on the bottom of the culture dish.

HEK293 expressing the orexin 1 receptor were grown in standard DMEM (GibcoBRL) cell culture medium supplemented by 10 % fetal bovine serum (GibcoBRL), 100 U/ml penicillin-streptomycin (GibcoBRL), and 0.1 mg/ml hygromycin B (Invitrogen). A day before transfection, cells were plated on 3cm plates (Nunc) containing a coverslip for Ca^{2+} imaging experiments and on 10 cm plates (Greiner) for immunoprecipitation studies, in a confluence of 10 % and 40%, respectively. Fugene6 was mixed with DNAs encoding for Gqα, G11α, Giα, Goα, Gsα and spread on the cells. Plates were incubated for 24-30 h prior to experimental use.
Gene transfer for chimeric G proteins with baculovirus: Transient expression of the Gα constructs using the baculovirus vector was done by seeding HEK-OX1R cells in 6-well plates (Greiner) at 20 % confluence. After two days, half of the medium (1 ml) was replaced with a high titer virus stock (10^7 pfu/ml) originating from Sf9 cell infection. The transduction was allowed to proceed for 3 h whereafter the medium was changed with fresh medium. Plates were incubated for 24-28 h prior to experimental use.

4.3. Preparation of homogenates, lysates and slices

4.3.1. Preparation of hippocampal homogenate

Rat hippocampi were hand homogenized with a teflon-coated homogenizer in 0.32 M sucrose, 10 mM HEPES KOH (pH 7.0), 1 mM EGTA (ethyleneglycol tetraacetic acid), 0.1 mM EDTA (ethylenediamine tetraacetic acid), 0.5 mM PMSF (phenylmethylsulfonyl fluoride), protease inhibitor cocktail (Bohringer Mannheim), 1 μM microcystin, 1 μM okadaic acid, and 1 mM sodium orthovanadate (2 ml/hippocampus). The homogenate was centrifuged for 10 min at 500xg, and the supernatant was collected and subsequently centrifuged for 20 min at 20 000xg at 4°C. The pellet containing the synaptic proteins was resuspended in 1% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCI, 0.5 mM PMSF, protease inhibitor cocktail, 1 μM microcystin, 1 μM okadaic acid, and 1 mM sodium orthovanadate and incubated for 30 min at 37°C. Following solubilization, large membrane fragments were removed by centrifugation at 1000xg for 5 min. The resulting supernatant is a crude hippocampal homogenate that contains synaptic proteins. Protein concentrations were determined by a Bio-Rad protein assay using bovine serum albumin as the standard.

4.3.2. Cross-linking of intact hippocampal slices

Hippocampal slices were cross-linked with 4% paraformaldehyde (Braun and Madison, 2000). After crosslinking, hippocampal slices were hand homogenized (0.25 ml/slice) in 1% Triton X-100, 10 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCI, 10 mM glycine, and 0.5 mM PMSF and mixed for 1 hr at 4°C. Sample buffer was added to the solubilized slice (final concentration 1 % SDS, 42 mM Tris [pH 6.8], 7% glycerol, and 7% β-mercaptoethanol). Prior to SDS-PAGE, these samples were incubated at 37°C, 80°C or 100°C for 10 min.

4.3.3. Preparation of HEK cell lysates

Immunoblots for Syntaxin 1A and Gβ in HEK Cells: tsA-201 cells were transfected as described above. Following the 2-day incubation at 28 °C, the DMEM medium was washed off and 2 ml of trypsin-EDTA (Life Technologies, Inc.) was added prior to incubation for 3 min. 8 ml of PBS buffer was added, and the cells were pelleted by centrifugation at 3000x*g* for 2 min. The supernatant was removed, and the pellet was resuspended in 3 ml of homogenization buffer (50 mM HEPES-Tris, pH 7.3, 8% sucrose, 100mM NaCl, 1mM EDTA, 10mM dithiothreitol, 0.1 mM PMSF, 30 mg of leupeptin, 30 mg of pepstatin, 3 mg of aprotinin, 0.1% Triton X-100), followed by three rapid freeze-thaw cycles between a dry ice-methanol slurry and 37 °C water. The preparation was centrifuged at 5000xg for 5 min to remove cell debris, and this supernatant was then spun at 100,000x*g* for 1 h. The pellet was then treated with Laemmli sample buffer (100 mM Tris-HCl, pH 6.8, 200 mM β-mercaptoethanol, 20% glycerol, 4% SDS, 0.2% bromphenol blue), and shaken for 2 h at 4 °C. The proteins were denatured at 95 °C for 1 min and loaded for SDS-PAGE.

Lysis of HEK293 cells for orexin receptor 1 immunoprecipitation: the cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.3) and scraped in ice-cold PBS. The cell pellets were frozen at -70°C. For whole cell lysate preparations, a pellet of approximately 5 x 10^6 cells was resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.4 % digitonin (Sigma), protease inhibitor mixture (Roche) and incubated at +4°C with rotation. Lysate was centrifuged for 10 minutes at 12 000xg and the supernatant collected and mixed with an equal amount of lysis buffer without digitonin. To make membrane preparations, a pellet of approximately 10^7 cells was first resuspended in 10 mM Tris-HCl pH 7.4, 10 mM EDTA, and protease inhibitor mixture, then hand homogenized in a glass coated homogenizer (Duall) with 20-30 strokes. The crude lysate was centrifuged for 5 minutes at 500xg. The supernatant was collected and subsequently centrifuged for 20 minutes at 40 000xg (+4°C). The pellet containing the cell membranes was then resuspended and treated as a whole cell preparation.

4.4. Subcellular fractionation

4.4.1. Isolation of hippocampal synaptosomes

Synaptosomes were isolated from the hippocampus by differential and discontinuous Percoll gradient centrifugation. Hippocampi were homogenized in 0.32 M sucrose, 5 mM HEPES, 0.1 mM EGTA (Teflon coat homogenizer) and centrifuged for 1000xg at 4°C. The supernatant was layered directly onto Percoll gradients, and centrifuged at 15 000xg. The synaptosomal fraction was washed, pelleted and resuspended in balanced salt solution (128 mM NaCl, 2.4 mM KCl, 1.2 mM $MgSO_4$, 1.2 KH_2PO_4, 10 mM HEPES, 10 mM D-glucose, pH 7.4) and mixed with Laemmli sample buffer.

4.4.2. Isolation of hippocampal caveolae

Rat hippocampi were lysed in 2 ml of morpholine-ethanesulfonic acid (MES) buffered saline (MBS: 25 mM MES, pH 6.5, 150 mM NaCl, 1 mM Pefabloc, 1 mM Na_2VO_4, 10 µg/ml of aprotinin and leupeptin) including 1 % Triton X-100, adjusted to 40 % sucrose and placed at the bottom of the ultracentrifuge tube. A 5-30 % linear sucrose (in MBS) gradient was formed above the sample and centrifuged at 37000 rpm (SW41 rotor, Beckman) for 16 h at 4°C. Fractions of 1 ml were collected across the entire gradient and the proteins were precipitated with TCA (trichloroacetic acid) and analyzed by western blotting. Triton insoluble complexes were collected as an opaque band corresponding to caveolae migrating at 10 to 20 % sucrose, diluted 1:4 in MBS and pelleted at 37000 rpm for 1h at 4°C. The pellet was resuspended in MBS and Laemmli sample buffer and heated at 100°C for 10 min before electrophoresis.

4.5. In vitro binding assays

4.5.1. Preparation of fusion proteins

After induction of expression with 100 μM isopropyl-β-D-thiogalactopyranside for 5 h, the bacteria were suspended in phosphate buffered saline (PBS: 137 mM NaCI, 2.7 mM KCI, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4), supplemented with 0.05% Tween 20, 2 mM EDTA, and 0.1% β-mercaptoethanol, and lysed by two passages through a French Press (Spectronic Instruments) or sonication (Branson Sonifier) in the case of the 6XHis synprint protein. The fusion protein was recovered by binding of the GST domain to glutathione agarose beads (Sigma) or Probond Ni^{2+} agarose beads (Invitrogen) in the case of 6XHis synprint peptide. The fusion protein beads were washed extensively and finally resuspended in 0.5% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCI, and 0.5 mM PMSF. Recombinant CSP was purified from the GST fusion protein by cleavage with 0.2 μM thrombin in 0.5% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$ and 150 mM KCI and followed by incubation in 0.3 mM PMSF. The protein concentration of recombinant proteins was estimated by Coomassie blue staining of protein bands after SDS-PAGE using bovine serum albumin as a standard.

4.5.2. Fusion protein pull-down assay

Glutathione-immobilized fusion proteins were incubated in the presence or absence of hippocampal homogenate, Gβγ (Calbiochem), purified Hsc70, or recombinant CSP at 37°C for 30 min. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis.

4.6. Immunological assays

4.6.1. Antibodies

CSP polyclonal antibodies corresponding to amino acids 182-198 of rat CSP, or full-length recombinant CSP, were described previously (Braun and Scheller, 1995; Braun et al., 1996). Other antibodies were obtained commercially: anti-Ca^{2+} channel α_{1B} subunit (Chemicon International), anti-Ca^{2+} channel β1 (Transduction Labs), caveolin 1 polyclonal (Santa Cruz or Transduction Labs), anti-clathrin monoclonal (Transduction Labs), anti-FLAG M2 monoclonal (Stratagene), flotillin monoclonal (Transduction Labs), anti-GAP43 monoclonal (Sigma), anti-Gα monoclonal (Calbiochem), anti-Gq/11α polyclonal, anti-Gqα polyclonal, anti-G11α polyclonal, anti-Goα polyclonal (Santa Cruz), anti-Gi1/2α polyclonal, anti-Gsα polyclonal (Calbiochem), anti-Gβ monoclonal (Transduction Labs), goat anti-mouse and goat anti-rabbit (Santa Cruz or Amersham Pharmacia Biotech), anti-Hsc70 monoclonal (Sigma), IgG1 immunoglobulin (Sigma), nSec-1 polyclonal (Stressgen), anti-NSF polyclonal (StressGen), rabbit anti- OX_1R (Alpha Diagnostics), rsec8 monoclonal (Transduction Labs), anti-α/β SNAP (Stressgen), SNAP25 monoclonal (Sternberger Monoclonals), anti-synaptophysin monoclonal (Sigma), anti-synaptotagmin polyclonal, anti-SV2 monoclonal, anti-syntaxin monoclonal (Sigma), and VAMP2 polyclonal (Stressgen).

4.6.2. Immunoprecipitation

Immunoprecipitation was achieved by incubating detergent-solubilized (1 % Triton X-100) hippocampal homogenate with the indicated antibodies for 30 min at 37°C followed by protein A/G agarose beads (Santa Cruz Biotech) for 30 min, 22°C. Samples were washed, suspended in 30 µl of sample buffer, separated by SDS-PAGE, and subjected to immunoblotting.

In the case of FLAG immunoprecipitation, the lysate was added to the resin containing the antibody (anti-FLAG M2 affinity gel, Sigma), and incubated at +4°C for 2 hours. The immunoprecipitation samples were centrifuged for 15 seconds at 10 000 rpm and washed four times with washing buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, protease inhibitor mixture (Roche)). Beads were eluted in sample buffer (62.5 mM Tris-HCl pH 7.4, 25% glycerol, 2% SDS, 0.01 % bromophenol blue) or more gently by 3XFLAG peptide 150 ng/µl (Sigma) in the wash buffer for 30 min at +4°C and mixed with an equal amount of sample buffer. β-mercaptoethanol (5 %) was added to the supernatants.

4.6.3. Immunoblotting

Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.45 µm or 0.2 µm) or, in the case of orexin 1 receptor experiments, to PVDF (polyvinyldifluoride) membrane in 20 mM Tris, 150 mM glycine, and 12% methanol. Transferred proteins were visualized by staining with Ponceau S. Nitrocellulose membranes were blocked for nonspecific binding using 5% milk, 0.1 % Tween 20 in PBS solution and incubated overnight with primary antibody. The membranes were washed four times in the above milk/Tween/PBS solution and incubated for 30 min with goat anti-rabbit or goat anti-mouse IgG-coupled horseradish peroxidase. Proteins were visualized using chemiluminescence (Amersham ECL) or fluorescence (Amersham ECF). In some cases, proteins were silver stained with Silver Stain Plus (Bio-Rad). Immunoreactive bands were visualized following exposure of the membranes to Amersham Hyperfilm-MP, or via Storm Scan 860 (ImageQuaNT).

The EC_{50} was defined as the half-maximal binding of soluble CSP to the immobilized synprint region, based on pixel intensity obtained by imaging (Amersham ECF; Storm Molecular Dynamics) and analyzed by ImageQuant software (Molecular Dynamics). CSP was detected with anti-CSP polyclonal. The EC_{50} was estimated from plots of pixels versus concentration of protein added to beads.

4.7. Functional assays

4.7.1. Patch clamp recordings

Botulinum toxin treatment was achieved by incubating the cells in the presence of 1µg/ml of botulinum toxin C1 in DMEM for 12 h. Immediately prior to recording, individual coverslips were transferred to a 3 cm culture dish containing recording solution comprised of 20 mM $BaCI_2$ 1 mM $MgCI_2$ 10 mM HEPES, 40 mM Tetraethylammonium chloride (TEA-Cl), 10

mM glucose, and 65 mM CsCl (pH 7.2 with TEA-OH). Whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments) linked to a personal computer equipped with pCLAMP, version 6.0. Patch pipettes (Sutter borosilicate glass, BF150-86-15) were pulled (Sutter P-87 microelectrode puller), fire polished (Narashige microforge), and showed typical resistances of 3-4 MΩ. The internal pipette solution contained 108 mM CsMS, 4 mM $MgCI_2$, 9 mM EGTA, and 9 mM HEPES (pH 7.2). Data were filtered at 1 kHz, analyzed using Clampfit (Axon Instruments) and fitted using Sigmaplot 4.0 (Jandel Scientific). Error bars are standard error, unless stated otherwise. Numbers in parentheses reflect the numbers of experiments, and p values given reflect Student's t tests.

4.7.2. Ca^{2+} imaging

The experiments were performed as described in (Larsson et al., 2003). The coverslips with cells were treated with 4 µM Fura-2 AM (Molecular Probes) in a Na^+ based medium (137 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 20 mM HEPES, 10 mM glucose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM probenecid, pH 7.4), and incubated at +37°C for 15 minutes. The coverslip was attached to the bottom of a thermostated (+37°C) perfusion chamber. The cells were stimulated by perfusion with orexin A (Neosystem) diluted in the Na^+ based medium. The cells were excited by alternating 340 nm and 380 nm UV light with a filter exchanger in an InCytIM2 system (Intracellular Imaging, Cincinnati, OH) and a dichroic mirror (DM450, Nikon). The emission was measured through a 450 nm barrier filter with an integrating CCD camera. A new ratio image (340/380 nm) was achieved every second. The experiments were analyzed with Microgal Origin 6.0.

4.7.3. Measurement of cAMP

Cyclic AMP was measured using a CatchPoint Cyclic-AMP Fluorescent Assay Kit (Molecular Devices). HEK-OX1R cells were detached from the 6-well plates in PBS containing 0.5 mM EDTA, washed once with the Na^+-based medium (no probenecid) and suspended in an appropriate volume of the Na^+-based medium containing 0.5 mM IBMX. After 10 min preincubation with IBMX at 37°C, the reactions were started by adding cells to a 96-well plate containing premade solutions in a total volume of 150 µL/well. The reactions were stopped after 10 min by adding 50 µL stop solution included in the kit. 20 µL from each well were analyzed according to manufacturer's instructions and fluorescence was recorded in a FLUOstar plate reader (BMG LabTechnologies GmbH, Offenburg, Germany) using a 544 nm excitation filter and a 590 nm emission filter. Infected Sf9 insect cells were assayed at 26 h post infection using the similar assay procedure as with HEK-OX1R cells except that Na^+-based medium was insect cell specific and the incubations were done at room temperature.

5. RESULTS

5.1. Syntaxin facilitation of G protein modulation of N-type Ca^{2+} channels

5.1.1. Syntaxin independent G protein modulation of N-type Ca^{2+} channels

It is well established that N-type Ca^{2+} channels transiently expressed in human embryonic kidney tsA-201 (HEK) cells are subject to potent inhibition by $G\beta\gamma$ (Hamid et al., 1999; Zamponi and Snutch, 1998a; Zamponi et al., 1997; Herlitze et al., 1997; Ikeda, 1996). One characteristic feature of this type of inhibition is its reversal following a strong membrane depolarization (termed facilitation). We demonstrate that in the absence of exogenous $G\beta\gamma$, application of a strong depolarizing prepulse (150 mV, 50 ms) to N-type (α_{1B}, α_2-δ and β_{1b}) channels has little effect on peak current amplitude or current waveform. However, upon cotransfection with $G\beta_1\gamma_2$, peak current amplitude is increased by almost 2-fold subsequent to application of the prepulse, thus reflecting relief of the tonic inhibition of the channel induced by $G\beta_1\gamma_2$ (Publication **I**, Figure 1A).

The experiments of Stanley and Mirotznik (Stanley and Mirotznik, 1997) suggested that syntaxin 1A might be a prerequisite for G protein modulation of N-type Ca^{2+} channels, and if so, this would imply that HEK cells should endogenously express syntaxin 1A. To examine this possibility, we carried out an immunoblot of syntaxin 1A in HEK cells. We show that syntaxin 1A could only be detected when transfected, indicating that syntaxin 1A is not present endogenously in HEK cells (**I**, Figure 1B). We also demonstrate that syntaxin 1A expression is not triggered by overexpression of $G\beta\gamma$ or secondarily by expression of N-type Ca^{2+} channels as described recently for P/Q-type Ca^{2+} channels (Sutton et al., 1999). We could not detect any expression of other synaptic proteins, indicating that HEK cells lack many of the proteins responsible for vesicle release in neurons. Syntaxin 1A transfection did not mediate a detectable change in the amount of endogenous $G\beta\gamma$ present in HEK cells (**I**, Figure 1C). Our data demonstrate that the presence of syntaxin 1A is not required for N-type Ca^{2+} channels to undergo G protein mediated inhibition (**I**).

5.1.2. Syntaxin 1A promotion of G protein inhibition of N-type Ca^{2+} channels

Although syntaxin 1A is not a prerequisite for G protein modulation of N-type Ca^{2+} channels, it is possible that syntaxin 1A does modulate the effects of $G\beta\gamma$ on N-type Ca^{2+} channels. To examine this possibility, we cloned syntaxin 1A from rat brain and investigated its effect on transiently expressed N-type Ca^{2+} channels. N-type Ca^{2+} channels coexpressed with syntaxin 1A underwent a hyperpolarizing shift in steady state inactivation from -44.2 mV (n=11) to -61.9 mV (n=7) (**I**), similar to what has been previously reported for N-type channels expressed in *Xenopus* oocytes (Bezprozvanny et al., 1995) and thereby confirming functional syntaxin 1A expression in our experiments. However, an additional effect of syntaxin 1A was that the current waveform exhibited dramatically slowed activation and inactivation kinetics, and the average peak current amplitude decreased by nearly 1 order of magnitude (**I**, Figure

2A). Qualitatively, the current waveform in the presence of syntaxin 1A was reminiscent of that obtained upon coexpression of the channel with exogenous Gβγ, suggesting that syntaxin 1A might secondarily mediate a tonic G protein modulation of the channel. Consistent with this idea, application of a strong depolarizing prepulse resulted in a 2.2 ± 0.2-fold (n=10) increase in peak current amplitude. The magnitude of the prepulse effect was independent of the type of Ca^{2+} channel β subunit coexpressed (*i.e.* β_{1b}, β_{2a} β_3) and on the presence of the ancillary α_2-δ complex (**I**).

The prepulse facilitation was reduced to a 1.2 ± 0.04-fold (n=12) enhancement when cells were incubated with Botulinum toxin C1 (BTC1) for 12 h prior to recording, indicating that our observations were due to the presence of the syntaxin 1A protein rather than the preceding transcription events (**I**). To ensure that BTC1 did not directly interfere with G protein modulation of the channel, we investigated the effects of syntaxin 1A on N-type channels coexpressed with both syntaxin 1A and $G\beta_1\gamma_2$. Our data demonstrate that the effects of syntaxin 1A and $G\beta_1\gamma_2$ were not additive, and more importantly, BTC1 was ineffective in removing the tonic G protein inhibition (**I**, Figure 2A). Coexpression of the N-type Ca^{2+} channels with syntaxin 1A or $G\beta_1\gamma_2$ plus syntaxin 1A resulted in a degree of G protein inhibition that did not differ significantly from that obtained upon coexpression of $G\beta_1\gamma_2$ alone, and BTC1 selectively removed the tonic G protein inhibition induced by syntaxin 1A overexpression (**I**, Figure 2B). Overall, these data are consistent with a mechanism by which syntaxin 1A promotes tonic G protein modulation of N-type Ca^{2+} channels.

5.1.3. Syntaxin 1A interaction with Gβγ

One possible explanation for our observations is a mechanism by which syntaxin 1A binding to the channel increases the sensitivity of the channel to free Gβγ subunits, *i.e.* those that are not part of the Gαβγ trimers associated by seven helix transmembrane receptors. In this scenario, the cleavage of the syntaxin 1A protein would attenuate the enhancing effect, and the overexpression of exogenous Gβγ would mask the effect. To study in which way the binding occurs, we carried out a binding assay involving a GST-syntaxin 1A fusion protein. Upon incubation with rat hippocampal homogenate, the syntaxin 1A fusion proteins were able to precipitate Gβγ subunits in a syntaxin 1A concentration-dependent manner (**I**, Figure 3A). Similarly, increasing amounts of nSec-1, a protein known to tightly associate with syntaxin 1A, were precipitated from the homogenate as the GST-syntaxin 1A concentration was increased. On the other hand, GST-VAMP-2 beads were unable to associate with either of Gβγ or nSec-1, indicating that nonspecific binding of Gβγ to the GST beads did not occur. While these data are consistent with a direct interaction between syntaxin 1A and G proteins, this experiment did not rule out the possibility that Gβγ interacts with syntaxin 1A indirectly via one or more additional proteins.

To investigate this possibility, we examined the ability of GST-syntaxin 1A to interact with purified Gβγ subunits in the absence of other proteins. We demonstrate that the recombinant GST-syntaxin 1A was able to bind the purified Gβγ subunits, indicating that there is indeed a direct physical interaction between Gβγ and syntaxin 1A, independent of any intermediary proteins (**I**, Figure 3B). In summary, our data suggest a mechanism by which syntaxin 1A physically binds to Gβγ, bringing Gβγ into close vicinity with its target site on N-type Ca^{2+}

channel α_1 subunits and thereby optimizing Gβγ modulation of channel activity. To prove the hypothesis correct, syntaxin 1A should be able to concomitantly bind to the N-type channel and to Gβγ. To confirm this, we carried out an assay examining the interactions of syntaxin and Gβγ with fusion proteins directed against the channel synaptic protein interaction (synprint) site. The 6XHis-synprint site bound syntaxin 1A from rat hippocampal homogenate, but more importantly, Gβγ was also detected and reflected the increase in syntaxin 1A binding as the amount of homogenate was increased (**I**, Figure 3C). It has been previously shown that Gβγ does not bind directly to the N-type channel domain II-III linker (Zamponi et al., 1997) consistent with the idea that the Gβγ is coupled to the synprint site via syntaxin. Overall, these data further support the idea that syntaxin 1A may mediate a physical role in enhancing tonic G protein modulation of N-type Ca^{2+} channels, and it is likely that distinct regions on the syntaxin 1A molecule interact with the Ca^{2+} channel and Gβγ (**I**).

5.2. CSP regulation of G protein modulation of N-type Ca^{2+} channels

5.2.1. CSP interaction with G proteins and N-type Ca^{2+} channels

In order to investigate the possibility of an in vitro association between G proteins and CSP, GST fusion proteins consisting of full-length CSP or the amino terminus of CSP (amino acids 1-82, including the J domain) were used in an in vitro binding assay. Our data show that Gβ associated with recombinant CSP in vitro (**II**, Figure 1). Conversely, no interaction of Gβ was observed with the J domain, most likely indicating that Gβγ subunits specifically interact with the carboxyl terminus of CSP. Next, we investigated whether Hsc70, an abundant neural protein with ATPase activity, influenced the association of Gβγ with CSP. It has been previously shown that CSP interacts with and activates the ATPase activity of Hsc70 (Braun et al., 1996). The regulation of the assembly/disassembly of multimeric complexes such as presynaptic complexes is typical of this family of chaperone proteins. Our study demonstrates that Hsc70 as well as Gβ associated with recombinant CSP in vitro (**II**, Figure 2AB). While the interaction of CSP with Hsc70 was increased in the presence of ATP, the interaction of GSP with Gβ did not require ATP. Unlike Gβ, which interacted with the carboxy-terminal domain of the protein, Hsc70 interacted with the amino-terminal J domain of CSP. The interaction of CSP with Gβγ occurred both in the absence and presence of Hsc70, indicating that Hsc70 is neither required for nor does it interfere in the interaction between Gβ and CSP.

We note that previous reports have indicated that CSP-protein interactions are typically transient and difficult to detect (Braun et al., 1996; Leveque et al., 1998; Nie et al., 1999). The relatively stable nature of the CSP/Gβ complex is, therefore, unexpected, particularly in view of the notion that protein-protein interactions among molecular co-chaperone-chaperone proteins like CSP/Hsc70 are typically of transient nature (Silver and Way, 1993; Cyr et al., 1994). No interaction between CSP and other synaptic proteins were detected (**II**, Figure 2D). These results suggest that the Gβ/CSP, also with the assistance of Hsc70, may contribute to the function of CSP in synaptic transmission. To investigate which proteins CSP and Gβγ associate with in brain tissue, we performed immunoprecipitations with rat hippocampus. We show that Gβ and CSP co-precipitated with anti-Gβ monoclonal, anti-syntaxin monoclonal, and anti-Ca^{2+} channel β1 subunit (**II**, Figure 3AB). Other synaptic proteins were not observed to precipitate with CSP. Only minor amounts of Hsc70 precipitated with CSP, suggesting that

the CSP/Hsc70 complex is less stable than the CSP/Gβγ complex (**II**, Figure 3C). We also demonstrate that Gβ interacted with recombinant CSP, syntaxin, and the synprint motif of Ca^{2+} channels, but not the CSP J domain (**II**, Figure 4A). In contrast, Gα was observed to interact strongly with CSP and syntaxin, weakly with the synprint motif of the Ca^{2+} channel in an ATP-independent manner, and strongly with the CSP J domain in an ATP-dependent manner (**II**, Figure 4B). Hsc70 associated primarily with CSP and the amino-terminal J domain construct but also weakly with syntaxin and the cytoplasmic II-III loop synprint motif of Ca^{2+} channels (**II**, Figure 4C).

The observation that separate regions of CSP associate with Gα and Gβ raises the possibility that CSP may chaperone Gα/Gβγ interactions in addition to Gβγ/Ca^{2+} channel interactions. We also show that the recombinant CSP/GST was able to bind purified Gβγ subunits (**II**, Figure 5A), indicating that the CSP/Gβγ complex is indeed a direct physical interaction between Gβγ and CSP. As it was shown previously, syntaxin was also capable of interacting with Gβγ. However, this interaction appeared to be much weaker than that between CSP and Gβγ. Binding of Gβ to CSP was four times greater than binding to syntaxin. In contrast, Gβγ did not form a direct interaction with the synprint motif of the α_{1B} Ca^{2+} channel subunit or the CSP J domain (**II**, Figure 5A). These results indicate that Gβγ directly interacts with CSP and syntaxin and may be coupled to the synprint site of Ca^{2+} channels indirectly via CSP or syntaxin. CSP was also observed to directly interact with the synprint motif of the Ca^{2+} channel (**II**, Figure 5B). Rapid chemical cross-linking of intact hippocampal slices revealed that CSP and Gβγ exist as a complex in vivo, as they are detected in colocalized complexes (**II**, Figure 5C). Overall, our data strongly suggest that CSP is capable of binding to both the N-type Ca^{2+} channels and to Gβγ in vitro, that a CSP/Gβγ complex exists in brain, and that these interactions are a physiological feature of neurons (**II**).

5.2.2. CSP promotion of G protein inhibition of N-type Ca^{2+} channels

To test whether the physical interaction between CSP and the N-type channel domain II-III linker region resulted in functional effects on the channel, we coexpressed CSP with N-type (α_{1B}, α_2-δ and β_{1b}) Ca^{2+} channels in tsa-201 cells and assessed its effect on channel function via whole-cell patch clamp recordings. Upon coexpression with CSP, the channels exhibited a slowed current waveform compared to that typically observed with N-type Ca^{2+} channels (**II**, Figure 6A). Kinetic slowing observed in the presence of CSP is indicative of tonic inhibition of the channels by Gβγ, and indeed, following application of a strong depolarizing prepulse (PP), peak current amplitude became dramatically increased, consistent with removal of a G protein-mediated inhibitory effect (Bean, 1989). To further examine this possibility, we cotransfected N-type channels together with CSP and the carboxy-terminal fragment of the β-adrenergic receptor kinase (βARK), an effective Gβγ sponge (Koch et al., 1994). We show that the degree of PP relief was greatly attenuated in the presence of the βARK fragment, further implicating the involvement of Gβγ in this effect (**II**, Figure 6A). In addition, the PP effect exhibited other hallmarks of G protein inhibition: PP relief developed monoexponentially as a function of PP duration, reinhibition following the PP occurred rapidly, and the degree of PP relief was dependent on the test potential (**II**, Figure 6B).

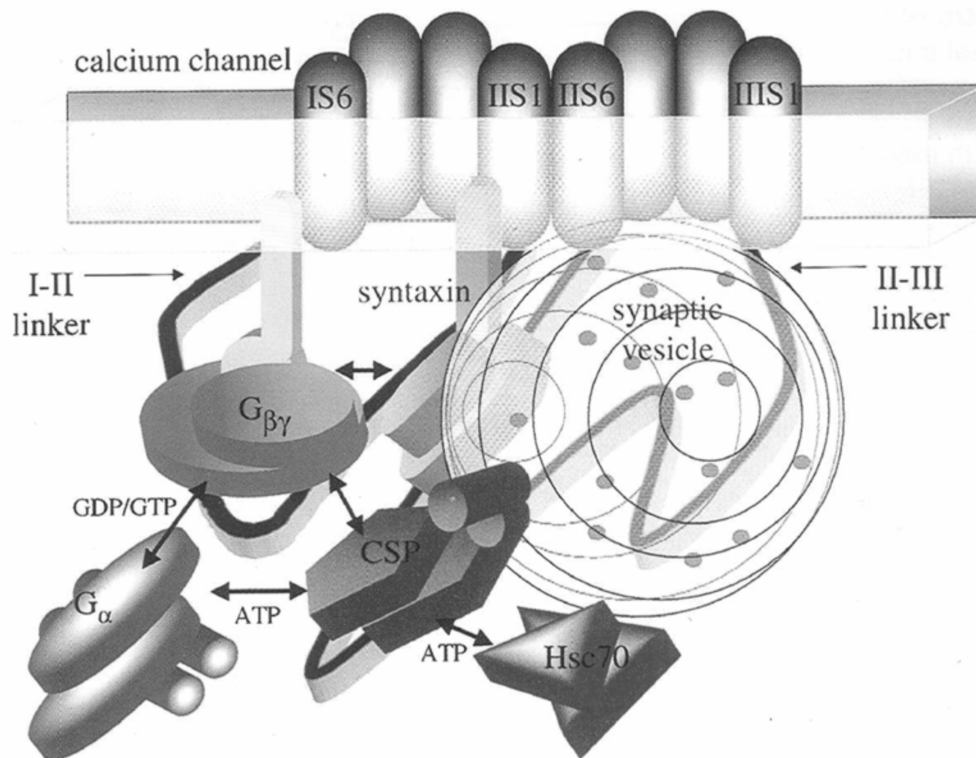


Figure 5. Model depicting the role of the syntaxin and CSP/Hsc70 chaperone machine in the regulation of G protein modulation of Ca^{2+} channel activity. The synaptic vesicle protein CSP, and the membrane bound synaptic protein syntaxin, by physically binding to the domain II-III linker region of the N-type Ca^{2+} channel subunit and to the Gβγ protein dimer, may help target Gβγ to its site of action on the N-type channel domain I-II linker. In addition, CSP may participate in the assembly/disassembly of the Gαβγ complex.

We also demonstrate that coexpression of CSP precluded an additional inhibitory effect of transiently overexpressed Gβγ subunits as well as the tonic G protein inhibition of N-type Ca^{2+} channels mediated by syntaxin 1A (**II**, Figure 6C). The observed effects did not appear to depend on GDP/GTP exchange because inclusion of GDPβS in the patch pipette did not abolish the CSP-induced G protein inhibition. To assess whether the CSP-mediated G protein inhibition of N-type Ca^{2+} channel activity was dependent on an interaction between CSP and the channel rather than a general CSP-induced activation of G protein signaling in tsa-201 cells, we coexpressed the complete synprint region of the α_{1B} II-III linker along with the channel and CSP to competitively inhibit CSP binding to the channel. We observed a dramatic reduction in the degree of CSP-induced PP facilitation upon coexpression of the synprint domain. In contrast, modulation of channel activity by exogenously expressed Gβγ was not affected by the presence of the synprint domain, indicating that the synprint domain did not generally interfere with G protein inhibition. A construct corresponding to the L-type channel II-III linker had no effect on the CSP-mediated G protein inhibition. Taken together, our electrophysical experiments indicate that the interactions between CSP and the N-type Ca^{2+} channels result in a robust tonic inhibition of channel activity by G protein βγ subunits (**II**).

5.3. Caveolin association with synaptic proteins in nerve terminal caveolae

5.3.1. Caveolin interaction with synaptic proteins

We investigated *in vitro* association between SNAP25 and caveolin1 by GST-SNAP25 binding assays. Our data demonstrate that caveolin1 associated with SNAP25 to a greater extent at 37°C than when the incubation was carried out on ice (**III**, Figure 1). In addition, the binding of caveolin1 to SNAP25 was increased in the presence of 2 mM ATP but was unchanged in the presence of the nonhydrolizable ATP homologue ATPγS. $CaCl_2$ (0.5 mM) was not observed to alter the interaction between SNAP25 and caveolin1. These results indicate that the interaction between SNAP25 and caveolin1 is dependent on temperature and ATP hydrolysis. Since it is likely that a 4-aminopyridine (4-AP) -induced rise in intracellular Ca^{2+} concentration in intact hippocampal slices initiates the formation of the SNAP25/caveolin1 complex, we evaluated the effects of divalent chelators EDTA/EGTA on the SNAP25/ caveolin1 interaction *in vitro.* We show that the association of SNAP25 with caveolin1 was reduced in the presence of 5 mM EDTA or 10 mM EGTA, and abolished at 10 mM EDTA or 20 mM EGTA, respectively (**III**, Figure 2). Our results demonstrate that divalent cations are essential for the association of caveolin1 and SNAP25 *in vitro* (**III**).

The EDTA sensitivity of the SNAP25/caveolin1 interaction may reflect a $MgCl_2$ requirement for ATP hydrolysis suggesting ATPases (e.g., kinases or chaperones) are important in the interaction. Notably, SNAP25 (Risinger and Bennett, 1999; Shimazaki et al., 1996), syntaxin (Risinger and Bennett, 1999; Foletti et al., 2000), VAMP2 (Hirling and Scheller, 1996) and caveolin1 (Davy et al., 2000) are reported substrates for protein kinases (Lin and Scheller, 2000),(Smart et al., 1999b) while SNARE complexes are disassembled with the ATPase NSF (Brunger, 2000; Lin and Scheller, 2000). We examined the association of syntaxin and VAMP under conditions that promoted (i.e., ATP) and inhibited (i.e., EDTA) the association of caveolin with SNAP25. We show that recombinant SNAP25/GST was able to bind native VAMP and syntaxin under all conditions examined (±EDTA, EGTA). In contrast, caveolin1 binding to SNAP25 is abolished in the presence of EGTA and EDTA (**III**, Figure 2). These results indicate that hippocampal VAMP and syntaxin can interact with recombinant SNAP25/GST to form the SNARE interactions in either the absence or presence of caveolin1 suggesting that caveolin1 is neither required nor does it interfere in the interaction between SNARE proteins *in vitro*(**III**).

5.3.2. Synaptic proteins localization in hippocampal caveolae

To assess whether caveolin1/SNARE interactions reflect the presence of SNAREs in caveolae, we biochemically isolated a caveolar fraction, a cytoskeletal fraction and a synaptosomal fraction from rat hippocampus. The SNARE family of proteins SNAP25, syntaxin and VAMP were abundant in the caveolae fraction as well as the synaptosome fraction (**III**, Figure 3). Established caveolae proteins such as caveolin1, flotillin, GAP43, and G proteins were observed to co-fractionate in the caveolar preparation. Presynaptic Ca^{2+} channels and the soluble NSF attachment protein αSNAP are absent from the caveolar fraction but present in the nerve terminal (synaptosomes) preparation. The presence of established caveolae proteins demonstrates the integrity of the caveolae fraction. Several of

the proteins present in the caveolar fraction including caveolin1, SNAP25, syntaxin, VAMP2 and CSP may also be prone to post-translational modification. The presence of SNAREs in hippocampal caveolae is consistent with that reported for endothelial caveolae (McIntosh and Schnitzer, 1999; Lafont et al., 1999; Schnitzer et al., 1995). SNAREs have also been reported to be enriched in lipid clusters of PC12 cells (Lang et al., 2001; Chamberlain et al., 2001).

5.3.3. Caveolin interaction with SNAREs

Caveolin1 was observed to interact with syntaxin/GST as well as SNAP25/GST (**III**, Figure 4A). It has been previously reported that caveolin1 does not associate with VAMP2/GST (Braun and Madison, 2000). Like the SNAP25/caveolin1 interaction, the syntaxin/caveolin1 interaction was enhanced in the presence of ATP. To further examine the possibility that ATP dependent processes influence the interaction between SNAP25 and caveolin1, the caveolin1/SNAP25 interaction was evaluated in the presence of alkaline phosphatase. Removal of protein phosphate by alkaline phosphatase reduced the association between caveolin1/SNAP25 and caveolin/syntaxin. Also, less caveolin was observed to associate with SNAP25 in the absence of phosphatase inhibitors than in the presence of phosphatase inhibitors (1 μM microcystin, 1 μM okadaic acid, 1 mM sodium orthovanadate). To directly examine the possibility that phosphorylation underlies the increase in caveolin/syntaxin and caveolin/SNAP25 binding, we performed our *in vitro* binding assay in the presence of [γ^{32}]ATP. Native VAMP, recombinant syntaxin, recombinant SNAP25, native nsec-1, and native syntaxin were observed to undergo phosphorylation (identification of ^{32}P phosphorylated proteins) (**III**, Figure 4B). Caveolin was not observed to be phosphorylated under these conditions. Immunoblot analysis did not detect any phosphotyrosine incorporation. Thus, our results suggest that the interaction of SNAP25 and syntaxin with caveolin1 is enhanced by serine/threonine phosphorylation of SNAREs.

To investigate if the caveolin/SNARE interaction is direct, we examined the ability of fusion proteins consisting of full length caveolin 1α and caveolin 1β to interact with recombinant SNAP25 and syntaxin. The recombinant caveolin 1α and caveolin 1β were both able to bind SNAP25 and syntaxin, indicating that the caveolin/SNAP25 and caveolin/syntaxin complexes are indeed direct physical interactions between caveolin and SNAREs (**III**, Figure 5AB). In contrast to the association of the native protein, ATP did not dramatically increase association of the recombinant proteins. These results indicate that the increase in binding of SNAP25 and syntaxin in the presence of ATP involves an additional component present in homogenate but absent from recombinant protein (e.g., kinases, chaperones). We also show that when rat hippocampal homogenate was incubated with caveolin 1α, 1β, 2 or 3, both SNAP25 and syntaxin were observed to bind all caveolin family members (**III**, Figure 5C). Overall, our data strongly suggest that caveolin1 is capable of binding to SNAREs *in vitro,* that the caveolin/syntaxin and caveolin/SNAP25 interactions are direct, and that the interactions are enhanced by phosphorylation (**III**).

Table 3. Summary of results obtained by studies with synaptic proteins, G proteins and N-type Ca^{2+} channels. *Plus* indicates that the proteins in question interact, whereas *minus* indicates that no interaction was observed. *ATP* indicates that positive results depend on phosphorylation. *Cat* indicates that positive results depend on presence of divalent cations, namely Ca^{2+}. We have also demonstrated electophysiologically the role of CSP and syntaxin regulating the activity of presynaptic Ca^{2+} channels: syntaxin and CSP modulate G protein mediated N-type Ca^{2+} channel inhibition.

	Protein	Associates with:									Locate in:	
		syntaxin	SNAP25	VAMP2	II-III linker synprint	β1	Cav1	CSP	CSP Jdomain	Cav1	caveolae	synapto-somes
Synaptic core complex proteins	Syntaxin1A		+		+			+		+ATP/cat	+	+
	SNAP25	+		+				-		+ATP/cat	+	+
	VAMP2		+								+	+
N-type calcium channel subunits	Synprint motif of II-III linker	+						+				
	β1							+			-	+
G protein subunits	Gα	+			+			+	+ATP			
	Gβγ	+		-	+	+		+	-		+	+
Core complex associated proteins	CSP	+	-		+	+					+	+
	NSec-1	+		-				-			+	+
	Hsc70	+			+			+ATP	+ATP			
	GAP43							-			+	+
	NSF							-			+	+
	Flotillin							-			+	-
	α/βSNAP										-	+
Caveolae forming proteins	Caveolin1	+ATP/cat	+ATP/cat								+	+
	Caveolin2	+	+									
	Caveolin3	+	+									

5.4. G protein coupling properties of orexin 1 receptor

In order to establish the interaction of the orexin 1 receptor (OX_1R), we used immunoprecipitation with HEK293 cells stably expressing epitope-tagged OX_1R receptor with a triple FLAG peptide in its C-terminus (OX_1R-FLAG). This construct had the ability of orexin A (OX-A) to mobilize Ca^{2+} in the HEK293 cells stably expressing the OX_1R-FLAG construct (**IV**, Figure 1A) in a manner dependent on extracellular Ca^{2+} as shown previously in a variety of cell lines (Holmqvist et al., 2002; Kukkonen and Akerman, 2001; Lund et al., 2000). We immunoprecipitated the OX_1R-FLAG with FLAG antibody and detected the receptor with OX_1R antibody (**IV**, Figure 1B). We demonstrated that Gqα/G11α proteins co-immunoprecipitated with OX_1R as detected using a Gqα/G11α selective antibody (positive in 15/15 experiments) (**IV**, Figure 2). OX_1R bound fairly exclusively to G11α (positive in 3/4 experiments). Binding of OX_1R to Gqα was rather negative (positive in 2/11 experiments), possibly because of the lower expression level of Gqα in HEK293 cells. Giα and Gsα also co-immunoprecipitated with OX_1R (positive in 6/11 and 2/3 of experiments, respectively). Binding of Goα to OX_1R was negative (positive in 0/6 experiments). Because the availability of G-proteins may dictate the coupling efficiency to G-proteins, we also tested the G protein interactions with OX_1R in the presence of over-expressed, transiently transfected G protein alpha subunits in OX_1R-FLAG cells. In this case, G11α, Gqα, Giα, Gsα, and Goα co-immunoprecipitated with the OX_1R. Excessive G protein alpha subunits did not show any nonspecific binding when tested with Gqα and Giα. This result indicates that OX_1R has the intrinsic ability to bind multiple G proteins (**IV**).

In order to investigate the coupling of OX_1R to Gα subunits, we constructed Gsα proteins with alternative G protein sequences at the C-terminus, a region known to interact with receptors. Using this approach, receptors activating a particular G protein would be linked by the chimeric G protein to activation of adenylate cyclase, and thus increased cAMP synthesis. We validated the constructs using muscarinic receptors previously shown to couple to specific G-proteins in this system. The G protein constructs were expressed in HEK293 cells expressing the OX_1R-FLAG. A significant stimulation of cAMP synthesis was obtained with Gqα/G11α and G16α, but not with Goα, Giα, G12α or Gsα (**IV**, Figure 3). Taken together, our data show that OX_1R binds to Gqα/G11α (more specifically to G11α than Gqα) and to a lesser extent to Giα and Gsα, but not to Goα (**IV**).

6. DISCUSSION

6.1. Modulation of presynaptic Ca^{2+} channels

The modulation of presynaptic Ca^{2+} channel activity by intracellular messenger pathways and G protein $\beta\gamma$ subunits has been well documented (Dunlap and Fischbach, 1981; Bourinet et al., 1996; Stea et al., 1995; Swartz, 1993; Swartz et al., 1993; Herlitze et al., 1996; Ikeda, 1996; Zamponi et al., 1997; Dascal, 2001; Miller, 1998). Upon activation of G protein-coupled seven-helix transmembrane receptors, both N-type and P/Q-type channels undergo a pronounced voltage-dependent inhibition (Ikeda and Schofield, 1989; Bean, 1989; Boland and Bean, 1993; Mintz and Bean, 1993; Hille, 1994). G$\beta\gamma$ binds to the Ca^{2+} channel α_1 subunit with 1:1 stoichiometry, which results in a stabilization of the closed states of the channel (Patil et al., 1996; Zamponi and Snutch, 1998a; Hamid et al., 1999). This effect can be reversed by strong membrane depolarization, resulting in an apparent facilitation of Ca^{2+} currents after application of a depolarizing prepulse (Bourinet et al., 1996; Bean, 1989; Zamponi and Snutch, 1998a).

G$\beta\gamma$ interacts with the Ca^{2+} channel α_1 subunit at the I-II linker and the carboxyl tail regions (Zamponi et al., 1997; De Waard et al., 1997; Herlitze et al., 1996). Syntaxin 1A, the cytoplasmically oriented membrane protein that is involved in synaptic vesicle release, is known to physically bind to Ca^{2+} channels (Sheng et al., 1994; Yokoyama et al., 1997). Therefore, we wanted to study whether syntaxin 1A facilitates the co-localization of G$\beta\gamma$ and the calcium channel, as previously suggested (Stanley and Mirotznik, 1997). We present evidence that syntaxin 1A augments, but is not required for, G protein modulation of transiently expressed Ca^{2+} channels (**I**). Overall, our data are consistent with a model in which syntaxin 1A physically localizes free endogenous G$\beta\gamma$ subunits to the vicinity of the N-type Ca^{2+} channel α_1 subunit, thus increasing the effective local G$\beta\gamma$ concentration near the channel and thereby facilitating the interactions between the channel and the G protein.

There have been reports supporting a role for CSP in exocytosis or regulation of Ca^{2+} channel activity. The close proximity of fusion-competent synaptic vesicles to voltage-gated Ca^{2+} channels that trigger release at the active zone supports a possible CSP/Ca^{2+} channel interaction. Several lines of evidence suggest that CSP may indeed modulate Ca^{2+} channel activity (Gundersen and Umbach, 1992; Umbach and Gundersen, 1997; Ranjan et al., 1998). In addition, the idea is further strengthened by the report that CSP binds to α_{1A} subunits of P/Q channels (Leveque et al., 1998). Also, it has been shown that overexpression of CSP suppresses the decrease of evoked release induced by the overexpression of syntaxin, suggesting that CSP could regulate a syntaxin-containing Ca^{2+} channel complex (Nie et al., 1999). In contrast, CSP has also been suggested to have a direct role in Ca^{2+} independent exocytosis (Chamberlain and Burgoyne, 1998; Brown et al., 1998; Graham and Burgoyne, 2000). To test the hypothesis that CSP influences the excitation-secretion coupling pathway by forming direct protein-protein interactions with syntaxin and G proteins and thereby secondarily influencing Ca^{2+} channel activity, we examined the interaction of CSP with proteins known to contribute to presynaptic inhibition.

Our data indicate that CSP promotes a tonic inhibition of N-type Ca^{2+} channel activity (**II**). The in vitro binding observations, together with transient expression of key proteins in HEK

cells and rapid chemical cross-linking of intact hippocampal slices, strongly support a mechanism in which CSP promotes the inhibition of N-type Ca^{2+} channels by endogenously present Gβγ. CSP, when in close proximity to the active zone, associates with Gβγ and Ca^{2+} channels, favoring a Gβγ/Ca^{2+} channel complex and possibly preventing the formation of the Gα/Gβγ complex. We show Gα to interact with the J domain of CSP, whereas Gβγ interacts with the C terminus. Interaction of either binding site of CSP with G proteins elicits robust tonic inhibition of N-type channel activity (Miller et al., 2003b). However, CSP J-domain and CSP C-terminus inhibition of Ca^{2+} channels occur through different mechanisms. Ca^{2+} channel inhibition by CSP C-terminus, thus involvement of Gβγ, is blocked by co-expression of the synprint site of N-type Ca^{2+} channel, indicating that this inhibition is dependent on a physical interaction with the channel. Taken together, CSP-mediated promotion of Gβγ binding to the N-type Ca^{2+} channel domain I-II linker region (De Waard et al., 1997; Herlitze et al., 1997; Yokoyama et al., 1997) results in a tonic inhibition of presynaptic Ca^{2+} channels.

Syntaxin has been shown to bind to the synprint motif of the Ca^{2+} channel (Rettig et al., 1996; Sheng et al., 1996). Since CSP also binds to the same II-III cytoplasmic loop of presynaptic Ca^{2+} channels, CSP may also chaperone interactions between Ca^{2+} channels and synprint interacting proteins such as SNAP25 and syntaxin. The CSP-containing complexes are influenced by ATP, and therefore, are dynamic in nature. The regulation of Ca^{2+} channels by chaperones like CSP represents a novel mechanism for the fine tuning of Ca^{2+} entry into presynaptic nerve terminals, and thus, the control of neurotransmitter release and synaptic efficacy. Recently, CSP has been shown to play a role in pathological conditions as the action of CSP in inhibition of Ca^{2+} channels is blocked by mutant huntingtin (Miller et al., 2003a).

The regulation of neurotransmission by voltage-dependent Ca^{2+} channels is a highly dynamic process, which is modulated by complex interactions between cytoplasmic messenger molecules, SNARE proteins, and multiple types of voltage-dependent Ca^{2+} channels (Wheeler et al., 1994). For example, G protein inhibition of N-type Ca^{2+} channels due to the activation of seven helix transmembrane receptors inhibits synaptic transmission, whereas PKC-dependent phosphorylation of the N-type channel α_1 subunit increases channel activity and antagonizes G protein and syntaxin 1A binding to the channel (Yokoyama et al., 1997; Hamid et al., 1999; Zamponi et al., 1997). Recently, G protein inhibition of N- type Ca^{2+} channels has been shown to be critically dependent on specific regions of the Gβ subunit (Doering et al., 2004). Interestingly, these regions are distinct from those involved in Gβγ signaling to other effectors. Therefore, the specificity for G protein signaling to Ca^{2+} channels may rely on unique Gβ structural determinants. Also, a specific Gβ subunit contains a precise locus of two amino acids that allows the subunit to sense the negative charge of a phosphate group on the N-type Ca^{2+} channel α_1 subunit. Thus, this molecular detector of PKC-dependent phosphorylation provides a molecular basis for G protein-PKC cross-talk in modulation of N-type Ca^{2+} channels (Doering et al., 2004).

The two major Ca^{2+} channel subtypes found at presynaptic nerve terminals (i.e. P/Q-type and N-type channels) are differentially modulated by G proteins and PKC (Bourinet et al., 1996; Stea et al., 1995; Zamponi et al., 1997). In addition, it has been reported that entry of Ca^{2+} ions through certain (P/Q type) α_{1A} Ca^{2+} channel isoforms triggers the expression of syntaxin 1A via Ca^{2+}-dependent gene transcription (Sutton et al., 1999). Therefore, a potential feedback mechanism by which expression of a specific Ca^{2+} channel type regulates the

activity of another may contribute to the fine tuning of neurotransmission. Both syntaxin (Stanley and Mirotznik, 1997) and CSP contribute to channel regulation by G protein βγ subunits. At some point, the extent of N-type channel modulation is dependent on the Gβ subtype (Arnot et al., 2000; Ruiz-Velasco and Ikeda, 2000). Thus, nature has devised a multitude of avenues for the precise control of Ca^{2+} entry into presynaptic nerve terminals and, therefore, the control of neurotransmission (Wheeler et al., 1994).

6.2. Caveolae function in sorting out the signals

Caveolins are a family of proteins that form the principle structure of invaginations of the plasma membrane called caveolae, thereby organizing and concentrating specific molecules in discrete regions of the plasma membrane of most cell types (Razani et al., 2002). Caveolae are enriched in proteins important for signal transduction, such as G protein coupled receptors (e.g. the OX1 receptor (unpublished observations)), receptor tyrosine kinases, components of the Ras mitogen activated protein kinases pathway, Src family tyrosine kinases, PKCs, and nitric oxide synthase (Anderson, 1998; Okamoto et al., 1998). Caveolin has been shown to interact with SNARE proteins. The SNAP25/caveolin1 complex displays several important cellular properties: the complex is sensitive to changes in synaptic strength (Braun and Madison, 2000; Bock and Scheller, 1999) caveolin1 and SNAP25 demonstrate widespread localization along the neuronal plasmalemma (Braun and Madison, 2000); (Fernandez-Chacon and Sudhof, 1999) and caveolin1 and SNAP25 co-enrich in hippocampal nerve terminals and co-localize in hippocampal cultures (Braun and Madison, 2000). However, little is known about the role of caveolin in neurotransmission. In fact, until very recently, caveolin was thought to be absent from nervous tissue (Braun and Madison, 2000; Davy et al., 2000; Megias et al., 2000; Cameron et al., 1997; Ikezu et al., 1998; Galbiati et al., 1998; Mikol et al., 1999). We examined the interaction of caveolin1 with core complex synaptic proteins in the hippocampus. Our data demonstrate that caveolin1 has direct interactions in vitro with syntaxin and SNAP25 (**III**). The interactions are enhanced by ATP hydrolysis and phosphorylation is involved. We also show that SNAREs are present in caveolae isolated from the hippocampus.

In CNS, also nonneuronal cells have been shown to have regulated, vesicular exocytosis (Zhang et al.,2004; Bezzi et al., 2004). As there is no united opinion on the role of caveolae in neurons, the interactions detected with caveolin 1 may reflect those encountered in astrocytes. Thus, our results do not specify the regulatory protein-proteins interactions to occur in neuronal or nonneuronal supportive cells. However, taken together with previous results (**I**, **II**), there is no reason to question caveolin 1 interactions with synaptic machinery proteins. Based on current knowledge, it is impossible to know whether the SNAP25/caveolin1 and syntaxin/caveolin1 complexes are presynaptic or postsynaptic complexes since SNAP25 and caveolin1 are widely distributed in neural plasmalemma in hippocampal neurons (Braun and Madison, 2000; Foletti et al., 2000; Galli et al., 1995; Garcia et al., 1995). In principle, the interaction of SNAREs with caveolin1 could represent a key site that controls either the process of neurotransmitter release or postsynaptic receptor localization, mobility and trafficking. Despite considerable effort, the specific roles of the presynaptic and postsynaptic elements of the synapse that are thought to contribute to changes in synaptic transmission have been difficult to separate and characterize.

Although the underlying mechanisms are widely thought to reside within the postsynaptic cell, available evidence does not rule out participation of the presynaptic terminal in altered synaptic strength (Malenka and Nicoll, 1999; Malinow et al., 2000; Luscher et al., 2000). Given that caveolin contributes to the formation of signal transduction hotspots in the membrane, a role for presynaptic protein kinases in the induction of LTP (Smart et al., 1999b) supports our results demonstrating that the SNAP25/caveolin1 and syntaxin/caveolin1 complexes involve phosphorylation. In agreement with our results, numerous current reports have shown that synaptic proteins are phosphorylated by different kinases including SNAP25 (Risinger and Bennett, 1999; Shimazaki et al., 1996) syntaxin (Risinger and Bennett, 1999; Foletti et al., 2000; Hirling and Scheller, 1996; Schlegel et al., 2001) VAMP2 (Hirling and Scheller, 1996; Nielander et al., 1995) nSec-1 (Fujita et al., 1996) and caveolin1 (Glenney, Jr. and Soppet, 1992).

The functional consequences of phosphorylation on synaptic transmission are not yet established. Recent reports have identified discrete subdomains of phosphorylated syntaxin along the axonal plasma membrane in rat brain (Foletti et al., 2000) and have found SNAREs to be concentrated in cholesterol subdomains (Lang et al., 2001; Chamberlain et al., 2001). In conclusion, these findings suggest that caveolin is a SNARE accessory protein and that SNAREs and caveolin1 may be components of a much larger complex which may include kinases and receptors involved in signaling pathways active during the onset of potentiation. The sequence of events that lead to prolonged changes in synaptic strength such as those that occur in LTP and long-term depression (LTD) are not yet completely understood (Malenka and Nicoll, 1999; Malinow et al., 2000; Luscher et al., 2000). Changes in the proteins involved in regulation of synaptic vesicle release may underlie changes in synaptic strength. In addition, it is becoming increasingly evident that many of the proteins directly implicated in synaptic transmission do not act individually, but rather, function as part of larger multimeric protein complexes in a cyclic and dynamic fashion. Many experiments are needed to determine the detailed synaptic protein complex assembly/disassembly that takes place during synaptic transmission.

6.3. Postsynaptic Ca^{2+} signaling mediation by a peptidergic receptor

We wanted to study GPCR mediated Ca^{2+} signaling mechanisms, namely the G protein binding spectra of a peptidergic orexin 1 receptor (OX_1R). Orexins are hypothalamic peptides known to activate G protein coupled orexin receptors OX_1R and OX_2R (Sakurai et al., 1998; de Lecea et al., 1998) and have a role in the regulation of sleeping and feeding. They are also found outside the central nervous system in neurons and neuroendocrine cells involved in energy metabolism as well as feeding-associated processes. Orexins display excitation in both the CNS and PNS (Kukkonen et al., 2002). Functional experiments suggest that OX_1R and OX_2R couple to a variety of G proteins in native and recombinant OX_1R or OX_2R expressing cells (Randeva et al., 2001). The main cellular response to orexins in neurons is a robust elevation of intracellular Ca^{2+} (Kukkonen et al., 2002), which requires extracellular Ca^{2+} (van den Pol et al., 1998; van den Pol et al., 2001). In addition to this response, high concentrations of orexins activate PLC and release Ca^{2+} from internal stores. The same response is also seen in the recombinant systems (Sakurai et al., 1998; Smart et al., 1999a; Holmqvist et al., 2002).

In cAMP assays or labeled GTP binding experiments, OX_1R and OX_2R have both been shown to couple to multiple G proteins. OX_1R has been shown to couple preferentially to pertussis toxin-insensitive G proteins (e.g. Gqα/G11α), while OX_2R couples to both pertussis toxin-insensitive and sensitive (i.e. Giα, Goα) G proteins (Zhu et al., 2003). OX_1R has also been shown to couple to adenylate cyclase activation (i.e. possibly Gsα) (Malendowicz et al., 1999))(Mazzocchi et al., 2001). Recently, OX_1R has been shown to regulate adenylyl cyclase activity via multiple mechanisms (Holmqvist et al., 2004). The data concerning the coupling of the OX_1R is complicated by the fact that this receptor may also form heterodimers that promote coupling to Giα type G proteins (Hilairet et al., 2003). The versatility of orexin-mediated G protein pathways raises a need to study interactions between the receptors and the G proteins directly. We focused on studying the G protein interactions of the OX_1R. Our data demonstrate that OX_1R couples primarily to Gqα/G11α and the G16α family of G proteins (**IV**). We also show that OX_1R couples to Giα and Gsα proteins, but not to Goα (**IV**). Our data is consistent with previous studies on orexin mediated Ca^{2+} signaling (Kukkonen et al., 2002; Kukkonen and Akerman, 2001; Lund et al., 2000). Many receptors are known to couple to G16α protein, the function of which is not completely understood.

We did not detect the inhibition or facilitation of cAMP formation when stimulated with OX-A. OX_1R influence on adenylyl cyclase may be dependent on the concentration of the agonist, and thus, it needs to be determined for each cell line specifically. We did not perform this, since we were interested in OX_1R mediated Ca^{2+} signaling, and thus, used the concentrations of the agonist that contribute to Ca^{2+} influx and Ca^{2+} release (1 nM and 10-100 nM of orexin A in HEK293 OX_1R -FLAG cells, respectively). We wanted to investigate whether the different Ca^{2+} pathways activated by OX_1R may be explained by differential coupling to G proteins. Interestingly, the OX_1R appeared to preferentially interact with G11α over Gqα (**IV**). In some studies, Gqα and G11α have been shown to activate different pathways. G11α has been reported to preferentially couple with pathways leading to activation of Ca^{2+} influx rather than release from stores (Macrez-Lepretre et al., 1997; Hoang et al., 2003). This may be of interest concerning the ability of orexin receptors to couple to Ca^{2+} influx in the absence of store discharge (Lund et al., 2000).

The assessment of the coupling of specific G-protein subclasses to distinct receptors may be difficult for several reasons. Often, effector mechanisms are regulated by several other factors than G-protein α subunits. Gβγ, Ca^{2+} and protein kinases have profound effects on effectors such as adenylyl cyclase or PLC isoforms. In addition, over-expression of receptors can change the ability of receptors to couple to G-proteins (Kukkonen et al., 2001; Nasman et al., 2001). Furthermore, the formation of receptor heterodimer complexes may alter the coupling as shown for the OX_1R and cannabinoid receptor (Hilairet et al., 2003). We suggest OX_1R exhibits multiple coupling depending on the cell type and the conditions of the signaling environment. Regarding the specific routes of Ca^{2+} signaling in neurons, however, we can not predict the underlying G protein pathway mechanisms. Gqα/G11α mediate important pathways in intracellular Ca^{2+} signaling. Also Giα, which may be involved in neuronal orexin signaling, can have various influences on Ca^{2+} signaling. Giα may co-participate in PLC mediated Ca^{2+} signaling (Mark et al., 2000) and may cause activation of different pathways depending on the Ca^{2+} response profile. Diverse Ca^{2+} pathways, whether activated by the same or different sets of G proteins, may contribute to differential Ca^{2+} signaling (e.g. localized/generalized Ca^{2+} elevation). As shown by the previous data, Gβγ may also contribute to differential Ca^{2+} signaling pathways (Cordeaux et al., 2000). Moreover, OX_1R

has been shown to couple to GIRK channels (Chalecka-Franaszek et al., 2000), another indication of Gβγ action.

We are not able to discriminate between the pre- and postsynaptic actions in our system. In neurons, some of the orexin-mediated actions may be explained by presynaptic signaling. To investigate this, we need to acquire native OX_1R expressing cells that are scarcely expressed in the hypothalamus. Most of the G protein coupled receptors have presynaptic receptor modifications, as shown in Table 1. For peptidergic receptors, the phenomenon is not yet understood, but part of their actions, may contribute to presynaptic signaling. Since the peptide transmitters co-localize with classical transmitters mediating the metabotropic, or ionotropic signaling, the action of peptidergic signaling is mostly regulatory. However, the peptidergic systems establish important regulatory functions in the body, the dysfunction of which leads to many pathological conditions also observed in the orexinergic system (e.g. narcolepsy). Thus, the peptide transmitter systems offer a target for drug therapies.

6.4. Regulation of synaptic transmission

As demonstrated by our data and previous synaptic transmission studies, neuronal signaling is a highly regulated process (Jarvis and Zamponi, 2001). As we have shown, the plasma membrane core complex and synaptic vesicle associated proteins that drive the fusion of the membranes interact with a variety of proteins. We demonstrated interaction with G proteins as well as voltage dependent Ca^{2+} channels, the activation which is the ultimate synaptic vesicle exocytosis factor. As we indicate, this activation can be regulated by G proteins that directly bind to the channel. We have investigated how this inhibition by G proteins is actually modulated by synaptic core complex proteins, namely syntaxin and VAMP2, and by a chaperone protein, CSP. We show that only the membrane bound protein syntaxin and the vesicle bound protein CSP directly contribute to G protein modulation. However, many of the synaptic proteins interact directly with Ca^{2+} channels (Zamponi, 2003). At the synapses, chaperones like CSP are important regulators of the dynamic complexes, allowing the high fidelity of the process. Dysfunction of these regulators leads to severe problems in regulated synaptic transmission (Miller et al., 2003a).

Many of the regulatory steps are determined by location. We show that caveolin proteins play a significant role in synaptic transmission by directly interacting with a wide variety of synaptic proteins. We show an important role for the synaptic core complex protein SNAP25 through its interaction with caveolin. Other synaptic proteins VAMP2 and syntaxin do not demonstrate the caveolin interaction. However, many of synaptic proteins can be found in caveolae, the localization which is a dynamic process. Localization in and departure from caveolae are associated with many signal transduction-regulated phenomenon, such as receptor desensitization and cellular trafficking. As it was discussed before, caveolae are not present in every cell type and in this case, some other mechanism is involved in organizing the molecular partners in a strict order.

This study also strengthens the versatility of G protein mediated coupling. As seen with a peptidergic receptor, a single GPCR may couple to multiple G proteins and thus, to a variety of signal transduction pathways. This phenomenon has an important role in physiological signaling. Presynaptic inhibition of VGCCs may follow the similar principle. Some of the

GPCRs that mediate the presynaptic inhibition are found only in the presynaptic terminals. The specificity of signaling may also be achieved by coupling to specific subtypes of Gα or Gβγ. This naturally may influence the outcome of receptor activation and thus, contribute to specific signaling, e.g. in the case of presynaptic inhibition. In presynaptic signaling, the identity of the Gα is likely a critical determinant in coupling specificity between GPCRs and their molecular effectors, as demonstrated with different Gi/oα subunits (Straiker et al., 2002). Inhibition of presynaptic Ca^{2+} channels is also dependent on the Gβ subunit isoform (Cooper et al., 2000). In addition, the degree of inhibition by a particular Gβ subunit is strongly dependent on the specific Ca^{2+} channel β subunit (Feng et al., 2001). The R-type Ca^{2+} channels are differentially modulated by Gq/11α coupled muscarinic receptors (Bannister et al., 2004). Muscarinic acetylcholine receptors evoke both inhibition and stimulation of R-type Ca^{2+} channels, mediated by Gβγ and Gα subunits, respectively. In contrast to stimulation, the inhibition depends on receptor subtype, thus Gβγ subunit. In addition, the inhibition is notably enhanced during pharmacological suppression of PKC, suggesting the cross-talk between Gβγ mediated inhibition and PKC mediated stimulation similar to that described for N-type Ca^{2+} channels. These suggest a possible mechanism by which the cellular or subcellular expression pattern of G protein subunits and VGCC β subunits may regulate the G protein sensitivity of presynaptic Ca^{2+} channels expressed at different locations, throughout the brain and possibly within a neuron.

This study strengthens the fact that neuronal signaling is highly regulated by a variety of versatile processes. The emergence and propagation of the signal is governed by various mechanisms, some of which are extensively discussed in this thesis. The electrically conductive membranes, synthesis and storage of transmitters, uptake of transmitters by nerve terminals or glia, release of transmitters, stimulation or inhibition of transmitter release by presynaptic receptors, transmission of signal by postsynaptic receptors and other postsynaptic proteins are the sites for signal regulation. These sites also provide a target for drug therapy. Change in the balance of protein environment involved in these processes may drastically alter the neuronal signaling. Therefore, the up- and down-regulation of certain processes are not just an inevitable phenomenon, but also a necessary event to keep the signaling appropriate. Also, the compensatory mechanisms in neurons are remarkable. Even removal of a dominant Ca^{2+} entry pathway by knocking out P/Q-type Ca^{2+} channels does not greatly attenuate transmission (Piedras-Renteria et al., 2004). In neurons, there are no independent factors or messengers. The signaling molecules are highly complexed, and pass message to one another in a parallel and radial fashion. None of the members are useless, but some of them are irreplaceable.

As discussed before, a variety of GPCRs play modulatory roles in presynaptic as well as postsynaptic neuronal signaling. Novel pathways, contributing to regulation of synaptic transmission, are discovered. The knowledge of presynaptic modulation of synaptic vesicle release has gathered together molecular systems that were not thought to interact. The modulation of postsynaptic signaling has recruited new members it was not thought to accompany. The finding, that G proteins mediate multiple divergent Ca^{2+} signaling pathways, has made the exploration of regulation of synaptic transmission a growing challenge. The complexity is ever increased by novel findings and the inconstancy of cellular environment.

7. SUMMARY

This study was undertaken to examine the molecular mechanisms of neurotransmitter release and G protein-mediated calcium signaling. We investigated the interactions between G protein subunits and voltage gated calcium channels underlying the presynaptic regulation of neurotransmission. We also investigated the subcellular location of these constituents and the effect of localization on the formation of protein complexes. We also focused on G protein binding properties of a peptidergic receptor representing divergent calcium signaling pathways. The main findings are indicated below.

Syntaxin 1A, a protein of the presynaptic vesicle release complex, was shown to mediate a crucial role in the tonic inhibition of N-type Ca^{2+} by G protein subunit βγ. The Ca^{2+} subunit linker, Gβγ and syntaxin 1A were shown to interact directly with each other, leading to inhibition of presynaptic calcium channel, and thus, the fine tuning of synaptic transmission. CSP, the synaptic vesicle protein known to play an important role in exocytosis, was shown to interact with N-type Ca^{2+} channels and G protein subunits α and βγ, underlying the tonic inhibition of the presynaptic calcium channel.

Caveolin, the scaffolding protein forming plasma membrane signaling domains, was shown to interact with synaptic proteins, namely the core complex protein SNAP-25, in a phosphorylation enhanced way. Also, a variety of synaptic proteins were shown to localize in caveolae, indicating that caveolae play an important role in the regulation of transmission. A set of synaptic proteins investigated was shown to have a specific effect on the regulation of synaptic transmission, since not all the synaptic proteins exhibit the same properties.

Orexin 1 receptor was shown to mainly couple to G proteins that contribute to phosphoinositide turnover. OX_1R was also shown to have the ability to couple to adenylyl cyclase stimulatory/inhibitory G proteins. The multiple coupling may explain some of the orexin signaling diversity, as obtained especially with calcium signaling. As a summary, the study shows some novel G protein-mediated calcium signaling pathways that take part in, one way or another, the regulation of synaptic transmission.

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