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DISSERTATION

Fine-tuning of neurotransmitter release via a G protein-mediated modulation of voltage-gated ion channels

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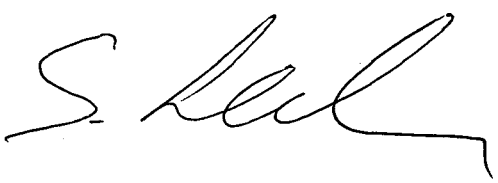

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Deutsche Kurzfassung der Dissertation

Azetylcholin und ATP sind die Haupt-Neurotransmitter im sympathischen Nervensystem. Beide entfalten Ihre Wirkung durch die Aktivierung ionotroper und/oder metabotroper Rezeptoren. Die Aktivierung ionotroper Rezeptoren bildet die Grundlage für die schnelle Kommunikation zwischen Nervenzellen, wohingegen die Aktivierung metabotroper, G Protein-gekoppelter Rezeptoren, generell die Effizienz der synaptischen Übertragung beeinflusst. Die Aktivierung G-Protein gekoppelter Rezeptoren löst eine Reihe zellulärer Antworten aus, unter anderem eine Modulation von spannungsabhängigen Ionenkanälen. In der vorliegenden Arbeit wurde untersucht, welchen Einfluss eine solche Modulation von Ionenkanälen, im Besonderen die Modulation von N-Typ Ca^{2+} Kanälen und M-Typ K^+ Kanälen durch muskarinische Azetylcholinrezeptoren beziehungsweise P2Y Nukleotid-rezeptoren, auf die Freisetzung von Noradrenalin aus sympathischen Neuronen hat.

Im ersten Teil der Arbeit wurde der Einfluss der M-Kanal-Modulation durch Muskarin-rezeptoren in Primärkulturen der oberen Halsganglien der Ratte untersucht. Derzeit sind fünf Muskarinrezeptor Subtypen (M_1 – M_5) bekannt. Durch den Einsatz der Muskarin Rezeptor Antagonisten Pirenzepin und Muskarin Toxin 7 sowie des Antiepileptikums Retigabin, in Radiotracer Freisetzungs Experimenten zur Messung der Noradrenalin-freisetzung und in Patch-Clamp Experimenten zur Messung der M-Kanal Modulation, konnte gezeigt werden, dass die durch M_1 Rezeptoren vermittelte Hemmung des M-Typ Kaliumkanals die Grundlage für die durch muskarinische Agonisten induzierte Noradrenalinfreisetzung ist.

Der zweite Teil der Arbeit widmete sich der Frage, ob und durch welchen Mechanismus der kürzlich entdeckte $P2Y_{12}$ Nukleotidrezeptor spannungsabhängige Calciumkanäle moduliert und dadurch die Noradrenalinfreisetzung aus PC12 Zellen beeinflusst. Durch den Einsatz verschiedener P2Y Rezeptor Agonisten und Antagonisten - wie zum Beispiel des antithrombotischen Wirkstoffes AR-C69931MX - konnte gezeigt werden, dass die Aktivierung von $P2Y_{12}$ Rezeptoren zu einer Hemmung der Adenylylzyklase und zu einer Hemmung von N-Typ Calciumkanälen führt. Die Hemmung der Ca^{2+} Kanäle ist der entscheidende Schritt in der durch $P2Y_{12}$ Rezeptoren vermittelten Reduktion der Noradrenalinfreisetzung. Des weiteren weisen Versuche mit heterolog überexprimierten G-Protein $\beta_1\gamma_2$-Dimeren und dem „Gβγ-scavenger" α-transducin darauf hin, dass die Hemmung des N-Typ Calciumkanals auf zwei unterschiedlichen Mechanismen beruht: einem spannungsabhängigen Mechanismus der vermutlich auf der direkten Interaktion von $G\beta_1\gamma_2$ mit der Calcium Kanal α_1-untereinheit basiert, und einem spannungsunabhängigem Mechanismus der ebenfalls βγ-Dimere benötigt, deren Identität jedoch zur Zeit noch unklar ist. Darüber hinaus zeigen die Daten, dass eine Erhöhung des intrazellulären cAMP Spiegels die Aktivierungskinetik des durch G Proteine gehemmten Ca^{2+} Kanals beschleunigen, was die durch $P2Y_{12}$ Rezeptoren vermittelte Hemmung der Noradrenalinfreisetzung stark vermindert – ein bislang weitgehend unbekanntes Phänomen.

Summary

Acetylcholine and ATP are the major transmitters in the sympathetic nervous system. Both exert their actions by activating ionotropic and/or metabotropic receptors. The activation of ionotropic receptors forms the basis for the fast communication between neurons, whereas the activation of metabotropic, G protein-coupled receptors, is associated with the short- and long-term modulation of neuronal activity. G protein-coupled receptors initiate branching cascades of intracellular events, including the modulation of voltage-gated ion channels. In the present work I investigated, how such a modulation of ion channels affects the release of neurotransmitters from sympathetic neurons.

In the first part of the work I investigated the modulation of M-type potassium channels by muscarinic acetylcholine receptors (mAChRs) in primary cultures of rat superior cervical ganglion neurons. So far five different mAChR subtypes, termed M_1 –M_5, have been identified. By using the muscarinic receptor antagonists pirenzepine and muscarinic toxin 7, as well as the anticonvulsive drug retigabine, in radio-tracer release experiments to measure ^{3}[H]noradrenaline release and in patch clamp experiments to measure M-current modulation, we could show that activation of M_1 mAChRs triggers transmitter release from sympathetic neurons, and that the basis for this stimulation is the inhibition of M-type potassium channels.

The second part of the work focuses on the question, if and by which mechanism the recently identified $P2Y_{12}$ nucleotide receptor modulates voltage-gated Ca^{2+} channels and inhibits transmitter release from PC12 cells, which are ontogenetically related to sympathetic neurons. By using various P2Y receptor agonists and antagonists, such as ADP and AR-C69931MX, we could show that the activation of $P2Y_{12}$ receptors mediates an inhibition of adenylyl cyclase and an inhibition of N-type Ca^{2+} channels. Moreover, we could show that activation of $P2Y_{12}$ receptors reduces depolarization-evoked noradrenaline release and that the crucial step for this effect is the inhibition of voltage-gated Ca^{2+} channels. In addition, PC12 cells were generated that stably overexpress G protein $\beta_1\gamma_2$-dimers and the "G$\beta\gamma$-scavenger" α-transducin, respectively. Patch clamp experiments performed with these cells revealed that the $P2Y_{12}$ mediated inhibition of N-type calcium channels involves two distinct signalling pathways: a voltage-dependent mechanism, based on the presumed direct interaction of $\beta_1\gamma_2$-dimers with the calcium channel α_1-subunit, and a voltage-independent mechanism that appears to require $\beta\gamma$-dimers composed of different β and γ isoforms. Furthermore we show that an elevation of intracellular cAMP levels accelerates the activation kinetics of the inhibited Ca^{2+} channels. This accelerated channel activation markedly reduces the $P2Y_{12}$ mediated inhibition of noradrenaline release.

1 Introduction

1.1 General principles of chemical neurotransmission

In primitive organisms, cells communicate with each other through chemical messengers that are secreted into the extracellular environment and bind to surface receptors in other cells. Neurons use a similar but highly modified and more sophisticated form of chemical signaling, which is faster, more precise and more flexible. Neurons secrete *neurotransmitters* at sites of functional contact called *chemical synapses*. At the chemical synapse a region of the membrane in the *presynaptic* cell that is specialized for rapid secretion is closely and firmly attached to a specialized region on the *postsynaptic* cell that contains a high density of *receptors* for the neurotransmitter. In this way communication in the nervous system is specifically directed from one cell to another. Electrical signals in the presynaptic cell cause the release of neurotransmitter that subsequently binds to surface receptors and triggers an electrical signal, the *synaptic potential*, in the postsynaptic cell. The synaptic potentials in turn may evoke an *action potential* which is conveyed along the axon to the nerve terminal to trigger the release of neurotransmitter onto the next cell. Hence, neurons transmit information throughout the nervous system by an alternating chain of chemical and electrical signals.

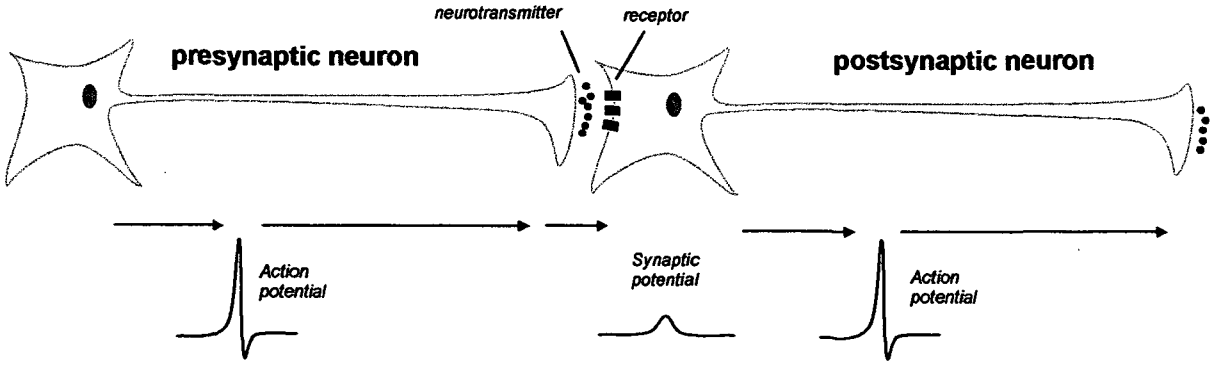

Figure 1-1, Schematic view of neuronal signalling.

Neurons convey information by a combination of chemical and electrical signals. At chemical synapses, electrical signals in the nerve terminal cause them to secrete neurotransmitters onto the postsynaptic cell. The neurotransmitters bind to postsynaptic receptors to cause electrical signals, called synaptic potentials, in the postsynaptic cell. The synaptic potentials trigger an action potential in the axon which is conveyed to the nerve terminal and triggers secretion of neurotransmitter onto the next cell.

1.1.1 Neurotransmitter release

Neurotransmitters are released from the presynaptic nerve terminal not as single molecules but as multimolecular packets called quanta. Each quantum is packaged in a synaptic vesicle and is released into the synaptic cleft by exocytosis at specialized release sites within the presynaptic terminal called the active zones. Exocytosis is controlled by a complex machinery of synaptic fusion proteins some of which are calcium sensitive. Thus the final step that leads to transmitter release is strictly dependent on an increase in intracellular calcium-concentration (Südhof, 2000; Rettig & Neher, 2002). The most common mechanism by which intracellular calcium levels are increased, and thus transmitter release is triggered, is a calcium influx through voltage gated calcium channels, mainly N-type and P/Q-type calcium channels, that open upon membrane depolarization when an action potential invades the presynaptic terminal. Hence calcium influx links electrical and chemical signaling. As mentioned above, transmitters are released at active zones. Such active zones are regions of the nerve terminal where voltage-gated calcium cannels, synaptic fusion proteins and other proteins that regulate transmitter release are localized in close proximity to each other (Westenbroeck et al, 1998). Moreover, active zones are directly opposing regions in the postsynaptic membrane where the appropriate receptors for the released transmitter are highly concentrated. This functional cluster of Ca^{2+} channels and fusion proteins not only assures the rapidity of information transfer, but also allows a rapid termination of the signaling process, since the local calcium concentration is quickly reduced by diffusion (Augustine et al, 2003). After the neurotransmitter has been released it is rapidly removed, to avoid an accumulation in the synaptic cleft, which would cause a prolonged stimulation and thus a pronounced desensitization of the postsynaptic receptors. This is achieved by three different mechanisms: first, the transmitter diffuses out of the synaptic cleft and is thereby diluted to ineffective concentrations; second, some transmitters like for example acetylcholine or ATP are degraded by specific enzymes in the synaptic cleft; and third, some transmitters like for example serotonin and amino acids are taken up either by the nerve terminal they have been released from, or into surrounding glia-cells where they are then degraded. The reuptake into the nerve terminals allows recycling, because the major proportion of the transmitter is repacked into synaptic vesicles and subsequently used again for transmission (Palfrey & Artalejo, 1998).

1.1.2 Receptors for Neurotransmitters

Neurotransmitter receptors are integral membrane proteins with a high affinity-binding site for their corresponding transmitter. They can be divided into two major classes according to the way they convert an extracellular signal into a cellular response: First, the ionotropic

receptors, or ligand-gated ion channels. These receptors are responsible for the fast synaptic action which takes less than a millisecond and thus forms the basis for the rapid communication in the nervous system. Such ionotropic receptors combine ligand binding site and ion channel in one single molecule or macromolecular complex. Thus binding of a transmitter to the receptor can directly, and thereby very fast, change the gating properties of the channel by causing conformational changes, resulting in a brief transmembrane current. The second group of receptors, the G-protein coupled- or metabotropic receptors (GPCR's), (Pierce et al, 2002) are associated with the so called slow synaptic action which mediates short- and long-term modulation of neuronal activity (Jan & Jan, 1997; Marder & Thirumalai, 2002). In contrast to the directly acting ionotropic receptors, interaction between G-protein coupled receptors and their target proteins is mediated by a third group of proteins, called a G-proteins. Such G-proteins initiate branching cascades of intracellular events that affect channels, pumps, enzymes, transcription factors and other proteins. To complete the picture, one should also mention a third group of receptors, namely the enzyme linked receptors. Enzyme linked receptors, mostly the tyrosine kinases, mediate mainly long-lasting or even permanent changes in neuronal activity. But since they are usually activated by hormones and growthfactors, they play only a secondary role in neurotransmission itself.

Ionotropic receptors, or ligand-gated ion channels, are specialized for rapidly converting extracellular chemical signals back into electrical signals at chemical synapses. They are concentrated in the plasma membrane of the postsynaptic cell in the region of the synapse and open transiently in response to the binding of a neurotransmitter, thereby causing a brief transmembrane current that produces a local change in the membrane potential, called synaptic potential. Like all other ion channels, ionotropic receptors exhibit selectivity for certain ions. This selectivity determines the nature of the postsynaptic response. Some neurotransmitters like acetylcholine, glutamate and serotonin open cation channels, causing an influx of sodium and calcium ions that depolarizes the postsynaptic membrane. This depolarization brings the membrane potential closer to the threshold for action potential firing, and these transmitters are therefore called excitatory neurotransmitters. By contrast, transmitters like γ-aminobutyric acid (GABA) or glycine open channels that are permeable to chloride ions and this may shunt action potential firing. Hence, these transmitters usually act as inhibitory neurotransmitters (Hille, 2001).

In contrast to ionotropic receptors that directly convert the incoming chemical signal into an electrical response, metabotropic receptors act indirectly by activating heterotrimeric G-proteins which can in turn interact with a multitude of effectors, like enzymes or ion channels (Hamm, 1998). Another striking difference between ionotropic and metabotropic receptors is that all G-protein coupled receptors, despite the chemical diversity of signal molecules that bind to them, share a common molecular structure. The receptors all have a large extracellular glycosylated N-terminal domain, seven membrane spanning hydrophobic helices that are connected by hydrophilic loops and an intracellular C-terminal domain. The C-terminal domain and the intracellular loops interact with the G-proteins and it seems as if the third loop was the major determinant for G-protein selectivity (Wess, 1998). Binding sites for small ligands are usually located deep inside a pocket formed by the seven membrane spanning segments, whereas binding sites for large ligands, such as peptides, are formed by the large N-terminal region and only a few transmembrane segments. As mentioned above, G-proteins are heterotrimers consisting of an α, β, and γ subunit. The α subunit is responsible for GTP and GDP binding and for GTP hydrolysis, whereas the β and γ subunits are associated in a tightly linked $\beta\gamma$ complex. G-proteins are generally referred to by the nature of their α subunits. So G_S contains the α_S subunit; G_q contains α_q; and so on. Today we know 16 different α subunits, 5 β and 12 γ subunits but all of the known $\alpha\beta\gamma$-trimers operate via the same general mechanism. In its inactive state the G-protein has GDP bound to its α subunit. Upon interaction with an activated receptor, GDP dissociates and GTP is bound instead, thus activating the G-protein. This displacement is accompanied by the dissociation of the α subunit from the $\beta\gamma$ complex and both α and $\beta\gamma$ can then interact with and activate effector systems. The lifetime of an active α subunit is highly limited since it carries an intrinsic GTPase activity, and so after some time the bound GTP is hydrolyzed to GDP. GTPase activity is usually regulated by a class of proteins called *regulators of G-protein signaling* (RGS). Following hydrolysis of GTP, the α subunit reassociates with the $\beta\gamma$ complex, thus reversing the activation process and allowing the whole cycle to begin again (Pierce et al, 2002).

G-proteins can be assigned to four distinct subfamilies, namely G_s, G_i, G_q and G_{12}, each activating different effector systems. A given receptor typically activates only members of one subfamily, but for most ligands different receptor subtypes exist, each activating different G-proteins, thus allowing a certain ligand to exert a multitude of effects. The most important of these effects are (i) the regulation of adenylyl cyclase activity (Sunahara et al, 1996), (ii)

the activation of phospholipase C, (iii) the modulation of voltage gated potassium channels and of (iv) voltage gated calcium channels (VACC's) (Wickman & Clapham, 1995).

The modulation of ion channels will be described in the next chapter in more detail; here I just want to give a short description of the other effector systems mentioned above. Adenylyl-cyclase can be stimulated by G_s proteins or inhibited by G_i proteins. It is a membrane bound enzyme that synthesizes the second messenger cyclic AMP (cAMP) from ATP. Although cAMP can directly activate certain types of ion channels, in most cells it exerts its effects mainly by activating cyclic-AMP-dependent protein kinase (PKA). This enzyme catalyzes the transfer of the terminal phosphate group from ATP to specific residues of selected target proteins, thereby regulating their activity.

Another important second messenger is inositol 1,4,5-triphosphate (IP_3). It is generated by the membrane bound enzyme phospholipase C (PLC), which is mainly activated by G proteins of the G_q class. PLC cleaves phosphatidylinositol 4,5-biphosphate (PIP_2), a phospholipid that is present in the inner half of the membrane lipid bilayer, to generate IP_3 and diacylglycerol (DAG). IP_3 causes the release of calcium stored in the endoplasmic reticulum (ER) by activating endoplasmic IP_3 receptors (IP_3 gated calcium channels), thereby influencing the activity of a number of Ca^{2+} dependent enzymes. While IP_3 is a diffusible second messenger, DAG remains embedded in the membrane. DAG can be further cleaved by another membrane bound phospholipase, phospholipase A_2 (PLA_2) to release arachidonic acid (PLA_2 can also release arachidonic acid directly from PIP_2), which can act as a messenger in its own right, or, and this is the more important function, it can activate protein kinase C (PKC). PKC, just like PKA, phosphorylates a number of target proteins, e.g. ion channels, thereby regulating their function.

1.1.3 Voltage-gated ion channels

As mentioned above, ligand-gated ion channels convert chemical signals into an electrical cellular response, called synaptic potential. The membrane potential of a neurons cell body more or less reflects the sum of all excitatory and inhibitory synaptic potentials generated simultaneously at different synapses. If at any time the membrane potential at the initial segment, a region of the soma at the base of the axon, exceeds a certain threshold level, a stereotyped series of changes, known as the action potential, is initiated: The membrane rapidly depolarizes to become briefly inside-positive and then rapidly repolarizes to a potential near rest. In many cells, the repolarization continues beyond the original resting

potential, so that the membrane becomes transiently hyperpolarized, and then slowly returns to the original resting potential. This last phase is called after-hyperpolarization. Although the depolarization caused by an action potential decreases with distance from its origin, it is still strong enough to overcome the threshold for action potential generation in adjacent regions, thereby allowing the action potential to travel along the axon until it finally reaches the nerve terminal. The proteins that are responsible for the generation of such action potentials are the voltage-gated ion channels, in particular Na^+, K^+ and Ca^{2+} channels (Hille, 2001).

Voltage-gated ion channels form aqueous pores across the plasma membrane that facilitate the passive flow of ions down their electrochemical gradient. As the term "voltage-gated" indicates, these channels open and close in response to changes in the membrane potential. Besides the open and the closed state, with prolonged depolarization most voltage-gated ion channels can also enter a closed "inactivated" state, in which they are refractory to further opening until the has been repolarzied. Moreover ion channels exhibit ion selectivity, which means that they can select for a particular ion species over another. This selectivity is achieved by a ring of charged amino acid residues that is located deep inside the pore. Since the ring has a diameter of a few angstrom only, ions have to strip off their entire hydration shell to pass through. Dehydration requires energy, dependent on charge and size of the ion, and thus will only occur if the interaction between the "naked" ion and the selectivity filter offers an appropriate energetic compensation for the loss of the hydration shell (Armstrong & Hille, 1998).

Sodium and calcium channels are evolutionarily closely related and thus they share many structural and functional properties. Both, sodium and calcium channels, are composed of a pore forming α-subunit and a number of so called auxiliary or regulatory subunits that are not essential for conduction, but which play an important role in modulation of channel function. The pore forming α- (Na channel) or α_1- (Ca channel) subunit accounts for all the defining properties of the channel, like ion selectivity and voltage sensitivity. It contains four repetitive domains (I-IV) that are arranged around the central pore (figure 1-2B), each domain having a motif of six putative transmembrane segments (S1-S6) with an amphipathic loop between S5 and S6 called P-loop. The S4 segments serve as the voltage sensor and the four P-loops form the outer part of the pore and the selectivity filter (see figure 1-2A). In segment S4 every third residue is positively charged. Due to these positive charges, segment S4 moves outward and rotates upon changes in the membrane potential, thereby initiating a conformational change that opens or closes the pore. Na^+ and Ca^{2+}-channels open in response to membrane

depolarization causing an influx of sodium and calcium ions, respectively, that further depolarizes the membrane.

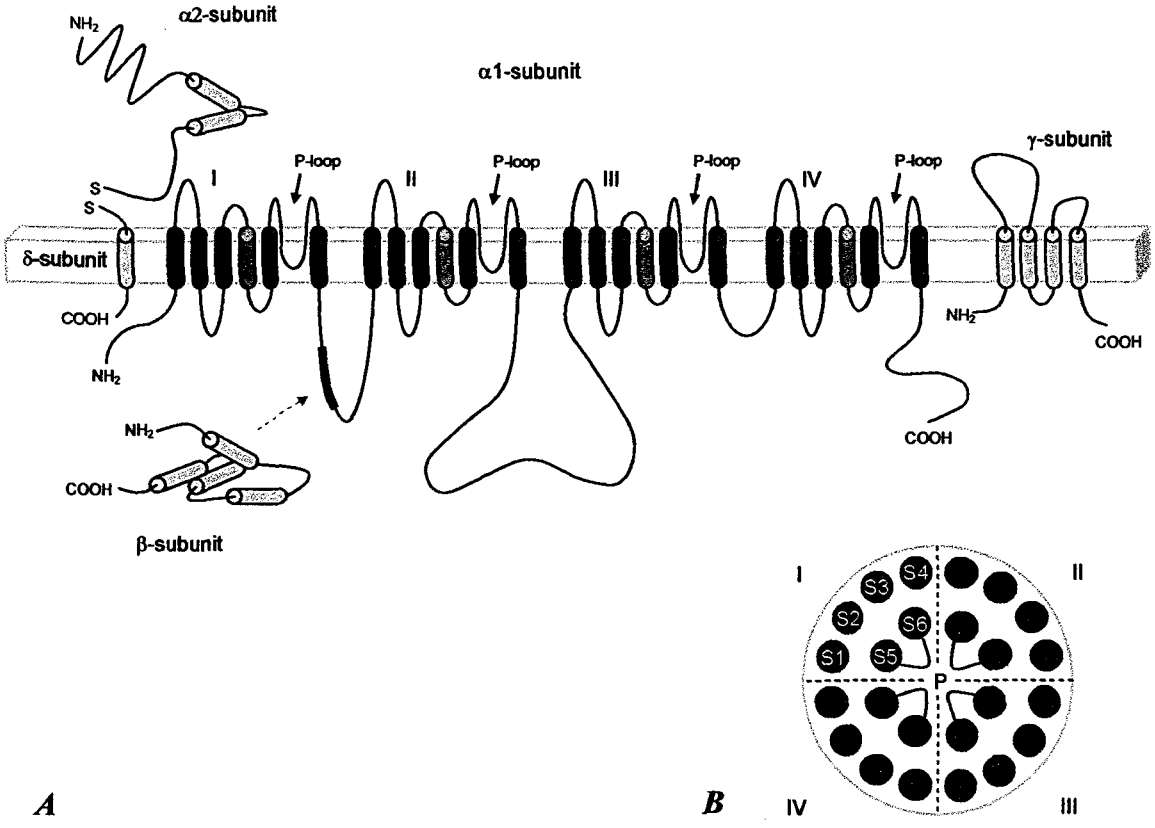

Figure 1-2, Subunit composition and proposed membrane topology of voltage-gated Ca^{2+} channels.
(A), the principal pore-forming α_1-subunit is depicted in blue. Regions of functional significance, such as the pore region (P-loop) and the putative voltage sensors (+) are indicated. Ca^{2+} channel β-subunits are cytoplasmic proteins that associate with the domain I-II linker region of the α_1-subunit. The α_2-δ-subunit is derived from a single gene, but is post-translationally cleaved into α_2-(extracellular) and δ-(membrane-spanning) subunits that are linked via disulfide bonds. The γ-subunit comprises four membrane-spanning regions. (B), schematically illustrates how the four domains of the α_1-subunit are arranged around the central pore.

Voltage-gated Na^+ channels (Yu & Catterall, 2003) exhibit very fast activation and inactivation kinetics and they are sensitive to block by tetrodotoxin (TTX) and saxitoxin (STX). In neurons, sodium channels are almost exclusively responsible for the fast depolarization during an action potential, and thus they are primarily found at the initial segment and in axon membranes. With respect to action potentials, the rapid inactivation of Na^+ channels plays an important role. If the channels would not inactivate, there would be a steady influx of sodium making it impossible to repolarize the membrane back to its initial level. Inactivation is mediated by a cytoplasmically located portion of the α-subunit that diffuses into the mouth of the inner vestibule of the pore thereby blocking conduction.

although the channel is sill in the open-conformation. This mechanism is often referred to as the ball-and-chain mechanism. Similar to sodium channels, voltage-gated Ca^{2+} channels also activate very fast, but they show much slower inactivation kinetics. Inactivation of calcium channels is mediated by the binding of Ca^{2+}/calmodulin to the long C-terminal cytoplasmic domain of the α-subunit. Hence inactivation of voltage-gated calcium channels depends on the intracellular calcium concentration. In neurons, a primary function of Ca^{2+} channels is to mediate calcium entry at the presynaptic nerve terminal that triggers the release of neurotransmitter, whereas in other cells, like cardiac pacemaker cells, they substantially contribute to the depolarizing phase of action potentials (Catterall, 2000).

In contrast to Na^+ or Ca^{2+}-channels, voltage-gated potassium channels display considerable diversity in terms of voltage dependence, rates of activation and inactivation, as well as in their structure and pharmacology. Most potassium channels are sensitive to block by Cs^+, Ba^{2+} and tetraethylammonium (TEA). Potassium channels like the delayed rectifiers are responsible for the repolarization during an action potential. Fast delayed rectifiers activate and inactivate rather quickly upon depolarization, and thus keep fast action potentials short, while the slow delayed rectifiers, like KCNQ1 or erg1 channels, aid the repolarization in cells with long action potentials, like cardiac cells. Other potassium channels time the interspike intervals during repetitive firing, like the A-type potassium channels that only open upon depolarization after a period of hyperpolarization or the Ca^{2+}-dependent K^+ channels. Moreover, some K^+ channels like the M-channel are already active near the resting potential and are further activated upon depolarization. Hence these channels oppose depolarizing signals and influence the responsiveness of the cell to synaptic inputs. A completely different class of potassium channels is the family of inwardly rectifying K^+ channels. In contrast to most other K^+ channels, these channels close in response to depolarization and are further activated upon hyperpolarization (Robbins, 2001).

The pore of a typical potassium channel is not formed by a single large peptide, but consists of separate protein subunits that are clustered to form the functional channel. At least 8 families of α-subunits, with a total of over 60 different subunits, have been identified so far. The largest of these families displays a subunit structure similar to the one of the repetitive domains in Ca^{2+} or Na^+-channels, with 6 transmembrane segments (TM), a voltage sensor (segment S4) and a P-loop between the segments S5 and S6 (referred to as 6TM-1P). But also other membrane topologies are possible: e.g. two transmembrane domains linked by a P-loop (2TM-1P), eight TM´s and two P-loops (8TM-2P), 4TM-2P and 7TM-1P. This large number

of different subunits together with the tetrameric architecture somehow explains the functional diversity of native K^+ channels, since a wide range of heterotetramers is possible using different subunits from within the same subfamily or even from different subfamilies (as realized recently; e.g. Sanguinetti et al, 1996). Even more functionally different channels are possible due to the co-assembly of nonpore-forming β-subunits. However, despite all the structural and biophysical differences, one can say that in general open K^+ channels stabilize the membrane potential. They draw the membrane potential closer to the equilibrium potential of potassium and farther from the firing threshold for action potentials. In neurons all types of K^+ channels are related to this stabilization. K^+ channels set the resting potential, keep fast action potentials short, time the interspike intervals during repetitive firing or just lower the effectiveness of excitatory inputs on a cell when they are open (for detailed reviews on K^+ channels see Coetzee et al, 1999; Garcia et al, 1997, Robbins, 2001).

1.2 The sympathetic nervous system

The autonomic nervous system regulates the function of all innervated effector tissues in vertebrate organisms with one exception, the skeletal musculature. It thus forms the major efferent component of the peripheral nervous system. Three major components contribute to the autonomic nervous system: (i) the sympathetic division originating in the thoracolumbar region of the spinal cord, (ii) the parasympathetic division which arises in craniosacral regions of the spinal cord, and (iii) the enteric nervous system, an independent nervous system entirely residing within the intestine. While the sympathetic and parasympathetic pathways originate in the central nervous system with neuronal somata located in cranial nerve nuclei and in the intermediate zone of the spinal cord, the enteric system is controlled by connections from the sympathetic and parasympathetic divisions. The most diverse of the autonomic systems is the sympathetic division which sends axons to all parts of the organism, whereas the parasympathetic nervous system supplies only a limited number of target organs. Each of the autonomic pathways is interrupted by ganglia that receive a cholinergic input via preganglionic neurons and contain the somata of postganglionic neurons, which give rise to axons. These postganglionic axons run in bundles into the effector organs, where single axons give rise to a considerable number of branches.

In sympathetic ganglia, preganglionic axons form synapses with the somatodendritic region of postganglionic neurons. One preganglionic axon may innervate a number of postganglionic neurons, and one postganglionic neuron may receive input from a number of preganglionic

axons. Thus, at the level of sympathetic ganglia, one can find neuronal convergence as well as divergence, and it appears therefore difficult to define specific pathways leading from the spinal cord directly to the effector organs. Nevertheless, postganglionic sympathetic neurons can be characterized by functional means and categorized into at least three groups: phasic, tonic and long-afterhyperpolarizing neurons. The electrophysiological characteristics of these divergent types of neurons are caused by the differential expression of various voltage-dependent K^+ channels. Phasic neurons are characterized by the presence of M-type K^+ channels, tonic neurons by small Ca^{2+}-activated K^+ channels, and long-afterhyperpolarizing neurons by the expression of small and longlasting Ca^{2+}-activated K^+ channels. Phasic and long-afterhyperpolarizing neurons usually receive a strong preganglionic input and appear to function primarily as relay neurons. In contrast, tonic neurons receive weak efferent as well as afferent inputs and may thus integrate signals in order to mediate sympathetic reflexes. Phasic neurons predominate in paravertebral ganglia and exert in many cases vasoconstrictor function, whereas tonic neurons prevail in prevertebral ganglia and regulate primarily the contraction of visceral smooth muscle cells (Jänig and McLachlan, 1992).

The transmitter released from the preganglionic axon terminals is primarily acetylcholine (Feldberg and Gaddum, 1934) which depolarizes postsynaptic neurons most commonly in two phases: an early one in the millisecond range, mediated by an activation of nicotinic receptors, and a later one in the range of seconds, mediated by an activation of muscarinic receptors (Brown, 1983). Aside of acetylcholine, ATP is released within the ganglia (Vizi et al, 1997), but it remained a matter of debate, whether ATP is really involved in ganglionic transmission: positive results have been obtained in enteric ganglia (Galligan and Bertrand, 1994), and negative results have been obtained in sympathetic ganglia (Inokuchi and McLachlan, 1995). In addition, several neuropeptides are present in autonomic ganglia, and it is mainly enkephalin, neurotensin, somatostatin, substance P, VIP and CGRP which have been detected in sympathetic ganglia. These peptides are *per se* not involved in ganglionic transmission (Furness et al, 1992), but may exert modulatory effects: for instance, enkephalins are believed to dampen ganglionic transmission, whereas substance P, neurotensin, and VIP rather mediate excitatory actions (Benarroch, 1994).

In sympathetically innervated effector organs, there are numerous bundles containing one or up to 100 postganglionic axons which are most commonly surrounded by Schwann cells. The axonal branches become varicose when they approach the cells that they innervate. Intervaricose axons are 0.1 to 0.4 μm in diameter and contain microtubules, whereas the varicosities themselves are larger and characterized by the presence of vesicles and

mitochondria. Originally, axonal varicosities were believed not to form specialized synaptic contacts with their effector cells, but to release their transmitters at various distances from the postsynaptic target quasi like local hormones. However, more recent evidence indicates that varicosities of sympathetic axons are in close contact with postsynaptic cells with a cleft of less than 100 nm and with only one sheet of basal lamina between pre- and postsynaptic structures. Nevertheless, typical presynaptic active zones and postsynaptic densities are most commonly lacking at these sympatho-effector junctions (Hirst et al, 1996). There are, however, indications from functional studies on cardiac pacemaker cells, which showed that added and neurally released transmitter, despite causing similar effects, activate different second messenger pathways (Hirst et al, 1996). This is thought to reflect the selective activation of postsynaptically clustered receptors coupled to one signaling pathway, on one hand, and activation of extrajunctional receptors linked to another signaling cascade, on the other hand.

Most varicosities of sympathetic axons contain noradrenaline and ATP as predominant neurotransmitters. Nevertheless, up to 15 % of the neurons in paravertebral and about 1 % of the neurons in prevertebral ganglia give rise to axons that use acetylcholine instead of noradrenaline as transmitter. The expression of either neurotransmitter phenotype is a multiple step process. Although the precise underlying mechanisms remain to be elucidated, it is obvious that sympathetic neurons express cholinergic markers early during development. At later stages, the innervated target organ determines which of the two neurotransmitters is produced. Sweat glands release a cholinergic differentiation factor that leads to the expression of choline acetyl transferase and consequently to the biosynthesis of acetylcholine in sympathetic neurons (Ernsberger and Rohrer, 1999).

As indicated above, Acetylcholine (ACh) and ATP are both ganglionic and sympatho-effector transmitters in the sympathetic nervous system. They exert their actions in similar ways, by activating ionotropic and/or metabotropic receptors. Both, acetylcholine and ATP cause depolarization (Connolly et al, 1993) and subsequent noradrenaline release from cultured rat sympathetic neurons (Boehm, 1994; Boehm et al, 1995), by activating ionotropic nicotinic acetylcholine receptors and P2X nucleotide receptors, respectively. The metabotropic actions of these transmitters are mediated by the so called muscarinic acetylcholine receptors (mAChRs) and by P2Y nucleotide receptors, respectively. Both, P2Y receptors and mAChRs have been shown to increase excitability or even stimulate noradrenaline release (Brown, 1983; Bofill-Cardona et al, 2000). Moreover, certain subtypes of mAChRs and P2Y receptors

are also found at the presynaptic nerve terminal where they mediate a feedback inhibition of sympathetic transmitter release (Boehm & Kubista, 2002). G protein-coupled receptors in general are known to modulate ion channel activity, and so do mAChRs and P2Y receptors. In the present work I investigated, how the modulation of voltage-gated ion channels, in particular Ca^{2+} channels and M-type potassium channels, by P2Y nucleotide and muscarinic actetylcholine receptors, respectively, affects the release of neurotransmitters from sympathetic neurons.

1.3 Voltage-gated calcium channels

Several different types of voltage-gated calcium channels that can be discriminated by their pharmacological and physiological properties have been described so far. Originally these channels were termed according to a single letter nomenclature that has been proposed in the late eighties by Richard Tsien (reviewed in Tsien et al, 1995). This nomenclature uses letters that have derived from certain current properties. For example, L-type currents are large and long-lasting, while T-type currents are tiny and transient. N-type currents were first found in neurons and P/Q-type currents in Purkinje cells. However, this nomenclature does not distinguish between channels and currents, and thus one will find, for example, L-type channels as well as L-type currents in the literature. With the advent of molecular biology, the channel proteins that underlie the different Ca^{2+} current were identified and it became apparent that currents with similar electrophysiological and pharmacological properties that have previously been assigned to the same channel protein, are in fact mediated by different channel proteins. As a consequence, more recently a new standard nomenclature that clearly distinguishes between channel and currents was introduced (Catterall et al, 2003; table 1-1).

The predominant species of calcium channels expressed in neurons are the $Ca_v2.2$ (N-type) and $Ca_v2.1$ (P/Q-type) channels. They are blocked by divalent transition metal ions (such as Ni^{2+}, Cd^{2+} or Co^{2+}) and by various snail and spider toxins, like for example ω-conotoxin GVIA that specifically blocks N-type currents or ω-agatoxin IVA that blocks P/Q-type currents, and exhibit single channel conductance and inactivation kinetics intermediate between those of the T- and L-type channels. N- and P/Q-type channels are most abundant within active zones of presynaptic nerve termini, where they are co-localized with the vesicular release machinery (Westenbroeck et al, 1998). Hence the release of neurotransmitter from presynaptic nerve termini depends crucially on calcium influx through these particular

channels. As a consequence, the modulation of the activity of N- and P/Q-type Ca^{2+} channels is a key mechanism by which synaptic efficacy is regulated.

Table 1-1, Calcium channel nomenclature

Channel	Current	Localization	Specific Antagonists
$Ca_v1.1$	L	Skeletal muscle transverse tubules	Dihydropyridins, phenylalkylamines, benzothiazepines
$Ca_v1.2$	L	Cardiac myocytes, endocrine cells, neuronal cell bodies	Dihydropyridins, phenylalkylamines, benzothiazepines
$Ca_v1.3$	L	endocrine cells, neuronal cell bodies	Dihydropyridins, phenylalkylamines, benzothiazepines
$Ca_v1.4$	L	Retina	Not established
$Ca_v2.1$	P/Q	Nerve terminal and dendrites	ω-agatoxin IVA
$Ca_v2.2$	N	Nerve terminal and dendrites	ω-conotoxin GVIA
$Ca_v2.3$	R	Neuronal cell bodies and dendrites	SNX-482
$Ca_v3.1$	T	Neuronal cell bodies and dendrites, cardiac myocytes	None
$Ca_v3.2$	T	Neuronal cell bodies and dendrites, cardiac myocytes	None
$Ca_v3.3$	T	Neuronal cell bodies and dendrites	None

Adapted from Catterall et al, 2003

1.3.1 Modulation of channel function

More than two decades ago, Dunlap & Fischbach (1978, 1981) first recognized G protein dependent modulation of voltage-gated calcium channels. They showed that noradrenaline (NA), serotonin (5-HT) and γ-aminobutyric acid (GABA) suppress Ca^{2+} currents of chick dorsal root ganglion neurons. Today, many neurotransmitters and hormones that exert their actions via G-protein coupled receptors are known to inhibit native calcium currents (Hille, 1994), basically via two major types of signalling pathways: (i) First, the so called membrane delimited pathways. Modulatory effects that are mediated by a membrane delimited pathway cannot be observed when currents are recorded in the cell-attached mode and the agonist is applied to the bath, which indicates that no diffusible cytoplasmic messengers are involved. In fact, these pathways are based on a direct interaction between G protein subunits and Ca^{2+} channels and thus are very fast in onset (see below). (ii) The second type of pathway involves the synthesis of diffusible second messengers that cause a voltage-independent inhibition of I_{Ca}. This effect is much slower in onset (2-60s) than membrane delimited pathways, involves α subunits of the $G_{q/11}$ protein family and is sensitive to high intracellular Ca^{2+} chelator (BAPTA) concentrations (Kammermeier et al, 2000; Haley et al, 2000; Bernheim et al, 1991).

It is activated by M_1 mAChRs (Bernheim et al, 1992) and by AT_1 angiotensin receptors (Shapiro et al, 1994).

However, the most widespread signalling pathway that mediates an inhibition of I_{Ca} is membrane delimited, and causes a pertussis toxin-sensitive (PTX) and voltage-dependent suppression of N-type Ca^{2+} currents. The majority of G protein-coupled receptors that have been reported to suppress I_{Ca}, including M_4 muscarinic acetylcholine receptors (Bernheim et al, 1992), α_2-adrenoceptors (Plummer et al, 1991), receptors for somatostatin (Shapiro & Hille, 1993), PGE_2 receptors (Ikeda, 1992), A_1 adenosine receptors (Zhu & Ikeda, 1993) and receptors for neuropeptide Y (Foucart et al, 1993) use this PTX-sensitive pathway. This type of inhibition is mediated by G proteins of the G_o class (Campbell et al, 1993; Caulfield et al, 1994) and causes a shift of the voltage dependence of gating which is accompanied by a slowing of activation kinetics. Another characteristic of this inhibition is that it can be relieved transiently (~100ms) by applying large depolarizing voltage pulses, before eliciting the Ca^{2+} current, a phenomenon known as "prepulse facilitation". It has been shown that the inhibition is based on a direct interaction of $G\beta\gamma$-dimers with the α_1-channel subunit (Herlitze et al, 1996; Ikeda, 1996, Zamponi & Snutch, 1998). The principal binding site for $G\beta\gamma$ is located within the I-II linker domain (Zamponi et al, 1997; DeWaard et al, 1997), but still other sites, like the domain I and the C-terminal domain, also seem to be necessary for full G-protein regulation of Ca^{2+}-channels (Zhang et al, 1996). Although several attempts have been made to reveal whether any particular combinations of $G\beta\gamma$ (sofar five β- and eleven γ-subunits have been identified) are responsible for the voltage-dependent inhibition of N-type channels, no clear results have been obtained (Garcia et al, 1998; Ruiz-Velasco & Ikeda, 2000; Herlitze et al, 1996). Most of the $\beta\gamma$ combinations tested in these studies were capable of producing voltage-dependent inhibition.

Besides the PTX-sensitive and voltage-dependent inhibition, several other membrane delimited pathways have been described in the past. For example, adrenergic inhibition of Ca^{2+} currents via α_2-adrenoceptors has been shown to involve an additional PTX-resistant and voltage-dependent (VD) inhibition (Shapiro et al, 1994). Moreover it could be shown that the PTX-sensitive component of this adrenergic inhibition is only partly voltage-dependent and is not exclusively mediated by $\beta\gamma$-dimers released from heterotrimeric G_o proteins, as has been thought previously (Caulfield et al, 1994; Ikeda, 1996). Delmas et al (1999) provided evidence that $\beta\gamma$-dimers derived from both, G_o and G_i, contribute to the PTX sensitive component, G_o mediating the voltage-dependent part and G_i a novel voltage-independent part,

both with fast onset rates. Another membrane delimited pathway that mediated a voltage-independent PTX-resistant inhibition of I_{Ca} has been reported for substance P (Shapiro et al, 1993, 1994) and pancreatic polypeptide (Wollmuth et al, 1995). A similar pathway has also been described more recently for muscarinic agonists (Kammermeier et al, 2000). The authors showed that M_1 mAChRs contribute a fast PTX-sensitive voltage-independent component to the muscarinic inhibition of I_{Ca}, and that the pathway underlying this inhibition involves both $G\alpha_{q/11}$-GTP and $\beta\gamma$ subunits. Moreover, Zhu & Ikeda (1994) found a PTX-resistant and voltage-dependent inhibition caused by secretin and vasoactive intestinal peptide (VIP). This inhibition was reduced by injection of anti G_s-antibodies and was sensitive to cholera toxin. Two recent studies by Filippov et al (1999, 2002) add even more diversity to the whole story. The authors found that inhibition of I_{Ca} mediated by heterologuously expressed $P2Y_6$ receptors is much more pronounced in perforated-patch (73%) as compared to whole-cell (53%) recordings. Moreover the inhibition observed in the perforated-patch configuration was hardly altered by PTX, while in whole-cell recordings it was reduced by 60% after treatment with PTX. Similar results were obtained when $P2Y_4$ receptors were overexpressed. In that case almost no inhibition (~10%) was observed in the disrupted whole-cell mode, while currents recorded in the perforated-patch mode were reduced by ~ 45% in a PTX-sensitive and voltage-dependent manner. These findings indicate that even the membrane-delimited, PTX sensitive pathway may require a soluble co-factor, and that modulation of I_{Ca} should rather be investigated in the perforated-patch than the whole-cell mode of the patch clamp technique. Taken together, the reports mentioned above show that most neurotransmitters activate not only a single pathway, but rather use a combination of pathways to inhibit Ca^{2+} channels either by activating a single receptor subtype that couples to multiple pathways, or by activating different receptor subtypes.

Calcium channels are multimolecular complexes composed of a pore forming α_1-subunit and a number of so called auxiliary or regulatory subunits that are not essential for conduction but which play an important role in the modulation of channel function. With respect to G-protein mediated modulation, the most important of these subunits is the β-subunit. Besides its ability to aid membrane trafficking of the α1-subunit, accelerate activation kinetics and increase current amplitude (for review see Arikkath & Campbell, 2003), it can also antagonize the modulatory effects mediated by G-proteins by binding to the alpha interaction domain (AID) on the intracellular loop between domain I and II, which overlaps with the binding site for Gβγ (Pragnell et al, 1994).

Another way of regulating G protein-dependent modulation is by phosphorylation. Protein kinase C (PKC) has been shown to phosphorylate a threonine (Thr422) residue within the βγ-binding site in the I-II linker domain, thereby antagonizing Gβγ mediated inhibition. Additionally, in some isoforms of N-type Ca^{2+}-channels there appears to be an intrinsic increase in channel activity, which might be due to a phosphorylation of either Thr422 or Ser435 (Hamid et al, 1999). However, this antagonism is only found for effects involving β_1 subunits but not for $\beta_{2,3}$ or $_4$. (Cooper et al, 2000).

As mentioned above, voltage-gated calcium channels are localized in close proximity to the vesicular release machinery. Biochemical studies revealed that some key proteins of the synaptic vesicle release complex, namely syntaxin1, SNAP25 and synaptotagmin1, physically interact with a region of the II-III linker domain of N- and P/Q-type calcium channels, called synaptic protein interaction or "synprint"-site (Sheng et al, 1994; 1996; 1997; Rettig et al, 1996). These physical interactions not only tether vesicles to channels, but they also seem to have an effect on channel activity (reviewed in Spafford & Zamponi, 2003). Co-expression of P/Q-type or N-type Ca2+ channels with either syntaxin1 or SNAP 25 markedy reduces the probability of channel opening (Bezprovanny et al, 1995, 2000), whereas expression of both, syntaxin1 and SNAP25, does not seem to have any effect (Zhong et al, 1999; Jarvis et al, 2001). Nevertheless syntaxin1 seems to be important in some way, since it has been shown to be required for the G protein-dependent modulation (Jarvis et al, 2000; Stanley & Mirotznik, 1997).

1.4 The M-current

The M-current, or M-type potassium current (I_M), was first described by Brown and Adams (1980) in bullfrog sympathetic neurons. It was termed M-current because it was found to be suppressed by muscarinic receptor activation. The M-current is a non-inactivating voltage-dependent potassium outward current with slow activation and deactivation kinetics (see figure 1-3A). M-channels are open in the subthreshold voltage range for action potentials and become further activated in response to membrane depolarization (see figure 1-3B). Hence, the M-current opposes depolarizing signals and influences the responsiveness of the cell to synaptic inputs. By contrast, closure or inhibition of the M-current causes depolarization and increases excitability. The suppression of I_M could be shown to underlie the slow excitatory postsynaptic potential (slow EPSP) in both frog (Adams & Brown,1982) and rat (Brown et al, 1995) sympathetic neurons that has previously be reported to be mediated by activation of muscarinic receptors (Kobayashi & Libet, 1968). I_M is much less sensitive to the potent K^+-channel blocker TEA than other channels and insensitive to other blockers, such as apamin and hexamethonium (Brown, 1988). Linopirdine (Aiken et al 1996, 1996; Lamas et al, 1997), a cognitive enhancer that improves memory and learning in rodents and primates, and its more potent and selective analogue XE991 (Wang et al, 1998) have been identified as blockers of the M-channel and thus provide useful tools for M-channel investigation.

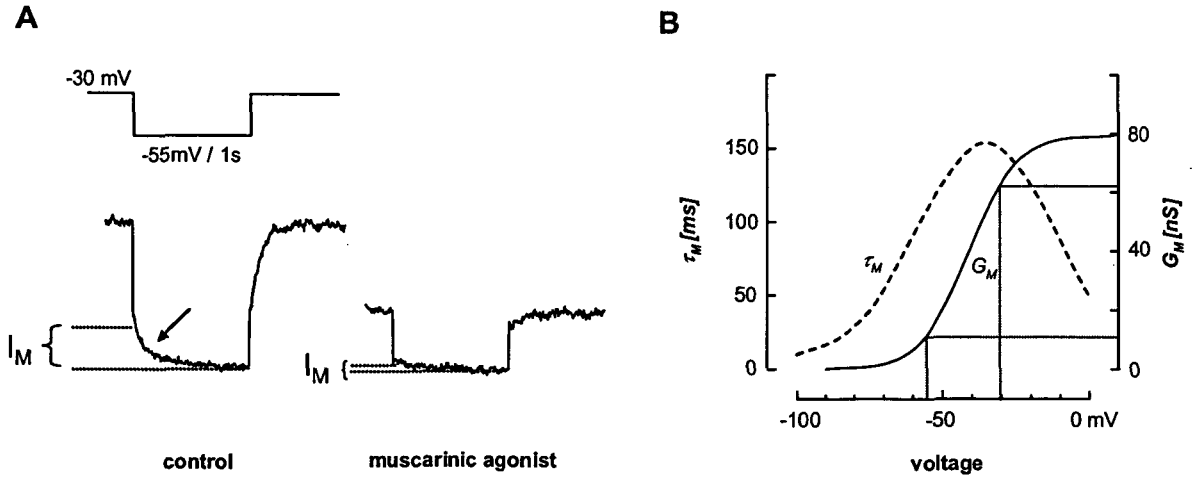

Figure 1-3, Some properties of the M-current.
(A), shows a typical M-current (control, left trace) recorded from rat SCG neurons. At -30 mV most of the M-channels are open and contribute a steady outward current to the net membrane current. Stepping from -30 mV to -55 mV causes closure of the M-channels (as indicated by the dotted lines in B). Since M-channels deactivate very slowly, the outward current at -55 mV declines in a time-dependent manner (marked ↓). The amplitude of this slow time-dependent decline in outward current is used as a measure for I_M (as indicated by the dotted lines). The right trace shows the same M-current suppressed by application of muscarinic agonist. (B) Voltage-dependence of the steady-state M-channel conductance (G_M) and time-constant for the M-current relaxation τ_M.

Another useful tool for M-channel research is the anti-convulsant drug retigabine that has recently been shown to be a potent M-channel opener. Retigabine enhances I_M by shifting the M-channel activation curve to more hyperpolarized potentials. This change in the voltage-dependence of activation is accompanied by a slowing of current deactivation and acceleration of current activation (Main et al, 2000; Rundfeldt & Netzer, 2000; Wickenden et al, 2000).

Since its discovery, I_M has been found in a variety of cell types and neural cell lines, including amphibian and mammalian paravertebral sympathetic ganglion neurons, amphibian and mammalian dorsal root ganglions, mammalian central neurons, the neuron like NG108-15 neuroblastoma glioma cells and rat pheochromocytoma PC12 cells (for detailed review see Brown &Yu, 2000). Besides muscarine, during the last two decades a number of other neuroactive substances have been reported to inhibit M-currents in various different cell types. In frog sympathetic neurons for example, activation of ryanodine (Bowden et al, 1999), substance P (Adams et al, 1983), LHRH (Pfaffinger, 1988), purinergic (Adams and Brown, 1982) and β-adrenergic (Akasu, 1988) receptors causes an inhibition of I_M. In rat sympathetic neurons, muscarinic and angiotensin II receptors (Shapiro et al, 1994) as well as bradykinin B_2 receptors (Jones et al, 1995) and $P2Y_6$ nucleotide receptors (Boehm, 1998) are coupled to the suppression of M-currents. In hippocampal pyramidal neurons, I_M was inhibited by activation of serotoninergic (Colino & Halliwell, 1987), opioid (Moore et al, 1994) and metabotropic glutamate (Harata et al, 1996) receptors. The widespread distribution and the multitude of receptors that regulate its function emphasize the important physiological role of the M-channel.

As the importance of the M-channel in controlling neuronal excitability in sympathetic neurons became more and more apparent, investigators focused on two elementary questions: The signal transduction pathway that mediates inhibition of I_M and the identity of the gene products comprising the M-channels.

The signal transduction pathway has long been a matter of investigation. The fact that such a large number of different receptors can regulate the M-current and that this suppression exhibits a marked delay and slow time course, have been taken as evidence that a diffusible second messenger couples receptor activation to channel inhibition. This assumption was further supported by the findings that muscarinic agonists or bradykinin could also inhibit M-

currents when recorded with the cell-attached patch technique from rat (Selyanko et al, 1992) or frog (Marrion, 1993) sympathetic neurons, while no inhibition was observed when agonists were applied to excised outside-out patches (Stansfeld et al, 1993). Many of the receptors that mediate inhibition of I_M are coupled to pertussis toxin-insensitive $G_{q/11}$ proteins that most commonly activate phospholipase C (PLC). Hence, second messengers downstream in the PLC signalling cascade like inositoltriphosphate (IP_3), diacylglycerol (DAG), arachidonic acid and intracellular Ca^{2+} have been investigated with respect to their role in M-current inhibition. The actions of bradykinin (Cruzblanca et al, 1998) and UTP (Bofill-Cardona et al, 2000) were both significantly attenuated by inhibition of phospholipase C (U-73122), blockade of IP_3 receptors with xestospongin C, buffering of intracellular Ca^{2+} with BAPTA and by depletion of internal calcium stores with thapsigargin, suggesting a role for the following signal transduction cascade: receptor $G_{q/11}$ phospholipase C IP_3 IP_3 receptor Ca^{2+} release M-channel blockade. However, the muscarinic inhibition via M_1 receptors was hardly altered by any of these manipulations and thus the results obtained from these studies raised even more questions instead of revealing a common pathway that leads to suppression of I_M. Additionally the role of several common protein kinases like protein kinase C (PKC), protein kinase A (PKA), MAP kinase and myosin light chain kinase (MLCK) has been investigated, but again no clear cut results were obtained (for detailed review see Marrion, 1997 or Brown & Yu, 2000). Only recently (Suh & Hille, 2002) the identity of the mysterious second messenger has been revealed. Suh and Hille found that activation of $G_{q/11}$ activates PLCβ which causes depletion of membrane PIP_2 levels. The decrease in PIP_2 concentrations results in an inhibition of I_M via an unknown mechanism, although it is tempting to speculate that a direct PIP_2-M channel interaction is involved, in analogy to other K^+ channels (Huang et al, 1998; Hilgemann et al, 2001). Upon removal of agonist, PIP_2 synthesis is initiated by phosphoinositide 4-kinase-mediated phosphorylation of phosphatidyinositol, thereby restoring M channel activity.

The molecular correlate of the M-channel has eluded investigators until recently. In 1998 two novel genes encoding potassium channel subunits termed KCNQ2 and KCNQ3, mutations of which cause most cases of benign familial neonatal convulsions (BFNC), a form of congenital epilepsy, have been identified (Biervert et al, 1998; Singh et al, 1998). KCNQ2/KCNQ3 heterotetramers were shown to form functional channels that produce an M-like current (Wang et al, 1998; Hadley et al, 2000; Shapiro et al, 2000). Using linopirdine and XE991 sensitivity mapping, as well as a comparison of biophysical parameters and mRNA

expression, it was concluded that KCNQ2 and KCNQ3 heteromultimers are the molecular correlate to the M-channel in rat SCG (Wang et al, 1998; Cooper et al, 2000).

KCNQ2 and KCNQ3 are members of the KCNQ gene family that comprises five genes, termed KCNQ1 to KCNQ5, all encoding subunits with the typical 6TM-1P motif. When expressed heterologuously, all five subunits have been shown to form functional homomultimers as well as a variety of functional heteromultimers that generate "M-like" currents as defined kinetically and pharmacologically (Hadley et al 2000; Selyanko et al, 2000; Schroeder et al 2000). KCNQ1 K^+ channels are important for repolarization of cardiac action potentials and for K^+ secretion in the inner ear. KCNQ1 subunits form heteromeric complexes with small regulatory subunits of the KCNE family, in particular with KCNE1 (Barhanin et al, 1996) that have been shown to conduct an I_{Ks} like current, a slow delayed rectifier K^+ current. It is thought that mutations in the KCNQ1 subunit are responsible for one of the most common forms of the long QT syndrome (LQT1) (e.g. Wollnik et al, 1997), which is also associated with a form of congenital deafness. KCNQ4 channel subunits are almost exclusively expressed in the inner ear, vestibular apparatus, and a number of nuclei in the central auditory pathway (Kharkovets et al, 2000). Although it is not quite clear that channels formed by KCNQ4 subunits are the molecular correlates to the predominant K^+ channels ($I_{K,n}$ and $I_{K,L}$) in outer hair cells, it could be shown that certain mutations in this subunit result in deafness (Kubisch et al, 1999).

1.5 P2Y receptors

Adenine and uridine nucleotides, such as ATP und UTP, are present in virtually all cells and may be released in response to various stimuli (Anderson & Parkinson, 1997). The extracellular nucleotide concentrations are precisely regulated by several groups of membrane-associated ectoenzymes that catalyze nucleotide degradation (NTPNDPNMP) or transphosphorylation reactions (ATP + UDP ADP + UTP) (Lazarowski et al, 2003). Once released, nucleotides exert their effects by activating membrane receptors known as P2 receptors.

P2 receptors are further subdivided into two major groups: ionotropic P2X receptors and metabotropic P2Y receptors. P2X receptors are ATP-gated cation channels that exhibit a substantial Ca^{2+} permeability and thus contribute to synaptic transmission (Robertson et al, 2001). P2Y receptors are members of the family of G-protein coupled receptors showing the typical motif of seven membrane spanning domains.

P2Y receptors consist of 308 to 377 amino acids with a molecular weight of 41 to 53 kDa after glycosylation. Positively charged amino acid residues in transmembrane regions 3,6 and 7 are believed to be involved in ligand binding through electrostatic interactions with the phosphate moiety of ATP (Van Rhee et al, 1995; Jiang et al, 1997). At least eight different mammalian DNA sequences have been identified that code for putative G protein coupled nucleotide receptors termed $P2Y_{1, 2, 4, 6, 11, 12, 13,}$ and $_{14}$. In contrast to P2X receptors P2Y receptors are not only activated by adenine nucleotides, but also by uridine nucleotides, as initially proposed by von Kügelgen et al in 1987.

The **$P2Y_1$** receptor was first cloned from chick brain (Webb et al, 1993). It is activated by ADP, 2MeSADP, 2MeSATP, ADPβS and ATP. It has been shown that ATP is a partial agonist at $P2Y_1$ receptors and that the response to ATP depends on the level of receptor reserve (Palmer et al, 1998) which explains the controversial observations made before with respect to the potency of ATP. Adenosine 3′,5′-diphosphate (A3P5P), adenosine 2′,3′-diphosphate (A2P5P) and adenosine 3′-phosphate 5′-phosphosulfate (A3P5PS) act as competitive antagonists at $P2Y_1$ receptors (Boyer et al, 1996). P2Y1 receptors are linked to G proteins of the Gq/11 class and hence activate phospholipase C which leads to IP_3 formation and Ca^{2+} mobilization. Moreover recombinant $P2Y_1$ mediates an inhibition of N-type calcium channels in neurons (Filippov et al, 2000). In blood platelets, activation of endogenous $P2Y_1$ receptors promotes platelet shape change and plays an important role in thrombus formation.

The **$P2Y_2$** (Lustig et al, 1993) and **$P2Y_4$** (Communi et al, 1995) receptors are sensitive to both, adenine and uridine nucleotides. At $P2Y_2$ receptors, UTP and ATP are equipotent agonists whereas at $P2Y_4$ receptors, the actions of ATP are species dependent: ATP is an agonist at rat $P2Y_4$, but an antagonist at human $P2Y_4$ receptors (Kennedy et al, 2000). They are virtually insensitive to ADP or UDP. There are no selective antagonists for these receptors, but still they can be discriminated by using a combination out of a series of non-specific P2Y antagonists reactive blue 2, suramin and PPADS that vary in their rank order of potencies at $P2Y_2$ and $_4$ (see table 1-1). Both, $P2Y_2$ and $P2Y_4$ are linked to $G_{q/11}$ proteins and thus mediate nucleotide-dependent increases in intracellular inositol phosphates. When expressed heterologously in sympathetic neurons, these receptors mediate an inhibition of N-type calcium channels as well as M-type potassium channels (Filippov et al, 1998, 2003).

The **$P2Y_6$** receptor (Chang et al, 1995) is preferentially activated by uridine nucleotides; most potently by UDP and only weakly or not at all by UTP (Communi et al, 1996). $P2Y_6$ is also linked to $G_{q/11}$ proteins and has been reported to be blocked selectively by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and its analogue H_2-DIDS (von

Kügelgen and Jacobson, 2001). When expressed heterologously in rat sympathetic neurons, activation of $P2Y_6$ mediates an inhibition of N-type Ca^{2+}-currents and M-type K^+-currents (Filippov et al, 1999). Moreover activation of endogenous $P2Y_6$ receptors in rat sympathetic neurons causes an inhibition of M-type potassium channels (Boehm 1998) and triggers noradrenaline release by activation of protein kinase C (Vartian et al, 2001).

$P2Y_{11}$ (Communi et al, 1997) is most potently activated by ATP and 2MeSATP, while ADP is almost inactive. Moreover it is insensitive to uridine nucleotides. $P2Y_{11}$ couples alternatively to G_s or G_q proteins and thus stimulates both, phospholipase C and adenylyl cyclase.

The **$P2Y_{12}$** receptor, cloned from rat platelets (Hollopeter et al, 2001), is activated by ADP, 2MeSADP, ATP, 2MeSATP and ADPβS. It is linked to $G_{i/o}$ proteins and mediates an inhibition of adenylyl cyclase (Hollopeter et al, 2001). Prior to its cloning, $P2Y_{12}$ was termed *$P2Y_T$* or *$P2Y_{ADP}$*. Under these pseudonyms, $P2Y_{12}$ has long been known to be expressed in platelets and megakaryoblastic cells. The $P2Y_{12}$ receptor plays a major role in thrombus formation. As mentioned above, activation of the $P2Y_1$ receptor causes platelet shape change, but no aggregation occurs unless the $P2Y_{12}$ receptor is activated concomitantly. Only coactivation of the two receptors initiates signaling pathways that ultimately trigger the activation of glycoprotein IIb/IIIa, which in turn promotes high affinity binding to fibrinogen and platelet aggregation. Hence numerous selective antagonists for the $P2Y_{12}$ receptor have been developed that are used as antithrombotic drugs. The thienopyridine ticlopidine and its analogon clopidogrel are inactive and highly unstable *in vitro*, but irreversibly inhibit the $P2Y_{12}$ receptor after activation by hepatic metabolism (Savi et al, 2000). AR-C69931MX, (N6–[2–methylthioethyl]–2–[3,3,3–trifluoropropylthio]–β,γ-dichloromethylene-ATP (Ingall et al, 1999), an analogon of ATP, acts as a competitive antagonist at $P2Y_{12}$, and has been shown to prevent thrombus formation and increase bleeding times *in vivo* (Huang et al, 2000). In contrast to clopidogrel, AR-C69931MX is also effective in vitro; it selectively blocks ADP induced inhibition of adenylyl cyclase in platelets (Ohlmann et al, 2000). Another selelective antagonist is 2MeSAMP, which has been shown to selectively block ADP-induced inhibition of adenylyl cyclase in platelets (Jantzen et al, 1999) and ADP-induced K^+-currents in *Xenopus* oocytes expressing the human $P2Y_{12}$ receptor (Hollopeter et al, 2001).

The human **$P2Y_{13}$** was recently cloned from spleen (Communi et al, 2001) and shows many similarities to the $P2Y_{12}$ receptor. It is coupled to G_i proteins, responds to diphosphate adenine nucleotides (ADP, 2MeSADP, ADPβS) and has been shown to inhibit forskolin induced formation of cAMP when heterologously expressed in HEK293-EBNA (Zhang et al, 2002) or

in CHO-K1 cells (Communi et al, 2001). Differences have been reported with respect to antagonist potencies. At the recombinant human $P2Y_{12}$ receptor, AR-C69931MX acted as a competitive antagonist with an IC_{50} of 2,4 nM (Takasaki et al, 2001), while antagonism at human $P2Y_{13}$ was non-competitive with an IC_{50} of 0,6 nM (Marteau et al, 2003). Moreover clopidogrel was not active at all at the $P2Y_{13}$ receptor.

The UDP-glucose receptor was originally cloned from human myeloid cells (Chambers et al, 2000) and has recently been renamed to **$P2Y_{14}$** (Abbracchio et al, 2003). In recombinant systems it is activated by UDP-glucose, UDP-galactose and UDP-glucuronic acid but is insensitive to ADP, ATP or other nucleotides. Pertussis toxin completely abolished agonist responses, confirming the receptor to be coupled to G proteins of the $G_{i/o}$ class. The transduction pathways used by this receptor in native systems still remain to be defined.

Table 1-1, Pharmacological properties of P2Y receptors.

Receptor subtype	*G-protein-effector cascade*	*physiological agonists*	*antagonists*
$P2Y_1$	G_q PI-PLC I_{Ca}/K_M	ADP,ATP	MRS-2216*, MRS-2179*, A3P5P*, A2P5P*, A3P5PS*
$P2Y_2$	G_q PI-PLC I_{Ca}/K_M	ATP=UTP	RB2>suramin>PPADS
$P2Y_4$	G_q PI-PLC I_{Ca}/K_M	ATP,UTP	PPADS>RB2>suramin
$P2Y_6$	G_q PI-PLC I_{Ca}/K_M	UDP>>UTP	RB2>PPADS>suramin DIDS*, H_2-DIDS*
$P2Y_{11}$	G_q PI-PLC and G_s AC	ATP>ADP	
$P2Y_{12}$	G_i AC/others	ADPATP	AR-C69931MX*, 2MeSAMP*, clopidogrel*, ticlopidine*
$P2Y_{13}$	G_i AC/others	ADP,ATP	
$P2Y_{14}$	G_i AC/others	UDP-glucose	

Abbreviations: PI-PLC, phospholipase C; AC, adenylyl cyclase; RB2, reactive blue2; A3P5P, adenosine 3′,5′-diphosphate; A2P5P, adenosine 2′,3′-diphosphate; A3P5PS, adenosine 3′-phosphate 5′-phosphosulfate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid;
AR-C69931MX, (N6–[2–methylthioethyl]–2–[3,3,3–trifluoropropylthio]–β,γ-dichloromethylene-ATP; 2MeSAMP, 2-methylthioadenosine 5′-monophosphate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; * indicates a selective antagonist.

1.6 Muscarinic acetylcholine receptors

Acteylcholine exerts its action by the activation of either ionotropic nicotinic receptors or metabotropic muscarinic receptors (mAChRs). So far, five different muscarinic receptor subtypes, termed M_1 to M_5, have been identified, all showing the seven transmembrane domain structure typical of G protein-coupled receptors. The M_1, M_3 and M_5 mAChRs typically couple to $G_{q/11}$ proteins that activate the phospholipase C pathway, while M_2 and M_4 receptors are preferentially coupled to G_i proteins mediating an inhibition of adenylyl cyclase.

Although muscarinic receptors have long been recognized as potential drug targets for the treatment of disorders such as Alzheimers disease, chronic obstructive pulmonary disease, overactive bladder or chronic pain, only few more or less subtype selective antagonists have yet been discovered (see table 1-2). Hence, different subtypes can only be discriminated from each other by using a series, but not a single, subtype preferring antagonist (Caulfield & Birdsall, 1998). The same is true for agonists at muscarinic receptors. Although numerous substances, like muscarine, oxotremorine M, pilocarpin, arecolin and the choline esters carbachol and bethanechol, have been shown to activate muscarinic receptors, neither of these substances showed a high selectivity for one particular subtype.

Table 1-2, Antagonist affinity constants (pK_B) for mammalian muscarinic receptors

Antagonist	Receptor subtype				
	M_1	M_2	M_3	M_4	M_5
Atropine	9.0–9.7	9.0–9.3	8.9–9.8	9.1–9.6	8.9–9.7
Pirenzepine	7.8–8.5	6.3–6.7	6.7–7.1	7.1–8.1	6.2–7.1
Methoctramine	7.1–7.8	7.8–8.3	6.3–6.9	7.4–8.1	6.9–7.2
4-DAMP	8.6–9.2	7.8–8.4	8.9- 9.3	8.4–9.4	8.9–9.0
Himbacine	7.0–7.2	8.0–8.3	6.9-7.4	8.0–8.8	6.1–6.3
AF-DX 384	7.3–7.5	8.2–9.0	7.2-7.8	8.0–8.7	6.3
Darifenacin	7.5–7.8	7.0–7.4	8.4–8.9	7.7–8.0	8.0–8.1
Muscarinic toxin 3	7.1	<6	<6	8.7	<6
Muscarinic toxin 7	9.8	<6	<6	<6	<6

Adapted from Caulfield & Birdsall (1998)

Muscarinic receptors are primarily found in peripheral effector tissues, such as heart, exocrine glands, smooth muscle or blood vessels, in parasympathetic and sympathetic neurons, and in the central nervous system (Levey, 1993, Caulfield, 1993; Caulfield & Birdsall, 1998). Identification of the precise physiological roles of the individual mAChR subtypes has been rather difficult in the past because of the lack of subtype selective antagonists and thus reports about the function of certain subtypes has often been controversial. More recently some of these problems could be overcome by generating knockout mice that lack one of the five mAChR subtypes.

Significant levels of **M_1 mAChRs** are found in salivary glands, sympathetic ganglia and in all major brain areas, including cerebral cortex, hippocampus and striatum. M_1 heteroreceptors have been reported to facilitate dopaminergic transmission in the striatum (Gerber et al, 2001). In cerebral cortical neurons, M_1 mAChRs have been shown to be responsible for the

ACh-induced MAP kinase activation, a process that has been implicated in learning and memory (Hamilton & Nathanson, 2001; Hamilton et al, 1997). Moreover, activation of postsynaptic M_1 receptors in sympathetic ganglia suppresses the M-type potassium current (Marrion et al 1989; Bernheim et al, 1992).

In the periphery, **M_2 receptors** are most abundantly expressed in the heart and smooth muscle organs, whereas they are rather equally distributed throughout the brain. The brain M_2 receptors contribute to the centrally mediated anti-nociception (Gomeza et al, 1999). In heart, activation of M_2 receptors causes a decrease in the heartrate, probably via an inhibition of voltage gated calcium channels and activation of inwardly rectifying K^+ channels (reviewed in Caulfield, 1993). In smooth muscle, M_2 mAChRs mediate an activation of a Ca^{2+} dependent nonselective cation conductance (I_{Cat}) (Bolton & Zholos, 1997) and an inhibition of a Ca^{2+} dependent K^+ current, BK_{Ca} (Kotlikoff et al, 1992). Since both currents, I_{Cat} and BK_{Ca}, are Ca^{2+} dependent, M_2 activation will only show a little effect under resting conditions.

M_3 mAChRs are predominantly expressed in gastrointestinal, urinary bladder and airway smooth muscle. Activation of M_3 receptors in smooth muscle mediates Ca^{2+} mobilization and contraction via the phospholipase-Cβ pathway. The increase in Ca^{2+} causes an efflux of potassium through Ca^{2+} dependent K^+ channels (BK_{Ca}), which hyperpolarizes the cell and is thus thought to serve as an inhibitory feedback mechanism following stimulation. Since activation of M_2 inhibits BK_{Ca} and activates I_{Cat}, co-activation of this receptor could potentiate the M_3 mediated contraction (Ehlert, 2003).

M_4 mAChRs are preferentially coupled to G_i proteins and thus mediate mainly inhibitory effects. M_4 has been shown to mediate the fast, pertussis toxin-sensitive suppression of calcium currents in rat superior cervical ganglia (Bernheim et al, 1992). Moreover M_4 has been shown to inhibit neurogenic contraction in rabbit vas deferens (Budriesi et al, 2001). Like M_2, M_4 mAChrs have been reported to mediate anti-nociceptive effects (Sheardown et al, 1997; Duttaroy et al, 2000).

M_5 mAChRs are expressed at very low levels in different regions of the CNS and in several peripheral tissues (Eglen & Nahorski, 2000). Not much is known about the physiological role of M_5; recent evidence from studies using M_5 knockout mice suggests the M_5 receptors to reduce the rewarding effects of morphine (Basile et al, 2002) and to mediate acetylcholine-dependent dilation of cerebral arteries and arterioles (Yamada et al, 2001).

1.7 Postganglionic sympathetic neurons

1.7.1 Methodological considerations

Primary cultures of dissociated postganglionic sympathetic neurons are widely used to investigate receptors that evoke or modulate neurotransmitter release at the sympathoeffector junction. Dissociated postganglionic sympathetic neurons in cell culture develop only a small number of short dendrites (Furshpan et al, 1986; Lein et al, 1995) and a network of branching axons. The neuronal somata tend to form aggregates which are interconnected by axons that frequently occur in bundles. Within the regions of these aggregates of neuronal somata, numerous quasi synaptic contacts can be revealed by punctate staining with antibodies directed against synaptic vesicle proteins (Mochida et al, 1994; Zhou and Misler, 1995) or against syntaxin, a membrane protein participating in sympathetic transmitter release (Mochida et al, 1995). However, the branching axons with their presynaptic specializations are the sole sites of transmitter release as detected by amperometric recordings (Zhou and Misler, 1995) as well as by biochemical techniques (Przywara et al, 1993). The neuronal somata, by contrast, hardly store and do not release noradrenaline (Przywara et al, 1993; Boehm, 1999). It has also been shown in explant cultures of rat superior cervical ganglia that sprouting axons, growth cones, or axon terminals are the predominant sites of noradrenaline uptake and release (Vogel et al, 1972). Even though the neuronal cell bodies per se do not release transmitters, stimulatory or inhibitory effects at these somata and/or dendrites may contribute to the modulation of transmitter release from cultured neurons by receptor agonists and antagonists. Hence, when observing an effect that evokes or modulates stimulation-evoked transmitter release, one has to establish whether this effect is mediated by a presynaptic or by a postsynaptic receptor. This can be achieved by performing experiments in the presence of Na^+ channel blockers which prevent action potential propagation and thereby abolish signaling between neuronal somata and axonal varicosities (Allgaier and Meder, 1995; Kristufek et al, 1999a). To investigate modulatory effects, transmitter release can be evoked either by electrical field stimulation (EFS) or by depolarization with high potassium concentrations. Electrically evoked transmitter release requires action potentials and subsequent calcium influx through voltage-gated calcium channels at the nerve terminal and thus is abolished in the presence of tetrodotoxin (TTX), in the absence of extracellular calcium (Wakade & Wakade, 1998; Böhm et al, 1991) or in the presence of specific Ca^{2+} channel blockers like ω-conotoxin GVIA. By contrast, chemically induced (high K^+) transmitter release is usually insensitive to TTX (Allgaier & Meder, 1995; Boehm & Huck, 1996), but can also be blocked by ω-conotoxin. However, noradrenaline release induced by

K^+ concentrations < 40mM was found to be partially TTX sensitive (Kristufek et al, 1999). Hence, using a combination of these experimental techniques allows the discrimination between presynaptic and postsynaptic receptors.

1.7.2 Regulation of transmitter release from postganglionic sympathetic neurons via ionotropic and metabotropic receptors

In sympathetic neurons, a plethora of receptors, including both ionotropic and metabotropic receptors, has been reported to modulate the release of neurotransmitter.

Activation of nicotinic acetylcholine receptors (nAChRs) or P2X nucleotide receptors in postganglionic sympathetic neuron induces the release of neurotransmitter, which is not too surprising since acetylcholine and ATP are the major transmitters released from preganglionic nerve terminals. As expected, this effect is entirely Ca^{2+} dependent, but is only partly blocked in the presence of TTX or Ca^{2+} channel blockers, like ω-conotoxin GIVA (e.g. Boehm & Huck, 1995; Dolezal et al, 1996; Boehm, 1999; Sperlagh et al, 2000). The fact that transmitter release is also induced in the presence of TTX, indicates that the receptors responsible for this stimulation are also located at the axonal varicosities themselves, i.e. presynaptic receptors. Moreover, these results demonstrate that the Ca^{2+} required to trigger neurotransmitter release, enters the cell through the transmitter-gated channel itself rather than through voltage-gated Ca^{2+} channels, and thus the receptors must be located in close proximity to sites of release, i.e. in active zones. In addition to triggering exocytosis themselves, P2X receptors have also been shown to facilitate stimulation evoked transmitter release (Boehm, 1999; Sperlagh et al, 2000).

Interestingly, besides excitatory transmitters, also transmitters like GABA and glycine that usually exert inhibitory actions have been reported to stimulate or facilitate release in sympathetic neurons (Owens et al, 1996; Boehm et al, 1997). $GABA_A$ and glycine receptors are permeable to chloride and usually mediate a chloride influx that hyperpolarizes the membrane. However, neurons can accumulate Cl^- via Cl^- co-transporters, thereby shifting the equilibrium potential for Cl^-, so that open Cl^- channels will mediate chloride efflux and thus depolarize the membrane. Accordingly, the secretagogue action of glycine in sympathetic neurons can be blocked by furosemide, which selectively blocks Cl^- co-transporters (Boehm et al, 1997).

Among the many metabotropic receptors that have been reported to modulate neurotransmitter release from sympathetic neurons, only a few receptors have been shown to

mediate stimulation or facilitation. Most receptors rather mediate an inhibition of transmitter release via presynaptic mechanisms.

At least three metabotropic receptors that are located at the somatodendritic region have been shown to stimulate noradrenaline release from rat sympathetic neurons (Boehm et al, 1995; Boehm & Huck, 1997) or from chick sympathetic neurons (Greene & Rein, 1978) in culture: a muscarinic acetylcholine receptor (Greene & Rein, 1978), a bradykinin B_2 receptor (Boehm & Huck, 1997), and a uridine nucleotide-sensitive P2Y receptor (Boehm et al, 1995). Activation of all these receptors leads to the stimulation of PLC and subsequent formation of IP3 (Wakade et a., 1991; Cruzblanca et al, 1999) which can in turn mobilize Ca^{2+} from intracellular stores. However, a rise in intracellular Ca^{2+} does not necessarily induce transmitter release which has been shown in chick sympathetic neurons (Wakade et al, 1990). Furthermore the secretagogue action of bradykinin is not altered if internal calcium stores are depleted with thapsigargin (Boehm, 1997). Bradykinin B_2 receptors have been shown to simultaneously activate two different signalling pathways: one mediates an inositol trisphosphate- and Ca^{2+}-dependent inhibition of M-currents, while the other one leads to an excitation of sympathetic neurons independently of changes in M-currents through an activation of Ca^{2+}-insensitive protein kinase C (Scholze et al, 2001). Similar results were obtained for the uridine nucleotide sensitive $P2Y_6$ receptor. Activation of this receptor also mediates an IP_3 and Ca^{2+} dependent inhibiton of M-currents (Bofill-Cardona et al, 2000), but independently from this inhibition of I_M, the UTP evoked noradrenaline release is mediated by an activation of protein kinase C (Vartian et al, 2001). By contrast, the mechanisms underlying the secretagogue action of muscarinic agonists still remain elusive. Considering the fact that the inhibition of I_M is not the mechanism of choice by which B_2 and $P2Y_6$ receptors stimulate transmitter release, one may doubt that this inhibition is responsible for the transmitter release evoked by the activation of muscarinic receptors. However, the cognition enhancer linopirdine which is thought to be a selective blocker of M-type potassium channels has been shown to cause action potential dependent release of noradrenaline (Kristufek et al, 1999) and thus it will be interesting to see if the muscarinic suppression of I_M may also be sufficient to trigger release from sympathetic neurons or if another mechanism is used.

Aside of these somatodendritic receptors, postganglionic sympathetic neurons express a number of presynaptic G protein coupled receptors that control transmitter release (for detailed review see Boehm & Kubista, 2002). Among these only a limited number of

receptors have consistently been shown to mediate facilitation, including angiotensin AT_1 receptors, β-adrenoceptors, bradykinin B_2 receptors and muscarinic M_1 receptors. However, the majority of reports on presynaptic metabotropic receptors demonstrate that their activation causes inhibition of sympathetic transmitter release.

Such presynaptic inhibition via G proteins may occur primarily at two levels: first, at the level of excitation-secretion coupling, and second, at the level of vesicle exocytosis. Voltage-gated Ca^{2+} and K^+ channels are well known to be regulated by a variety of G proteins (Brown and Birnbaumer, 1990). An enhancement of repolarizing K^+ currents may shorten the depolarizing phase of action potentials and thus diminish ensuing Ca^{2+} influx. Moreover, receptor mediated activation of inwardly rectifying GIRK channels could lead to hyperpolarization of the cell, and consecutively to decreased depolarization-dependent Ca^{2+} influx and inhibition of transmitter release. Although such a mechanism has been shown to contribute to the inhibition of transmitter release in the hippocampus (Thompson et al, 1993), there is almost no evidence supporting the idea that alterations of K^+ channels are generally important in the presynaptic inhibition in sympathetic neurons. α_2-Adrenoceptors as prototypic examples of inhibitory presynaptic receptors were directly shown not to affect depolarization evoked K+ currents in sympathetic neurons (Bhave et al, 1990; Boehm & Huck, 1996).

Ca^{2+} influx into the cell via voltage-dependent Ca^{2+} channels is the crucial step between depolarization and vesicle exocytosis from nerve endings (Heidelberger et al, 1994). G protein coupled receptors regulate Ca^{2+} influx at the somatodendritic region and at the axons of sympathetic neurons (Dolezal et al, 1994 and 1996; Toth et al, 1993). The inhibition of voltage-dependent Ca^{2+} channels was demonstrated to be of major importance in the regulation of sympathetic transmitter release via presynaptic α_2-autoreceptors. In rat sympathetic neurons, the acetylcholine-mediated noradrenaline release is in part cadmium-insensitive, showing that this part is independent of voltage-dependent Ca^{2+} channels. This cadmium-insensitive release is not influenced by activation of α_2-adrenoceptors (Boehm and Huck, 1995). In sympathetic neurons of the chick embryo, transmitter release can be induced by electric field stimulation under conditions that do not allow the opening of N-type Ca^{2+} channels. Under these circumstances, α_2-adrenoceptors fail to modulate noradrenaline release. Thus, if the Ca^{2+} channels which are controlled by presynaptic receptors are blocked, receptor-mediated presynaptic inhibition is abolished. This indicates that α_2-autoreceptors reduce transmitter release only via an inhibition of voltage-dependent Ca^{2+} channels (Boehm and Huck, 1995 and 1996). Besides the α_2-adrenoceptor, numerous other presynaptic receptors have also been shown to inhibit voltage-gated calcium channels and inhibit

transmitter release, including NPY neuropeptide Y receptors (Toth et al, 1993; Oellerich et al, 1994), receptors for somatostatin (Boehm and Huck, 1996), muscarinic M_2 and M_4 receptors (Shapiro et al, 1999; Bernheim et al, 1992; Göbel et al, 2000; Budriesi et al, 2001), adenosine A_1 receptors (Zuh & Ikeda, 1993) and ORL1 opioid receptors (Larson et al, 2000; Allgaier et al, 1994).

However, G proteins may also act downstream of Ca^{2+} entry by direct interference with protein-protein interactions involved in transmitter release, as suggested by studies on permeabilised cells. Non-hydrolysable GTP analogs were found to alter catecholamine release from α-toxin permeabilised chromaffin cells and PC12 cells in a pertussis toxin-sensitive manner (Ahnert-Hilger et al, 1987; Ahnert-Hilger et al, 1992). Moreover a recent study on motorneurons puts forward the possibility that G protein mediated inhibition of transmitter release may be due to an interaction of βγ-subunits with the SNARE fusion machinery. In further support of a direct G protein-dependent inhibition of vesicle exocytosis, it was observed in central neurons that spontaneous release in either the presence of the Na^+ channel blocker tetrodotoxin (and thus in the absence of action potentials) or in the presence of the Ca^{2+} channel blocker Cd^{2+} can also be reduced by presynaptic receptor activation and via pertussis toxin-sensitive G proteins (e.g. Takahashi et al, 2001; Miller, 1998; Boehm and Betz, 1997). Nevertheless, in sympathetic neurons spontaneous transmitter release remains most commonly unaltered when inhibitory presynaptic receptors are activated (Brock & Cunnane, 1996; Trendelenburg et al, 2001).

Taken together, it appears straightforward to suggest that the G-protein mediated inhibition of voltage-gated calcium channels is the major mechanism underlying the presynaptic inhibition of sympathetic transmitter release.

1.8 Aim of the work

1.8.1 Part I – Muscarinic actylcholine receptors

In primary cultures of dissociated sympathetic ganglia of various species, acetylcholine (Boehm and Huck, 1995) and other agonists at nAChRs, such as nicotine (Greene and Rein, 1978; Boehm and Huck, 1996) and DMPP (Dolezal et al, 1994) trigger noradrenaline release. With respect to agonists at mAChRs, however, divergent results have been obtained. In cultures derived from chick embryos, muscarinic agonists, including methacholine and arecoline, triggered [^{3}H]noradrenaline release, and the stimulatory action of methacholine was antagonized by nanomolar concentrations of atropine (Greene and Rein, 1978). Bhave et al. (1988), in contrast, were unable to detect transmitter release from chick sympathetic neurons when challenged by an activation of muscarinic receptors, even though this stimulation clearly increased the intracellular levels of inositol phosphates. In cultures of rat and mouse sympathetic neurons, the muscarinic agonist oxotremorine M (OxoM) at 10 to 100 μM did trigger transmitter release (Nörenberg et al, 2000 and 2001). However, in these latter studies, antagonists have not been used, and oxotremorine M is known to quite potently activate nicotinic acetylcholine receptors (Reitstetter et al, 1994). Thus, the presence of release stimulating muscarinic receptors in primary cultures of postganglionic sympathetic neurons remained to be confirmed.

Apart from excitatory mAChRs as described above, sympathetic neurons in cell culture are known to express inhibitory mAChRs, activation of which reduces rather than stimulates noradrenaline release. In rat superior cervical ganglion (SCG) neurons (Boehm and Huck, 1995), for instance, atropine at nanomolar concentrations caused a marked increase (instead of a decrease, as above) in acetylcholine-evoked noradrenaline release. Kindred results have been obtained in rabbit hearts (Lindmar et al, 1968) and are indicative of release-inhibiting presynaptic mAChRs. The presence of such receptors in SCG neurons was confirmed by Koh and Hille (1997) who showed that pirenzepine-insensitive (i.e. not M_1) muscarinic receptors reduced noradrenaline release. Most recently, mAChRs of the M_2 subtype were found to reduce electrically evoked transmitter release in cultured paravertebral sympathetic neurons from neonatal mice (Göbel et al, 2000).

Transmitter release strictly depends on transmembrane Ca^{2+} influx. In sympathetic neurons, it is primarily voltage-gated Ca^{2+} channels of the N-type which contribute to this Ca^{2+} influx, and blockade of these channels prevents transmitter release. Furthermore, the G protein-mediated inhibition of these channels is believed to underlie the inhibition of sympathetic transmitter release via presynaptic receptors (Boehm and Kubista, 2002). In support of this

hypothesis, mAChRs were found to inhibit N-type Ca^{2+} channels (Bernheim et al, 1992) in rat SCG neurons, and the mAChR-mediated inhibition of transmitter release from these neurons was lost when N-type Ca^{2+} channels were blocked by ω-conotoxin GVIA (Koh and Hille, 1997).

In contrast to Ca^{2+} channel blockers, blockers of K_M channels, such as Ba^{2+} and linopirdine, were reported to induce instead of inhibit noradrenaline release from rat SCG neurons in a Ca^{2+}-dependent manner (Kristufek et al, 1999). Accordingly, the inhibition of these channels via M_1 mAChRs can also be expected to trigger transmitter release. In the first part of this thesis, I tested this hypothesis by using the muscarinic receptor agonist OxoM and the K_M channel opener retigabine.

1.8.2 Part II – Metabotropic P2Y nucleotide receptors

Although, ATP and noradrenaline have long been known to be the major transmitters released from postganglionic sympathetic neurons, autoinhibitory feedback modulation of sympathetic transmitter release was assumed to occur primarily via noradrenaline and α_2-adrenoceptors. Evidence for a similar role of ATP has been obtained only 15 years ago (Stjärne & Astrand, 1985; Fujioka & Cheung, 1987). Originally these effects were thought to be mediated by adenosine, a degradation product of ATP (Starke et al, 1989). However, using P2 receptor antagonists like, suramin, reactive blue 2 and PPADS, several studies on various tissues, including, the vas deferens (von Kügelgen et al, 1989, 1993, 1994), pancreas (Koch et al, 1998), kidney (Bohmann et al, 1997) or tail artery (Goncalves & Queiroz, 1996) revealed that P2 receptors, rather than adenosine receptors are responsible for the feedback inhibition by nucleotides (reviewed in Boehm, 2003). Generally, these inhibitory presynaptic receptors are activated by adenine, but not uridine, nucleotides. They are rather insensitive to prototypic P2X receptor ligands, such as suramin, but are quite potently blocked by reactive blue 2 and related substances. This frequently led to the conclusion that these presynaptic nucleotide receptors are P2Y receptors, but the particular receptor subtype has never been identified.

The rat phaeochromocytoma cell line PC12 is ontogenetically related to sympathetic neurons. These cells develop a neuronal phenotype upon treatment with nerve growth factor (NGF; Greene and Tischler, 1976), and express various types of neuronal VACCs (Liu et al, 1996). In rats, five P2Y receptors, namely $P2Y_{1,\ 2,\ 4,\ 6,}$ and $_{12}$, have been detected (Ralevic and Burnstock, 1998; Hollopeter et al, 2001). In PC12 cells, ADP was found to inhibit Ca^{2+} currents (I_{Ca}) in a PTX-sensitive and voltage-dependent (at least partly) manner, and the

pharmacological characteristics of the receptor involved were similar to those of previously described inhibitory presynaptic receptors, but at that time did not correspond to any of those of molecularly defined P2Y receptor subtypes (Vartian and Boehm, 2001). More recently, we were able to show that PC12 cells express $P2Y_{12}$ receptors, activation of which reduced the accumulation of cyclic AMP (Unterberger et al, 2002). Previously, the inhibition of adenylyl cyclase activity via $P2Y_{12}$ receptors had been reported for thrombocytes (Hollopeter et al, 2001), endothelial (Simon et al, 2001), and glioma cells (Jin et al, 2001), but not for neurons or neuroendocrine cells. The fact that the nucleotide sensitivities for both, the inhibition of I_{Ca} and the reduction in cyclic AMP were similar raises the question as to whether a single P2Y receptor subtype mediated both effects.

In order to clarify this, we used the selective $P2Y_{12}$ receptor antagonists, 2-MeSAMP and AR-C69931MX, and determined their effects on the inhibition of adenylyl cyclase and I_{Ca} by adenine nucleotides in PC12 cells. Since the inhibition of voltage-gated Ca^{2+} channels is a key event in the presynaptic inhibition of sympathetic transmitter release, we tested whether ADP might also reduce stimulation-evoked ^{3}[H]-noradrenaline release from PC12 cells, and tried to reveal the signal transduction mechanism underlying the inhibition of I_{Ca} by ADP.

2 Methods

2.1 Cell culture

2.1.1 PC12 cells

PC12 cells were plated onto collagen-coated (Biomedical Technologies Inc., Stoughton, MA, USA) culture dishes (NUNC, Roskilde, Denmark) and were kept in OptiMEM (Life Technologies, Vienna, Austria) supplemented with 0.2 mM L-glutamine (HyClone, Aalst, Belgium), 25.000 IU/l penicillin and 25 mg/l streptomycin (Sigma, Vienna, Austria), 5% fetal calf serum, and 10% horse serum (both Life Technologies, Vienna, Austria). OptiMEM supplemented with L-glutamine, penicillin and streptomycin is referred to as "complete medium" in the following chapters. Once per week, cell cultures were split, and the medium was exchanged twice per week. In order to increase I_{Ca} of PC12 cells through neuronal differentiation (Vartian and Boehm, 2001), the horse serum was reduced to 5%, fetal calf serum was omitted from, and recombinant human β-nerve growth factor (NGF, 50 ng/ml, R&D Systems Inc., Wiesbaden, Germany) was included in, the medium for 5 to 6 days. As shown in figure 2-1, PC12 cells develop a neuronal phenotype upon NGF treatment. Prior to the recording of I_{Ca}, PC12 cells were detached from culture dishes and replated at low density (about 30.000 cells per dish) in 35 mm dishes.

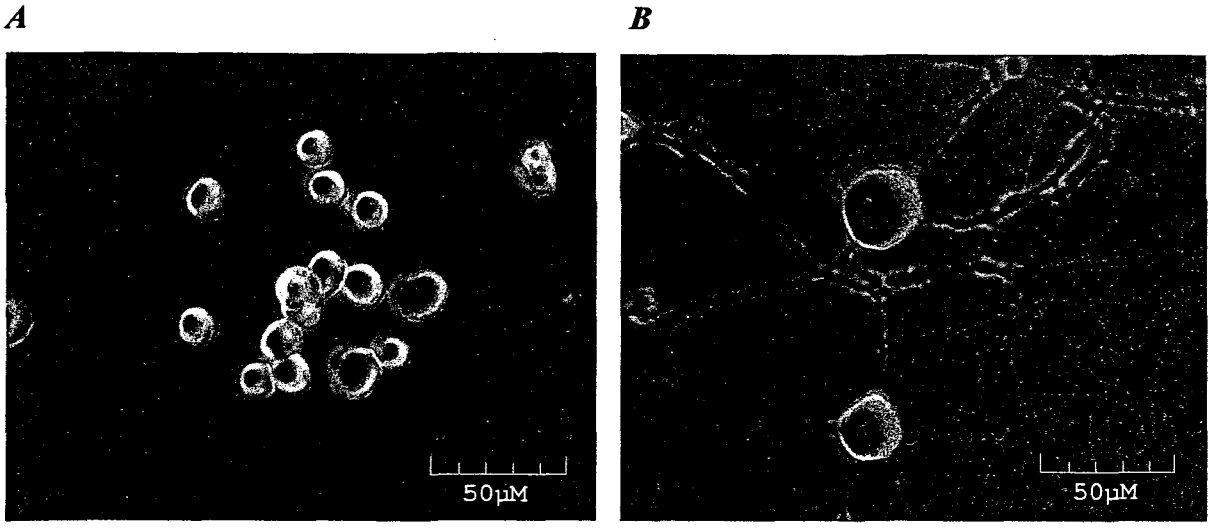

Figure 2-1 PC12 cells in the light microscope.

A shows a phase contrast image of wildtype PC12 cells. B shows a phase contrast image of differentiated PC12 cells after 5 days of treatment with human β-NGF.

2.1.2 *Primary cultures of rat superior cervical ganglion neurons.*

Primary cultures of dissociated SCG neurons from neonatal rats were prepared as described before (Boehm, 1999). Briefly, ganglia were dissected from 2 to 6 day old Sprague Dawley rat pups which had been killed by decapitation in accordance with the rules of the university animal welfare committee. Ganglia were cut into 3 to 4 pieces and incubated in collagenase (1.5 mg/ml; Sigma, # 9891) and dispase (3.0 mg/ml; Boehringer Mannheim, # 165859) for 20 min at 36 °C. Subsequently, they were trypsinised (0.25 % trypsin; Worthington, # 3703) for 15 min at 36 °C, dissociated by trituration, and resuspended in Dulbeccos modified Eagle's Medium (Gibco #041-01885M) containing 2.2 g/l glucose, 10 mg/l insulin, 25000 IU/l penicillin and 25 mg/l streptomycin (Gibco #043-05140D), 50 µg/l nerve growth factor (Gibco #0436050), and 5% fetal calf serum (Gibco #011-0620H). Thereafter, the cells were plated onto 5 mm discs for superfusion experiments and onto 35 mm culture dishes (Nunc #153066) for electrophysiological experiments, both coated with rat tail collagen (Biomedical Technologies Inc., Stoughton, MA, USA). All cultures were kept in a humidified 5% CO_2 atmosphere at 36 °C for 4 to 8 days. On day one after plating, the medium was completely exchanged, and after 3 to 5 days, the medium was exchanged again and the serum was omitted.

2.2 *Determination of cylic AMP*

The accumulation of cyclic AMP in PC12 cell cultures was determined as described before; since differentiation of PC12 cells did not alter the ADP-dependent inhibition of adenylyl cyclase, nor the expression of $P2Y_{12}$ receptors (Unterberger et al, 2002), these experiments were performed in cultures not treated with NGF. PC12 cells were incubated in medium containing 2.5 µCi ml^{-1} tritiated adenine to label the cellular purines. After 12 hours, the medium was replaced by a buffer (120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH) containing 100 µM of the phosphodiesterase inhibitor Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one] and 1 U ml^{-1} adenosine deaminase. Dishes were then kept at room temperature for about 90 minutes. Thereafter, the cells were incubated in the above buffer containing the adenosine A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS 21680) for 15 min at room temperature to stimulate adenylyl cyclase. ADP was present together with CGS 21680, and P2Y receptor antagonists were added 10 minutes before the addition of ADP. The stimulation period was terminated by exchanging the buffer for 1 ml of

2.5 % perchloric acid containing 100 µM non-labelled cyclic AMP followed by a 20 min incubation at 4 °C.

Cyclic AMP was separated from the other purines by a two column chromatographic procedure (Johnson et al, 1994). One tenth of each sample obtained as described above was used for the determination of the total radioactivity. The remaining 900 µl were neutralised by addition of 100 µl 4.2 M KOH and applied to Dowex 50 columns (AG 50W-X4; Bio-Rad, Vienna, Austria) which were then rinsed with 3 ml H_2O. The eluate obtained by the subsequent application of 8 ml H_2O was directly poured onto alumina columns (Bio-Rad, Vienna, Austria), which were then washed with 6 ml H_2O. Finally, cyclic AMP was eluted with 4 ml imidazole buffer (20 mM imidazole in 0.2 M NaCl; pH 7.45). Radioactivity within the samples obtained was determined by liquid scintillation counting.

The radioactivity in the fraction of cyclic AMP was expressed as percentage of the total radioactivity incorporated in the cells. Stimulation of PC12 cells with the adenosine A_{2A} receptor agonist CGS 21680 caused a reproducible increase in these values of cyclic AMP, but the extent of basal and stimulated cyclic AMP synthesis may vary between different preparations (Unterberger et al, 2002). Therefore, the values of cyclic AMP obtained in the presence of CGS 21680 were normalized to the values obtained in its absence within the same preparation (normalized to basal). To quantify the inhibition of stimulated cyclic AMP accumulation by ADP, values obtained in its presence were expressed as percentage of the values obtained in the presence of CGS 21680 only (% of control).

The extent as well as the concentration dependence of the nucleotide-induced inhibition of adenylyl cyclase may vary between different batches of PC12 cells (Unterberger et al, 2002; Vartian and Boehm, 2001). Therefore, changes in the concentration response curves for agonists in the presence of increasing antagonist concentrations were investigated in one batch of PC12 cells per one antagonist. All data represent arithmetic means ± s.e.mean; n represent numbers of culture dishes in cyclic AMP assays. Concentration-response curves were fitted to experimentally obtained data by the ALLFIT programme (DeLean et al, 1978).

2.3 Electrophysiology

2.3.1 I_{Ca} in PC12 cells

Whole-cell currents were recorded at room temperature (20-24°C) from PC12 cells 48 to 96 h after replating at low density, according to pulished procedures (Vartian and Boehm, 2001), using an Axopatch 200B amplifier and the Pclamp 6.0 hard- and software (Axon Instruments,

Foster City, CA, USA). Currents were low-pass filtered at 5 kHz, digitized at 10 to 50 kHz, and stored on an IBM compatible computer. Traces were analyzed off-line by the Clampfit program (Axon). Patch electrodes were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA, USA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) and filled with a solution consisting of (mM) CsCl (130), tetraethylammonium chloride (20), $CaCl_2$ (0.24), glucose (10), HEPES (10), EGTA (5), Mg-ATP (2), and Li-GTP (2), adjusted to pH 7.3 with KOH, to yield tip resistancies of 2 to 3 MΩ. The external bathing solution contained (mM) NaCl (120), tetraethylammonium chloride (20), KCl (3), $MgCl_2$ (2), $CaCl_2$ (5), glucose (20), HEPES (10), adjusted to pH 7.3 with KOH. This combination of solutions results in small liquid junction potentials of about +2 mV which, however, were neglected during I_{Ca} recordings. Drugs were applied via a DAD-12 drug application device (Adams & List, Westbury, NY, USA) which permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (Boehm, 1999).

Whole-cell I_{Ca} was elicited by 30 ms depolarizations from a holding potential of –80 mV to 0 mV at a frequency of 4 min^{-1}. Leakage currents were corrected for by using an on-line leak subtraction protocol which applies four hyperpolarizing pulses prior to the depolarization to 0 mV in order to determine the extent of leakage. I_{Ca} was quantified by measuring peak current amplitudes during the depolarization to 0 mV. To account for time-dependent changes in I_{Ca}, drug effects were evaluated by evoking currents in the presence of test drugs (B) and by comparing them to control currents recorded before (A) and after (washout, C) the application of the drugs, according to the equations: 200B / (A + C) = % of control current, or 100 - (200B/[A+C]) = % inhibition (Vartian and Boehm, 2001). The cells being investigated were continuously superfused with control solutions or with solutions containing agonists and/or antagonists at the concentrations indicated. To evaluate the effects of P2Y receptor antagonists (2-MeSAMP, AR-C69931MX), cells were first exposed to these agents for at least 30 s, and then ADP or other nucleotides were applied together with antagonists, again for at least 30 s. As the extent of inhibition of I_{Ca} exerted by nucleotides may vary between different cells, the inhibitory effects of different agonist concentrations, whether in the absence or presence of antagonists, were normalized to the inhibition caused by the very same agonist at 100 μM (relative inhibition of I_{Ca}; Vartian and Boehm, 2001).

To determine voltage-dependence of inhibition, currents were elicited by a double-pulse voltage protocol: cells were clamped at -80mV; every 15 seconds, a 35ms depolarization to 0mV (P1) was applied, followed by a 5 sec waiting period at -80mV, a 30ms prepulse to

+80mV, a 10 ms repolarisation to -80mV to close Ca^{2+} channels, and then another 35 ms depolarization to 0mV (P2).

The extent as well as the concentration dependence of the nucleotide-induced inhibition of I_{Ca} may vary between different batches of PC12 cells (Unterberger et al, 2002; Vartian and Boehm, 2001). Therefore, changes in the concentration response curves for agonists in the presence of increasing antagonist concentrations were investigated in one batch of PC12 cells per one antagonist. All data represent arithmetic means ± s.e.mean; n represent numbers of single neurons in electrophysiological experiments. Concentration-response curves were fitted to experimentally obtained data by the GraphPad Prism 3.1 programme (GraphPad Software, Inc).

2.3.2 M-current in rat SCG

Experiments were performed at room temperature (20-24°C) on the somata of single SCG neurons using the perforated-patch modification of the patch-clamp technique (Rae et al, 1991) which prevents rundown of I_M (see Boehm, 1998). Patch pipettes were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA, USA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany) and front-filled with a solution consisting of (mM) K_2SO_4 (75), KCl (55), $MgCl_2$ (8), and HEPES (10), adjusted to pH 7.3 with KOH. Then, electrodes were back-filled with the same solution containing 200 μg/ml amphotericin B (in 0.8 % DMSO) which yielded tip resistances of 1 to 3 MΩ. The bathing solution contained (mM) NaCl (140), KCl (6.0), $CaCl_2$ (2.0), $MgCl_2$ (2.0), glucose (20), HEPES (10), adjusted to pH 7.4 with NaOH. Tetrodotoxin (TTX; 0.5 μM) was included to suppress voltage-activated Na^+ currents. OxoM, retigabine and all other drugs were applied via a DAD-12 drug application device (Adams & List, Westbury, NY,USA) which permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (see Boehm, 1999). To investigate I_M, cells were held at a potential of -30 mV, and thrice per minute 1 s hyperpolarisations to -55 mV were applied to deactivate K_M channels; the difference between current amplitudes 20 ms after the onset of hyperpolarisations and 20 ms prior to re-depolarisation was taken as a measure for I_M. Amplitudes obtained during the application of test drugs (b) were compared with those measured before (a) and after (c) application of these drugs by calculating 200b / (a+c) = % of control or 100 - (200b/[a+c]) = % inhibition (see Boehm, 1998).

Results are presented as arithmetic means ± standard errors of the mean; n = number of single cells in electrophysiological experiments. Significances of differences between data points were evaluated by the nonparametric Mann-Whitney test, unless indicated otherwise.

2.4 Measurement of [^{3}H]noradrenaline release

[^{3}H]noradrenaline uptake and superfusion were performed as described (Boehm, 1999). Culture discs with dissociated neurons or PC12 cells were incubated in 0.05 µM [^{3}H]noradrenaline (specific activity 52.0 Ci/mmol) in culture medium supplemented with 1 mM ascorbic acid at 36 °C for 1 h. After labelling, culture discs were transferred to small chambers and superfused with a buffer containing (mM) NaCl (120), KCl (6.0), $CaCl_2$ (2.0), $MgCl_2$ (2.0), glucose (20), HEPES (10), fumaric acid (0.5), Na-pyruvate (5.0), ascorbic acid (0.57), adjusted to pH 7.4 with NaOH. Superfusion was performed at 25 °C at a rate of about 1.0 ml/min. Collection of 4 min superfusate fractions was started after a 60 min washout period to remove excess radioactivity.

To investigate noradrenaline release from rat SCG cultures evoked by OxoM, [^{3}H] overflow was induced by the inclusion of this muscarinic agonist in the superfusion buffer for 2 minutes, unless indicated otherwise. Tritium overflow was also elicited by the application of 60 monophasic rectangular electrical pulses (0.5 ms, 60 mA, 50 V/cm) at 1.0 Hz and by the presence of the nicotinic agonist DMPP for 2 min. Modulatory agents, such as TTX, $CdCl_2$, mecamylamine or retigabine were added to, or $CaCl_2$ was omitted from, the buffer after 50 min of superfusion (i.e. 10 min prior to the start of sample collection). The buffer then remained unchanged until the end of experiments.

To investigate the effect of ADP on stimulation-evoked release from PC12 cells, [^{3}H] overflow was induced twice at 72 min and 92 min, respectively, by 30 sec application of superfusion buffer containing 30mM K^+ (NaCl was reduced accordingly to maintain isoosmolarity). Modulatory agents, such as ADP, $CdCl_2$, CGS21680, forskolin or pCPT-cAMP, were added to the buffer as indicated in the figures. The buffer then remained unchanged until the end of experiments.

The radioactivity remaining in the cells after the completion of experiments was extracted by immersion of the discs in 2 % (v/v) perchloric acid followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Packard Tri-Carb 2100 TR). Radioactivity released in response to electrical field stimulation from rat

sympathetic neurons after labelling with tritiated noradrenaline under conditions similar to those of the present study had previously been shown to consist predominantly of the authentic transmitter and to contain only small amounts (≤15%) of metabolites (Schwartz and Malik, 1993). Hence, the outflow of tritium measured in this study was assumed to reflect the release of noradrenaline and not that of metabolites.

The spontaneous (unstimulated) rate of [^{3}H] outflow was obtained by expressing the radioactivity of a collected fraction as percentage of the total radioactivity in the cultures at the beginning of the corresponding collection period. Stimulation-evoked tritium overflow was calculated as the difference between the total [^{3}H] outflow during and after stimulation and the estimated basal outflow which was assumed to decline linearly throughout experiments. Therefore, basal outflow during periods of stimulation was assumed to equate to the arithmetic mean of the samples preceding and those following stimulation, respectively. The difference between the total and the estimated basal outflow was expressed as a percentage of the total radioactivity in the cultures at the beginning of the respective stimulation (% of total radioactivity; S%). The amount of OxoM-evoked tritium release may vary considerably between different SCG preparations. Therefore, tritium overflow in the presence of release modulating agents, such TTX, $CdCl_2$ or retigabine, was always compared with that obtained within the same SCG preparation in the absence of these drugs. To directly compare effects of different modulatory agents upon electrically and OxoM-evoked overflow, respectively, the values obtained in the presence of these modulators were expressed as percentage of the corresponding control values within the same preparation.

Results are presented as arithmetic means ± standard errors of the mean; n = number of cultures in release experiments. Significances of differences between data points were evaluated by the nonparametric Mann-Whitney test, unless indicated otherwise.

2.5 Subcloning of $G\beta_1\gamma_2$ into pIRES

$G\beta_1$ and $G\gamma_2$ were subcloned from pcDNA3 constructs, containing either $G\beta_1$ or $G\gamma_2$, into the biscistronic pIRES vector (BD Biosciences Clontech; Palo Alto, CA, USA) for stable expression in PC12 cells. The pIRES vector contains two multiple cloning sites (MCS A, MCS B) that are located on either side of the internal ribosomal entry site (IRES) from the encephalomyocarditis virus (ECMV), which allows translation of two consecutive open reading frames from the same messenger RNA.

$G\gamma_2$ was cut out of the pcDNA3 construct using EcoRI giving a fragment of approximately 450 base pairs. The pIRES plasmid was cut in MCS-A using EcoRI and subsequently the 5′-ends were dephosphorylated with shrimp alkaline phosphatase (SAP) to avoid re-ligation. $G\gamma_2$ was ligated into pIRES using rapid DNA ligation kit (Roche). 2µl of the ligation-reaction were used for the transformation of competent *E.Coli* cells. Since the γ_2-fragment and the vector had EcoRI restriction sites on both ends, the γ_2-insert could be ligated into the vector in two different orientations (I or II see figure 2-2). To determine which of the *E.Coli* colonies were transformed with a plasmid containing γ_2 in the correct orientation, colonies were screened with PCR, using a vector and an insert specific primer (vector specific-γ2fwd: 5′-TAA TAC GAC TCA CTA TAG GC-3'; insert specific γ2rev: 5'-GGA TGG TTC TCC TCC CTC TC-3'). Hence only positive colonies should give a PCR product of approximately 500bp (see figure 2-2).

PCR was performed as follows using "expand high fidelity PCR system" (roche):

- colonies were picked with a sterile pipette tip and transferred into 5µl H2O.
- 15µl of the following PCR reaction-mix were added:

1×	PCR-buffer
1.5mM	MgCl2
300nM	γ2fwd-primer
300nM	γ2rev-primer
200µM	of each dNTP
1Unit	Taq/Tgo DNA polymerase mix

- PCR was performed according to the following parameters:

1 cycle	2min/94°C
25 cycles	30sec/94°C – 30sec/52°C – 1min/72°C
1cycle	5min/72°C

PCR products were separated on a 1.5% agarose-gel containing ethidium-bromide (1.5% agarose; 0.1M Tris; 2.5mM EDTA; 0.1M boric acid). Plasmid DNA from two colonies that were tested positive by PCR was purified with HiSpeed Plasmid Maxi Kit (qiagen) according to the manufacturer's protocol and the result from PCR was confirmed by sequencing (VBC-genomics, Vienna).

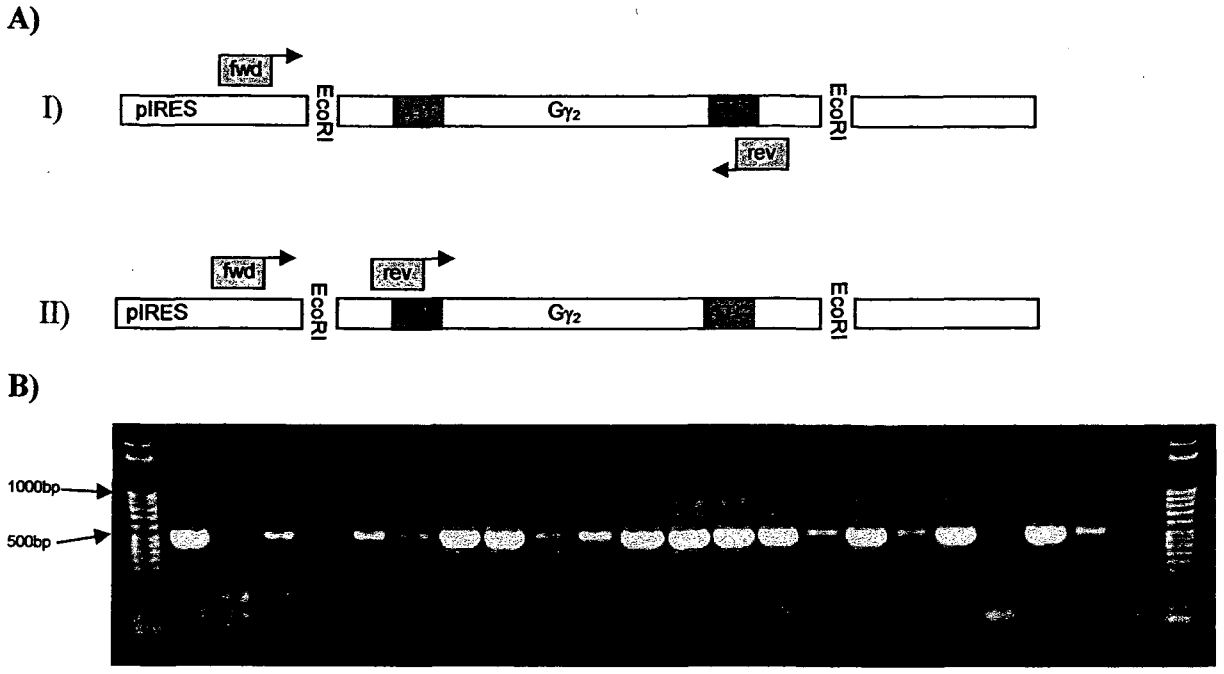

Figure 2-2 , *Bacterial colonies were screened by PCR*
(A) $G\gamma_2$ can be ligated into pIRES in two different orientations (I and II).Start and stop-codons are highlighted in green and red, respectively, indicating that only construct-I can be expressed to give a functional protein. Forward and reverse primers are highlighted in grey, indicating that PCR only works with construct-I. (B) In 10 out of 22 colonies that were screened, a PCR product of ~ 450 bp was detected, indicating that these colonies contained construct-I.

β_1 was isolated from the pcDNA3-β_1 construct using PCR in order to generate an additional XbaI restriction site on the 5'-end of the resulting β_1-fragment (see figure 2-3). PCR was performed as follows:

1×	PCR-buffer
1.5mM	MgCl2
10ng	pcDNA3-β1
300nM	β1-fwd primer (5'-CACTCTAGAGAGACCCAAGCTTGGT-3'
300nM	β1-rev primer (5'-TGATCAGCGAGCTCTAGCAT-3')
200µM	of each dNTP
1Unit	Taq/Tgo DNA polymerase mix

temperature profile:

1 cycle	2min/94°C
25 cycles	30sec/94°C – 30sec/52°C – 1min/72°C
1cycle	5min/72°C

The purified β_1-fragment (agarose gel 1.5%; GFX gel band purification kit, Amersham biosciences) and the pIRES-γ_2 construct were digested with XbaI, ligated as described above and transformed into competent *E.Coli* cells. Again two different orientations of β1 were possible (see figure 2-3 I and II). Plasmid DNA was isolated from bacterial colonies by

miniprep and screened by restriction digest with EcoRI (see figure 2-3C). Since the β1-fragment contains an EcoRI site near its 5'-end (see figure 2-3B), digest of the functional construct should give the following two fragments: a ~ 450bp fragment (γ_2) and a ~750bp fragment containing the IRES sequence that separates MCS-A and MCS-B. By contrast digest of the non-functional construct should give a ~450bp fragment (γ_2) and a ~ 1700bp fragment containing the IRES sequence and β_1.

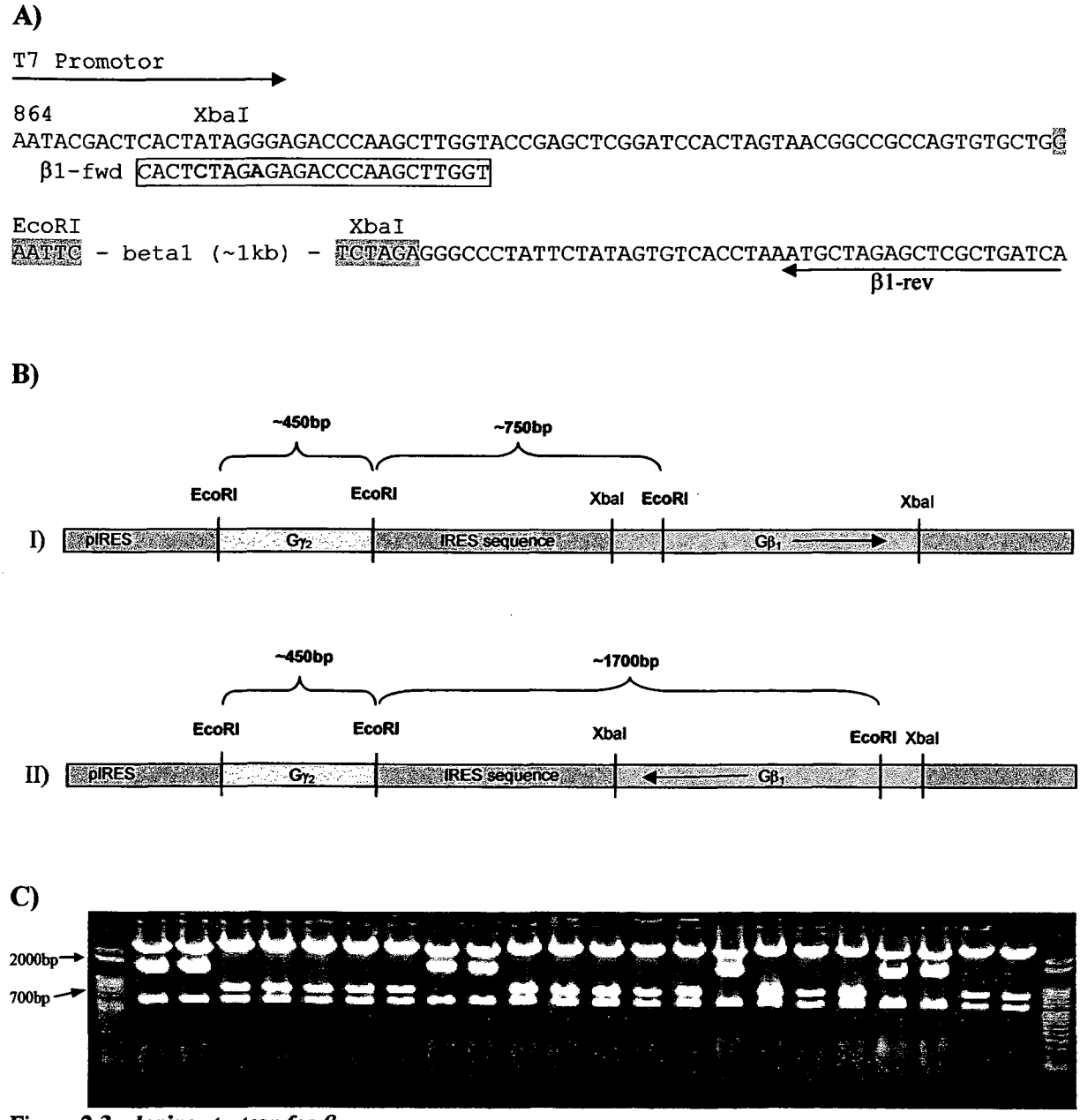

Figure 2-3, *cloning strategy for β_1*

(A) Sequence of the multiple cloning site in pcDNA3. The β1-fwd primer contains two mismatches (highlighted in red) in order to create an XbaI restriction site. The second XbaI site downstream of β_1 and the EcoRI site upstream of β_1 are highlighted in grey. (B) $G\beta_1$ can be ligated into pIRES-γ_2 in two different orientations (I and II). EcoRI digest of construct I gives 450bp and 750bp fragments respectively. By contrast, digest of construct II gives a 1700bp instead of the 750bp fragment. (C) 15 out of 22 colonies were tested positive for construct I as seen on the agarose gel.

2.5.1 Dephosphorylation/ligation

In order to dephosphorylate 5'-ends of linearized plasmids, ~50ng vector DNA were incubated with 1Unit SAP (shrimp alkaline phosphatase, Roche diagnostics GmbH, Mannheim, Germany) at 37°C for 10 minutes. After dephosphorylation SAP was inactivated at 65°C for 15 minutes. Insert DNA was added to the dephosphorylated vector at a molar ratio of 2:1 and ligated with rapid DNA ligation kit® (Roche diagnostics GmbH, Mannheim, Germany) for 10 minutes at room temperature. The ligation reaction was either stored at -20°C or immediately used for transformation.

2.5.2 Transformation of competent E.Coli

One-shot-Top10F'® competent *E.Coli* cells (invitrogen) were used for transformation. 50µl aliquots of the cells were thawed on ice. 2µl of the ligation reaction were added directly to the competent cells and mixed by gently tapping the vial. The DNA-*E.Coli* mixture was then incubated on ice. After 30 minutes on ice, cells were incubated at 42°C for 30 seconds, and subsequently 250µl of pre-warmed SOC-medium were added. The cells were then placed in a thermo-mixer at 37°C and 225 rpm. After 1 hour of incubation in the thermo-mixer, 10µl to 50µl of the transformed *E.coli* suspension were spread on LB/ampicillin-agar plates. The inverted plates were then incubated at 37°C overnight.

SOC-medium:

20g	bacto-tryptone
5g	yeast extract
0,5g	NaCl
3,6g	glucose
ad 1000ml	H_2O

2.5.3 Plasmid-miniprep (boiling prep)

Bacterial colonies were inoculated into 2ml LB-medium and incubated over night at 37°C/300rpm. 1,5 ml of the suspension cultures were harvested by centrifugation at 12000×g, resuspended in 300µl STETL-buffer (see below) and incubated on ice for 2 minutes. Samples were then placed in a water bath at 95°C for 2 minutes and then put back on ice for one minute. Thereafter, the bacterial lysate was centrifuged at 12000×g for 15 minutes and the resulting pellet was removed with a sterile toothpick. Plasmid DNA was precipitated by addition of 300µl isopropanol, collected by centrifugation (20min, 12000×g) and washed twice with 70% ethanol.

LB-medium:

10g	bacto-tryptone
5g	yeast extract
10g	NaCl
ad 1000ml	H_2O

STET-Buffer:

4g	sucrose
2.5ml	Triton X 100
2.5ml	Tris-HCl (1M, pH 8)
5ml	EDTA (0.5M, pH 8)
ad 50ml	H2O

STETL-Buffer:

1ml	STET
10µl	Lysozyme (50mg/ml)

2.6 Stable transfection of PC12 cells

Dishes (10cm ø) with approximately 80% confluent PC12 cells were prepared the day before transfection. 15µg plasmid DNA and 90µl TransFast™ (Promega corp., Madison, WI, USA) were mixed with 6ml OptiMEM complete medium without serum and incubated for 15 minutes at room temperature. The mixture was vortexed every 5 minutes to promote formation of cationic liposomes containing the plasmid. Growth medium was removed from PC12 cells and 6ml transfection mixture was added. Cells were incubated at 37°C for one hour and then gently overlaid with 12 ml complete medium containing 10% horse and 5% fetal calf serum. PC12 cells were kept in transfection medium for 48 hours and were then plated in selection medium (Optimem complete + 10 % horse serum + 5 % fetal calf sermon + 600µg/ml G418). The use of selection medium was continued for 3-5 weeks, with frequent changes of medium to remove dead cells and debris, until distinct colonies could be visualized. Colonies were picked by sucking them up with 50µl pipette and transferred in to 48 well dishes.

2.7 Materials

(-)-[7,8-^{3}H]noradrenaline was obtained from NEN (Vienna, Austria); amphotericin B, OxoM, pirenzepine, mecamylamine, bethanechol, and thapsigargin from Sigma (Vienna, Austria); TTX from Latoxan (Rosans, France); muscarinic toxin 7 (MT-7) from the Peptide Institute Inc. (Osaka, Japan); bulk chemicals were from Merck (Vienna, Austria). Retigabine was

kindly donated by Dr. B. Pechstein, Viatris, Frankfurt/Main, Germany. Water-insoluble drugs were first dissolved in DMSO and then diluted into buffer to yield final DMSO concentrations of up to 0.1% which were also included in control solutions. At these concentrations, DMSO did not affect any of the parameters investigated.

[2,8-^{3}H]adenine (specific activity 32 Ci mmol^{-1}) was obtained from NEN (Vienna, Austria). Na-ADP, Mg-ATP, Li-GTP, 4-(3-butoxy-4-methoxybenzyl)imidazoline-2-one (RO 20-1724), 3',5'-cyclic AMP, and adenosine 5'-O-(2-thiodiphosphate) (ADPβS) from Sigma (Vienna, Austria); 2-methylthio-AMP and -ADP, as well as 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS 21680) from RBI (Natick, MA, USA); tetrodotoxin from Latoxan (Rosans, France). 2-Chloro-N^{6}-methyldeoxyadensoine 3',5'-biphosphate (MRS 2216) was a gift of Dr. K.A. Jacobson (NIDDK, Bethesda, MD, USA). N^{6}-(2-methyl-thioethyl)-2-(3,3,3-trifluoropropylthio)-β,γ-dichloromethylene-ATP (AR-C69931MX) was kindly provided by AstraZeneca R&D Charnwood (Loughborough, UK). All primers were synthesized at VBC-genomics (Vienna, Austria).

3 Results

3.1 PART I – Muscarinic acetylcholine receptors

3.1.1 OxoM stimulates noradrenaline release and inhibits I_M with different concentration dependences.

To evaluate whether activation of muscarinic receptors may stimulate transmitter release from sympathetic neurons, cultures of dissociated rat SCG were loaded with [^{3}H]noradrenaline, superfused with a physiological buffer, and exposed to various concentrations of OxoM for 2 minutes. Figure 3-1A shows that 1 to 100 μM OxoM stimulate the release of increasing amounts of radioactivity. This effect reached a maximum at 30 to 100 μM where 2.64 ± 0.28 % (n = 9) of the cellular radioactivity were released by the muscarinic agonist. At these concentrations, OxoM also reduced currents through K_M channels, as evaluated by the reduction of I_M deactivation amplitudes (Figure 3-1C). This second effect reached a maximum of 85.3 ± 5.0 % (n = 6) inhibition at 10 μM. The two effects, stimulation of tritium release and inhibition of I_M, showed clearly diverging concentration dependences, as half-maximal effects were observed at 10 μM and 1 μM, respectively (Figure 3-1 B and D).

3.1.2 The stimulation of noradrenaline release by OxoM does not involve nAChRs.

OxoM has been found to activate nAChRs in Xenopus myocytes (Reitstetter et al, 1994) and in guinea pig celiac ganglion neurons (Xian et al, 1994). To verify whether nAChRs might contribute to the secretagogue action of OxoM in rat sympathetic neurons, tritium overflow was stimulated by electrical fields, OxoM (100 μM), and DMPP (100 μM) in either the absence or presence of 10 μM of the nicotinic antagonist mecamylamine (Figure 3-2A and B). Electrically evoked overflow was not affected by mecamylamine, whereas the overflow due to DMPP was virtually abolished. The release stimulating action of OxoM was somewhat reduced in the presence of mecamylamine, but this effect was not statistically significant ($p > 0.05$).

To reveal whether OxoM might at all be able to activate nAChRs in sympathetic neurons of the rat, currents through these receptors were determined. As shown in Figure 3-2 (C to E), OxoM did induce currents which were abolished by mecamylamine. However, to activate these currents concentrations of ≥100 μM OxoM were required (Figure 3-2 D), and currents induced by 100 μM OxoM amounted to only 4.9 ± 3.7 % of the currents evoked by 100 μM DMPP (Figure 3-2E). Thus, activation of nAChRs by OxoM does not occur at concentrations lower than 100 μM, and even at 100 μM, does not significantly contribute to the release stimulating action of OxoM.

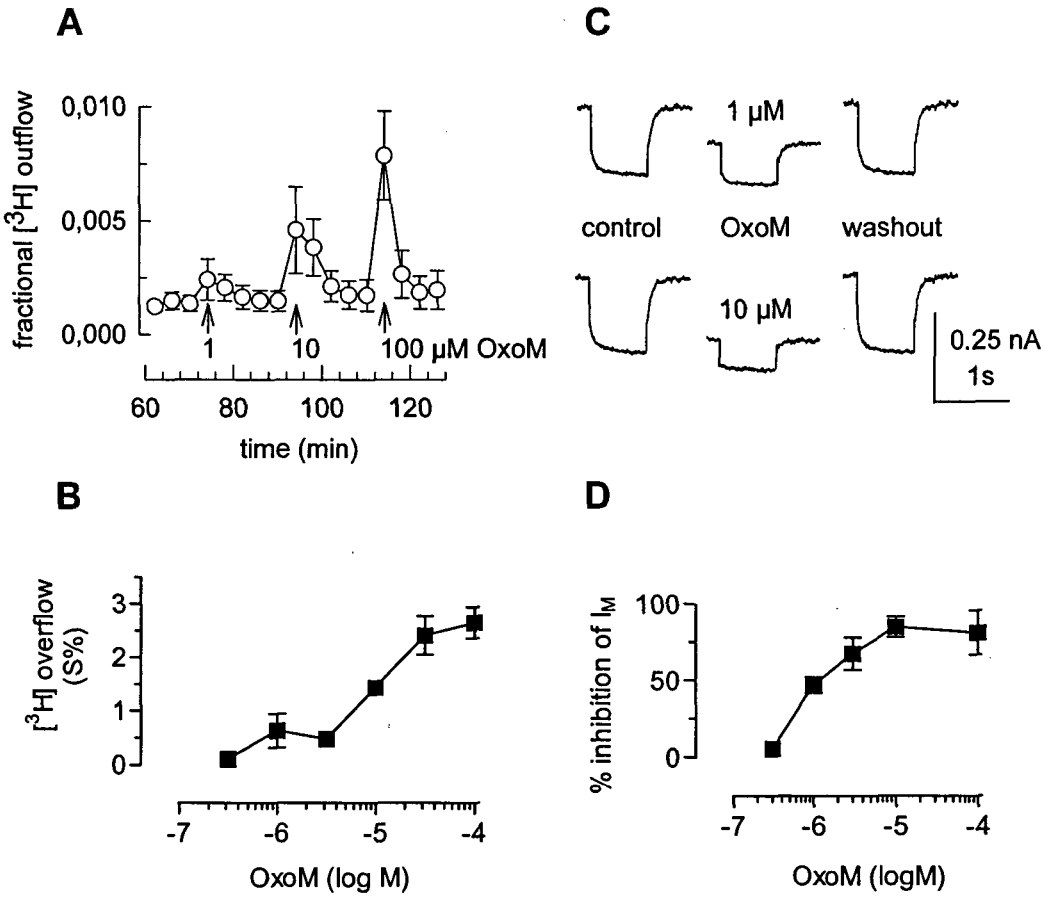

Figure 3-1. OxoM stimulates noradrenaline release and inhibits I_M in rat SCG neurons.

A, Primary cultures of rat SCG neurons were labelled with [³H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. 1, 10 and 100 µM OxoM were applied as indicated by the arrows, each for 2 min. The graph shows the time course of fractional [³H] outflow per min; n = 6. ***B*** *shows the concentration-dependence of tritium overflow evoked by the indicated concentrations of OxoM, applied as shown in A. OxoM-induced overflow is depicted as S% (% of total radioactivity; see Methods); n = 9.* ***C****, I_M was measured using the amphotericin B-perforated patch technique in a SCG neuron. The current traces shown were obtained by clamping the cell at –30 mV and by applying 1 s hyperpolarizing voltage steps to – 55 mV. The recordings were performed before (control), during (OxoM) and after (washout) the application of 10 or 100 µM OxoM.* ***D****, Concentration-dependence of the inhibition of I_M relaxation amplitudes by OxoM in experiments performed as shown in C; n = 6.*

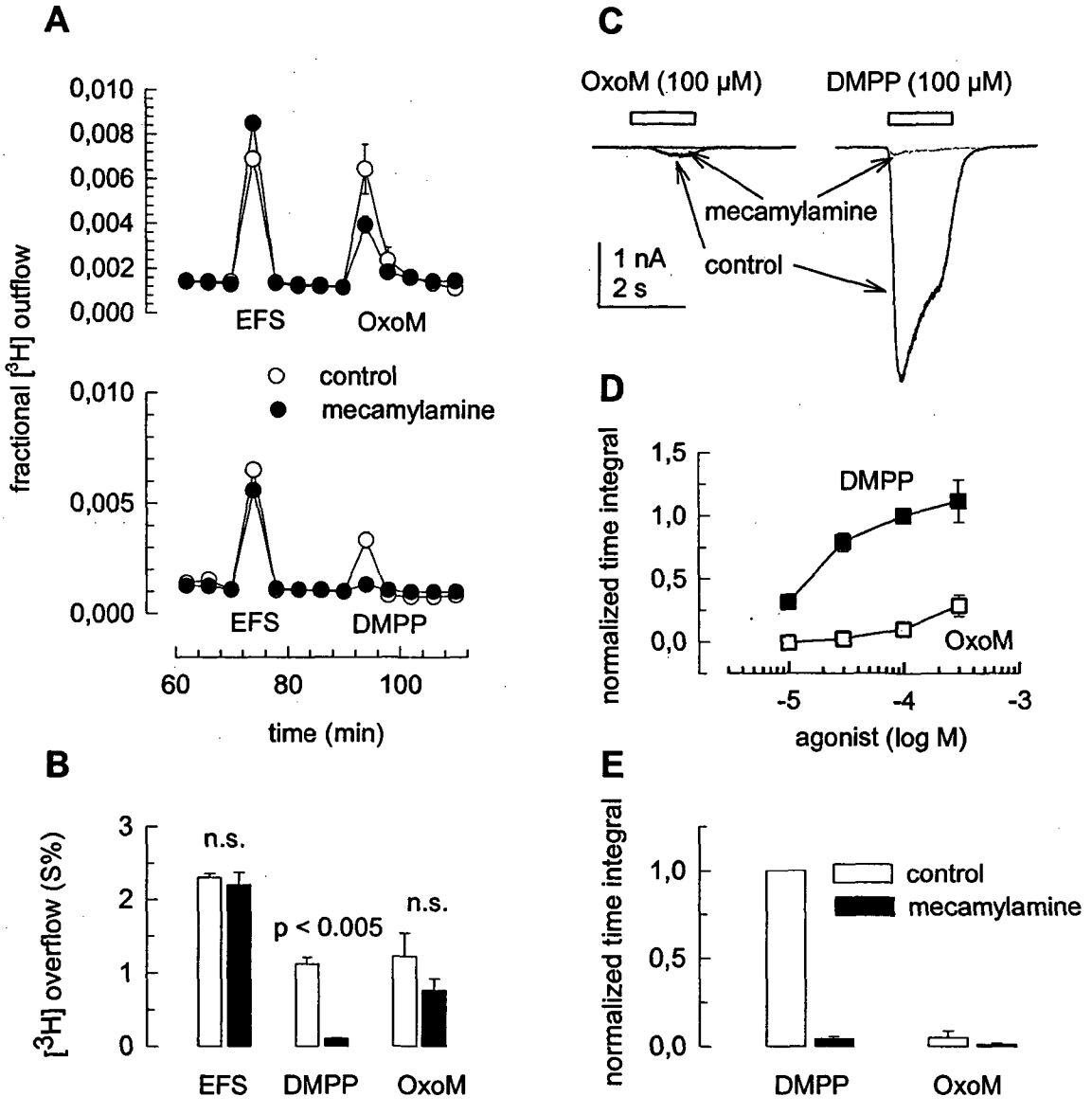

Figure 3-2. The stimulation of noradrenaline release by OxoM does not involve nAChRs.
A, Cultures were labelled with [³H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Tritium overflow was evoked by electrical field stimulation (EFS; 60 pulses, 1 Hz) starting at 72 min of superfusion and by application of either 100µM OxoM or 100µM DMPP for 2 min starting at 92 min of superfusion. 10 µM Mecamylamine were absent (○) or present (●) from min 50 of superfusion. The graph shows the time course of fractional [³H] outflow per min; n = 3. B shows the amount of tritium overflow (S%) induced by electrical field stimulation (EFS), 100 µM DMPP, or 100 µM OxoM in the absence (white bars) or presence (black bars) of 10 µM mecamylamine; n = 6 to 9. P values for the significance of difference between the results obtained in the absence and presence of mecamylamine are indicated above the bars; n.s. indicates no significant difference. C, The current traces shown, were recorded in a SCG neuron using the amphotericin B-perforated patch technique. The cells were clamped at –70 mV and currents were elicited by 1.5 s applications of either OxoM or DMPP (as indicated by the bars) in the absence (control) or presence of 10 µM mecamylamine as indicated by the arrows. D shows the concentration-dependence of time integrals of currents elicited by increasing concentrations of either OxoM or DMPP, recorded as described in C. Values obtained with different agonist concentrations were normalized to those obtained in the very same neuron with 100 µM DMPP (normalized time integral); n = 6. E shows the normalized time integral (determined as in D) of currents evoked by 100 µM DMPP or 100 µM OxoM in the absence (white bars) or presence (black bars) of 10 µM mecamylamine; n = 5.

3.1.3 *Pertussis toxin potentiates the stimulation of noradrenaline release by OxoM.*

Muscarinic receptors may be linked to proteins of either the Gi or Gq family (Caulfield and Birdsall, 1998). To investigate which types of G proteins are involved in the stimulation of tritium overflow by OxoM, cultures were treated with pertussis toxin (PTX; 100 ng/ml for 24 h) which abolishes signalling via the Gi protein family. In PTX-treated cultures, electrically evoked overflow remained unaltered, but OxoM-induced overflow was enhanced fivefold (Figure 3-3A and B). This indicates that OxoM-induced noradrenaline release is restricted by a mechanism that involves PTX-sensitive G proteins. In a separate series of experiments, SCG neurons were subsequently stimulated with 100 µM DMPP and 100 µM OxoM in either untreated or PTX-treated cultures. Due to the PTX-treatment, the tritium overflow triggered by OxoM was enhanced from 0.99 ± 0.21 to 5.68 ± 0.69 % of total radioactivity (S%; n = 6; $p < 0.01$), but the overflow induced by the nicotinic agonist DMPP remained unchanged (0.99 ± 0.12 % of total radioactivity in untreated and 0.86 ± 0.15 % of total radioactivity in PTX-treated cultures; n = 6; $p > 0.3$). This indicates that noradrenaline release induced by OxoM is restricted by a mechanism that involves PTX-sensitive G proteins, whereas other types of stimulation-evoked release are not.

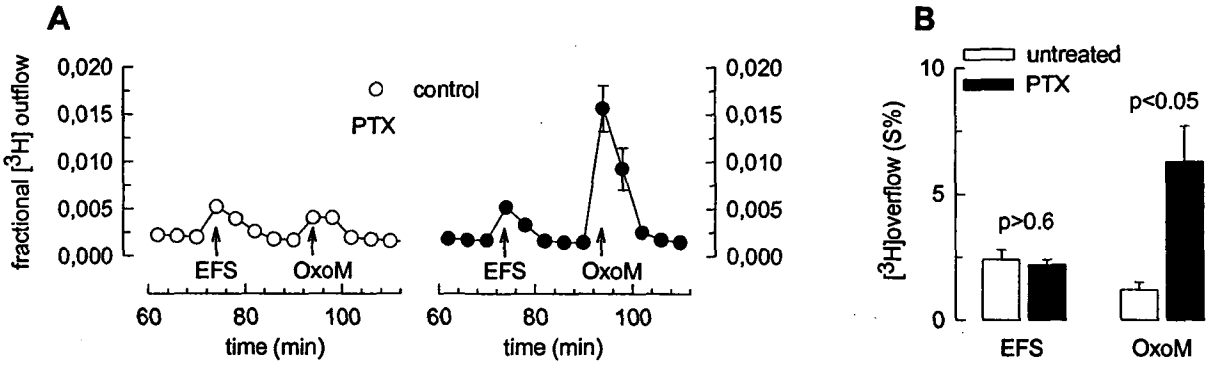

Figure 3-3. Pertussis toxin potentiates the stimulation of noradrenaline release by OxoM.
*A, Cultures were treated with 100 ng/ml pertussis-toxin (PTX) for 24h (•) or remained untreated (○). Thereafter, the cultures were labelled with [³H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Tritium overflow was evoked by electrical field stimulation (EFS; 60 pulses, 1 Hz) starting at 72 min of superfusion and by the application of 10 µM OxoM for 2 min starting at 92 min of superfusion. The graphs show the time course of fractional [³H] outflow per min; n = 3. **B** shows the amount of tritium overflow (S%) induced by electrical field stimulation (EFS) or 10 µM OxoM in untreated (white bars) or PTX-treated (black bars) cultures; p-values for the significances of differences between PTX-treated and non-treated cultures are indicated above the bars; n = 7 to 9.*

3.1.4 *Concentration-dependence and basic mechanisms of noradrenaline release induced by OxoM.*

To reveal whether this inhibitory PTX-sensitive mechanism might affect the concentration-dependence of OxoM-induced noradrenaline release, SCG cultures were treated with PTX or remained untreated, were superfused with a physiological buffer, and were exposed to various concentrations of OxoM for 2 minutes. In untreated cultures, the release stimulating effect of OxoM was half maximal at 10.9 $\pm$ 8.2 µM and reached a maximum of 3.1 $\pm$ 0.6 % of the total radioactivity (S%). In PTX-treated cultures, the maximal effect of OxoM was markedly enhanced and amounted to 8.0 $\pm$ 0.7 % of total radioactivity (S%). Furthermore, the concentration-response curve was shifted to the left, and the half maximal concentration was 2.1 $\pm$ 0.5 µM (Figure 3-4A). Thus, in PTX-treated neurons OxoM stimulates noradrenaline in the same range of concentrations as it inhibits I_M (see Figure 3-1D).

To investigate the mechanisms underlying the release stimulating action of OxoM in isolation, all subsequent radiotracer release experiments were performed in PTX-treated cultures. In the absence of extracellular Ca^{2+}, neither electrical field stimulation nor OxoM were able to induce tritium overflow (Figure 3-4B and C). Likewise, in the presence of 0.5 µM TTX to prevent action potential propagation, neither stimulus produced tritium overflow. And finally, the blockade of voltage-gated Ca^{2+} channels by 100 µM Cd^{2+} also abolished electrically as well as OxoM-induced [^{3}H] overflow (Figure 3-4C). Thus, OxoM triggers noradrenaline release via the same mechanisms as electrical field stimulation: generation of action potentials and subsequent transmembrane Ca^{2+} influx through voltage-gated Ca^{2+} channels.

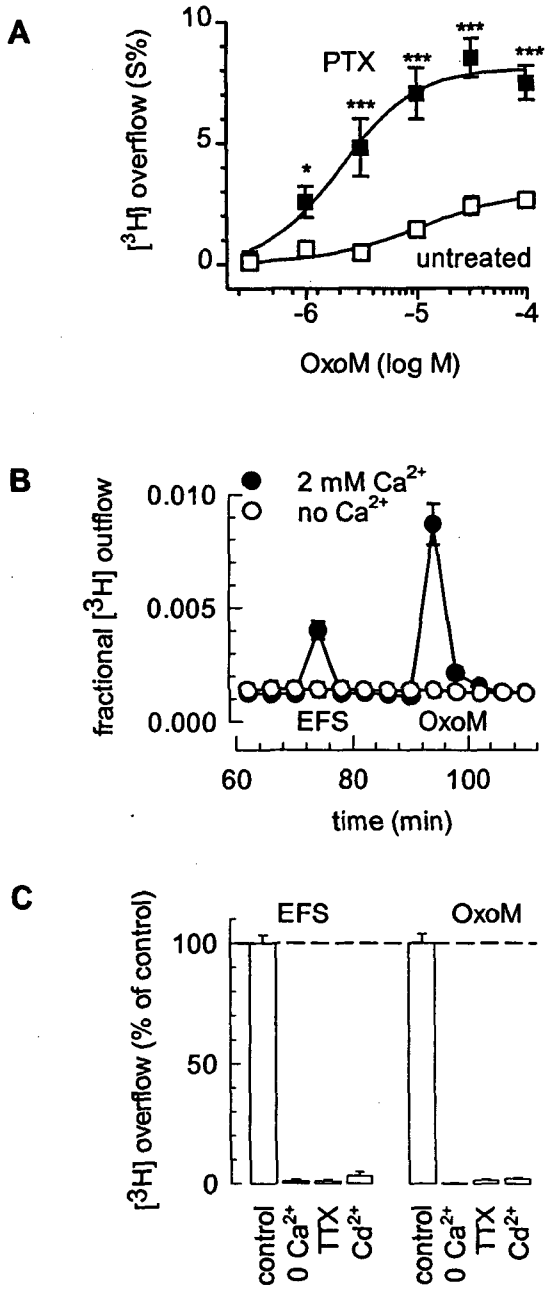

Figure 3-4. Concentration-dependence and basic mechanisms of noradrenaline release induced by OxoM. *Cultures were treated with 100 ng/ml pertussis-toxin (PTX) for 24h (●■ in A and all data in B and C) or remained untreated (□ in A). Thereafter, the cultures were labelled with [³H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. A shows the concentration-dependence of tritium overflow (S%) evoked by the indicated concentrations of OxoM (each applied for 2 min as shown in figure 3-1A) in cultures treated PTX for 24h (■) or in untreated cultures (□); n = 9; * $p<0.05$, *** $p<0.001$ vs the amount of tritium overflow in untreated cultures. B, From min 50 of superfusion onward, the buffer contained either 2 mM Ca^{2+} or no Ca^{2+}. Tritium overflow was evoked by electrical field stimulation (EFS; 60 pulses, 1 Hz) starting at 72 min of superfusion and by the application of 10 µM OxoM for 2 min starting at 92 min of superfusion. The graphs show the time course of fractional [³H] outflow per min; n = 3. C, From min 50 of superfusion onward, the buffer contained either no Ca^{2+} or 2 mM Ca^{2+}, either alone (control) or together with 1 µM tetrodotoxin (TTX) or 100 µM Cd^{2+}. Tritium overflow was evoked by electrical field stimulation (EFS) and OxoM as shown in B. The graph shows the amount of tritium overflow (S%) under the various conditions expressed as percentage of the overflow under control conditions; n = 6.*

3.1.5 *One mAChR subtype mediates both, the inhibition of I_M and the stimulation of noradrenaline release by OxoM.*

The muscarinic receptor that mediates the inhibition of I_M has been reported to belong to the M_1 subtype (Marrion et al, 1989; Bernheim et al, 1992). To investigate whether the induction of transmitter release by OxoM is mediated by the same receptor subtype, concentration response curves for both types of effect were obtained in the absence and presence of the M_1 preferring mAChR antagonist pirenzepine. OxoM inhibited I_M (in untreated neurons) and evoked noradrenaline release (in PTX-treated cultures) with half maximal effects at 0.8 ± 0.5 μM and 3.2 ± 0.7 μM, respectively. In the presence of pirenzepine (0.1 μM), both concentration-response curves were shifted to the right, and the half-maximal concentrations amounted to 3.9 ± 2.0 μM and 10.8 ± 14 μM, respectively (Figure 3-5A and B). Assuming an underlying competitive mechanism, one can calculate a pA_2 value for pirenzepine for these two types of effect by applying the equation $\log (CR-1) = pA_2 + \log [B]$ (where CR is the ratio of equieffective agonist concentrations in the presence and absence of the antagonist concentration B, respectively; Arunlakshana and Schild, 1959). These pA_2 values were 7.6 for the inhibition of I_M and 7.4 for the stimulation of noradrenaline release. Thus, pirenzepine interfered with the OxoM-dependent inhibition of I_M in the same range of concentrations as with the stimulation of noradrenaline release by OxoM.

In order to further characterize the receptor mediating these two types of effect, cultures were incubated in MT-7 (100 nM for 60 min) which selectively and irreversibly blocks M_1 mAChRs (Olianas et al, 2000). In cultures treated with MT-7, OxoM (10 μM) failed to cause significant changes in I_M and to evoke noradrenaline release. In sister cultures not treated with MT-7, however, OxoM clearly reduced I_M relaxation amplitudes and stimulated noradrenaline release (Figure 3-5C and D). In an additional set of experiments, the actions of OxoM were compared with those of the muscarinic agonist bethanechol which has been shown to selectively activate M_2 mAChRs in rat SCG neurons (Liu and Rittenhouse, 2003). While OxoM (10 μM) unequivocally reduced I_M relaxation amplitudes and stimulated tritium overflow, bethanechol (100 μM) failed to mimic either of these two effects (Figure 3-5E and F). Nevertheless, bethanechol (100 μM) did reduce electrically evoked tritium overflow by 16.9 ± 3.1 % ($n = 6$; $p < 0.09$) as shown above for 10 μM OxoM (Figure 3-3).

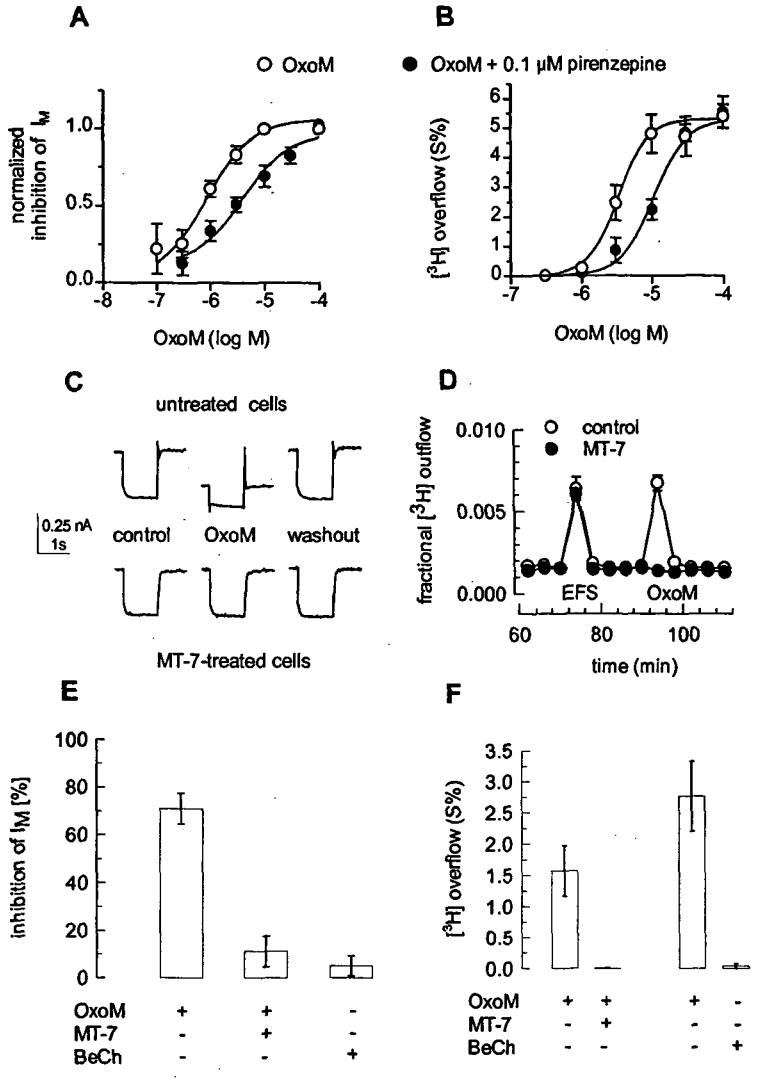

Figure 3-5. Characterization of the receptor mediating the stimulation of noradrenaline release and the inhibition of I_M by OxoM.

I_M was recorded from rat SCG neurons using the amphotericin B-perforated patch technique, and currents traces were obtained by clamping the cell at –30 mV and by applying 1 s hyperpolarizing voltage steps to –55mV once every 20 s (A, C, E). Alternatively, cultures were labelled with [^{3}H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected, and tritium overflow was evoked by electrical field stimulation (EFS; 60 pulses, 1 Hz) or by 2 min applications of the indicated concentrations of OxoM (B, D, F). A shows the concentration dependence of the inhibition of I_M by OxoM applied either alone (○) or in the continuing presence of 0.1 µM pirenzepine (●). The reduction of I_M relaxation amplitudes by various OxoM concentrations together with or without pirenzepine were normalized to the reduction achieved by 10 µM OxoM in the very same neuron; n = 3 – 11. B shows the concentration dependence of tritium overflow evoked by OxoM applied either alone (○) or together with 0.1 µM pirenzepine (●); n = 6. C shows representative current traces for the inhibition of I_M by 10 µM OxoM in neurons pretreated with 100 nM MT-7 for 1h (lower traces) and in untreated neurons (upper traces). D, cultures were treated with 100 nM MT-7 (●) during the labelling with [^{3}H]noradrenaline or remained untreated (○). Tritium overflow was evoked by electrical field stimulation (EFS; 60 pulses, 1 Hz) starting at 72 min of superfusion and by the application of 10 µM OxoM for 2 min starting at 92 min of superfusion. The graph show the time course of fractional [^{3}H] outflow per min; n = 3. E shows the inhibition of I_M by 10 µM OxoM or 100 µM bethanechol (BeCh) in untreated neurons or in neurons treated with 100 nM MT-7 for 1 h; n = 5 to 8. F, shows the amount of tritium overflow (S%) stimulated by 10 µM OxoM or 100 µM bethanechol (BeCh) in untreated cultures or in cultures treated with 100 nM MT-7 for 1 h; n = 10 to 12.

3.1.6 *Retigabine interferes with both, the inhibition of I_M and the stimulation of noradrenaline release by OxoM.*

The anticonvulsant retigabine has been shown to selectively enhance I_M in SCG neurons by shifting the voltage-dependent gating of K_M channels to more negative potentials (Tatulian et al, 2001). When retigabine was used in radiotracer release experiments, it suppressed the release stimulating effect of OxoM in a concentration-dependent manner, but left electrically evoked tritium overflow unaltered (Figure 3-6A). The inhibition of OxoM-induced [^{3}H] overflow was half maximal at 0.1 μM retigabine, whereas the overflow due to electrical stimulation was not affected by retigabine concentrations as high as 10 μM (Figure 3-6B).

To analyse the mechanisms of the functional antagonism between retigabine and OxoM with respect to noradrenaline release, both agents were also applied in perforated-patch recordings of I_M. As to be seen in the original recordings shown in figure 3-6C, OxoM reduced holding currents determined at a potential of –30 mV from 389.8 ± 68.8 pA to 146.6 ± 37.2 pA ($n = 7$; $p < 0.01$, paired t-test), on one hand, and the I_M relaxation amplitudes due to hyperpolarisation of the neurons to –55 mV from 120.0 ± 23.9 pA to 44.0 ± 15.8 pA ($n = 7$; $p < 0.001$, paired t-test), on the other hand. Retigabine (10 μM) augmented outward holding currents at –30 mV from 389.8 ± 68.8 pA to 661.1 ± 122.6 pA ($n = 7$; $p < 0.01$, paired t-test), but reduced I_M relaxation amplitudes (120.0 ± 23.9 pA before and 68.6 ± 12.4 pA in the presence of retigabine; $n = 7$; $p < 0.01$, paired t-test). In the presence of retigabine (10 μM), OxoM (10 μM) still reduced the holding currents at –30 mV from 661.1 ± 122.6 pA to 385.2 ± 89.6 pA ($n = 7$; $p < 0.01$, paired t-test), but failed to alter the relaxation amplitudes (68.6 ± 12.4 pA in the presence of retigabine alone and 76.6 ± 25.8 pA in the presence of retigabine plus OxoM; $n = 7$; $p > 0.5$, paired t-test). In order to resolve these apparent inconsistencies, slow ramp hyperpolarisations from –25 to –100 mV were applied in the absence and presence of retigabine. As shown in figure 3-6D, retigabine shifted the current-voltage relation of outward currents by about 20 mV to the left. Accordingly, hyperpolarisations from –30 to –55 mV are close to the activation threshold of I_M in the absence of retigabine and will thus lead to the closure of the channels. In the presence of retigabine, however, the activation threshold is at around –80 mV and, thus, at potentials of –55 mV K_M channels are far from closing. Therefore, the interaction between retigabine and OxoM with respect to I_M was further evaluated by changes in the holding current at –30 mV. Retigabine enhanced these holding currents in the absence and presence of OxoM with similar concentration-dependence, and in the presence of 10 μM retigabine plus 10 μM OxoM, the holding currents were identical to

those in the absence of both agents (Figure 3-6E). Thus, the inhibitory effect of OxoM on I_M was counteracted by increasing concentrations of retigabine.

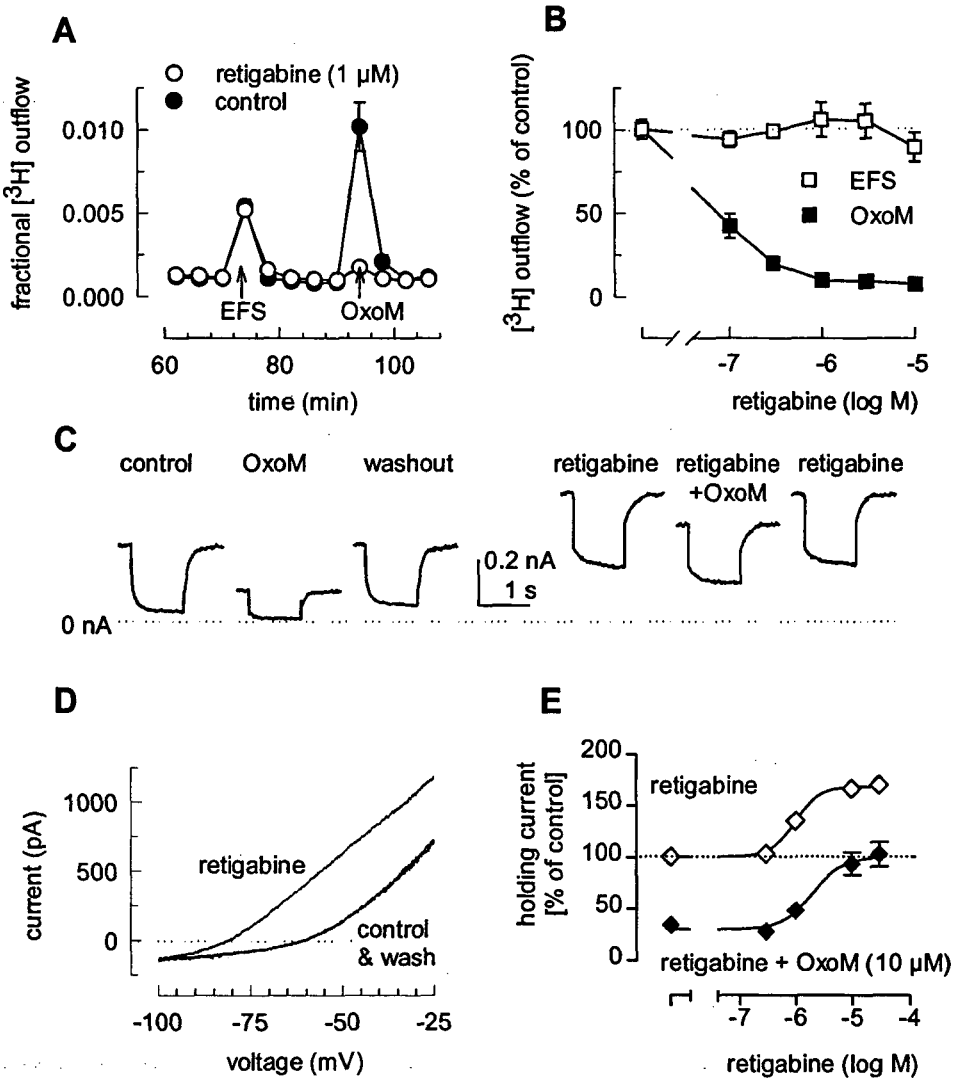

Figure 3-6. Effects of retigabine on noradrenaline release and on I_M.

A, cultures were labelled with [^{3}H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Tritium overflow was evoked by electrical field stimulation (EFS; 60 pulses, 1 Hz) starting at 72 min of superfusion and by the application of 10 µM OxoM for 2 min starting at 92 min of superfusion. From min 50 of superfusion onward, the buffer contained either the indicated concentrations of or no retigabine. A shows the time course of fractional [^{3}H] outflow per min in the absence (•) or presence (○) of 1 µM retigabine; n = 6. B shows the concentration-dependence of the effects of retigabine on [^{3}H]noradrenaline release induced by electrical field stimulation (EFS) or 10 µM OxoM. Tritium overflow in the presence of retigabine is depicted as percentage of the overflow in its absence (% of control); n = 6 to 9. C, I_M was measured using the amphotericin B-perforated patch technique in a SCG neuron. The current traces shown were obtained by clamping the neuron at –30 mV and by applying 1 s hyperpolarizing voltage steps to – 55 mV. The three traces on the left were recorded before (control), during (OxoM) and after (washout) the application of 10 µM OxoM alone. The traces on the right were recorded before (retigabine), during (retigabine + OxoM) and after (retigabine) the application of 10 µM OxoM in the continuing presence of 10 µM retigabine. D shows currents evoked by a ramp hyperpolarisation from –25 to –100 mV recorded before (control), during (retigabine) and after (washout) the application of 10 µM retigabine. E shows the changes in holding currents at –30 mV in the absence or presence of the indicated concentrations of retigabine applied alone (○) or together with 10µM OxoM (•). Current amplitudes in the presence of retigabine and/or OxoM were expressed as percentage of the amplitudes measured in their absence in the same neuron (% of control); n = 5-7.

3.1.7 *Retigabine prevents action potential discharge, but not depolarisation, in the presence of OxoM.*

To reveal as to how the above interactions between OxoM and retigabine at the levels of I_M might translate into changes in transmitter release, changes in membrane potentials were determined by perforated-patch current-clamp recordings. OxoM (10 μM) reproducibly depolarised SCG neurons from –54.6 ± 1.5 to -41.5 ± 2.5 mV and triggered action potentials in 8 out of 23 cells (Figure 3-7A and B). In the presence of retigabine (10 μM), neurons were hyperpolarized to -76.6 ± 0.7 mV and the addition of OxoM then depolarised the neurons to -67.1 ± 2.5 mV. In the presence of retigabine, however, OxoM always failed to trigger action potentials. Hence, retigabine did not abolish the depolarising action of OxoM, but only brought the membrane potential to more hyperpolarized values and thus further away from the action potential threshold.

This result raises the question as to whether OxoM depolarised the neurons in the presence of retigabine also by causing a closure of K_M channels. Since retigabine hyperpolarized the neurons by about the same value (22 mV, see above) as it shifted the voltage dependence of the outward currents (about -20 mV, see figure 3-6D), one may assume that the conductance of the K_M channels in current-clamped cells was the same in the absence and presence of retigabine (see also Tatulian and Brown, 2003). Hence, the effect of muscarinic receptor activation on the K_M channel conductance, and thus on the membrane potential, should be the same whether retigabine was present or not. In support of this hypothesis, the depolarisation elicited by 10 μM OxoM was almost identical in the absence and presence of 10 μM retigabine (Figure 3-7B).

If the depolarising action of OxoM is only due to the closure of K_M channels, the muscarinic agonist should loose this action once K_M channels are closed *a priori*. To test for this hypothesis, we first checked the hyperpolarizing effect of retigabine (10 μM) and then injected a hyperpolarizing current to bring the membrane to exactly the same potential again, but in the absence of retigabine (Figure 3-7C). This procedure can be expected to entirely close K_M channels (see the current voltage curve in figure 3-6D). When OxoM (10 μM) was applied during the injection of this hyperpolarizing current, its depolarising action was largely attenuated. For comparison, in the presence of retigabine OxoM depolarised the neurons by the same extent as in its absence (Figure 3-7C and D).

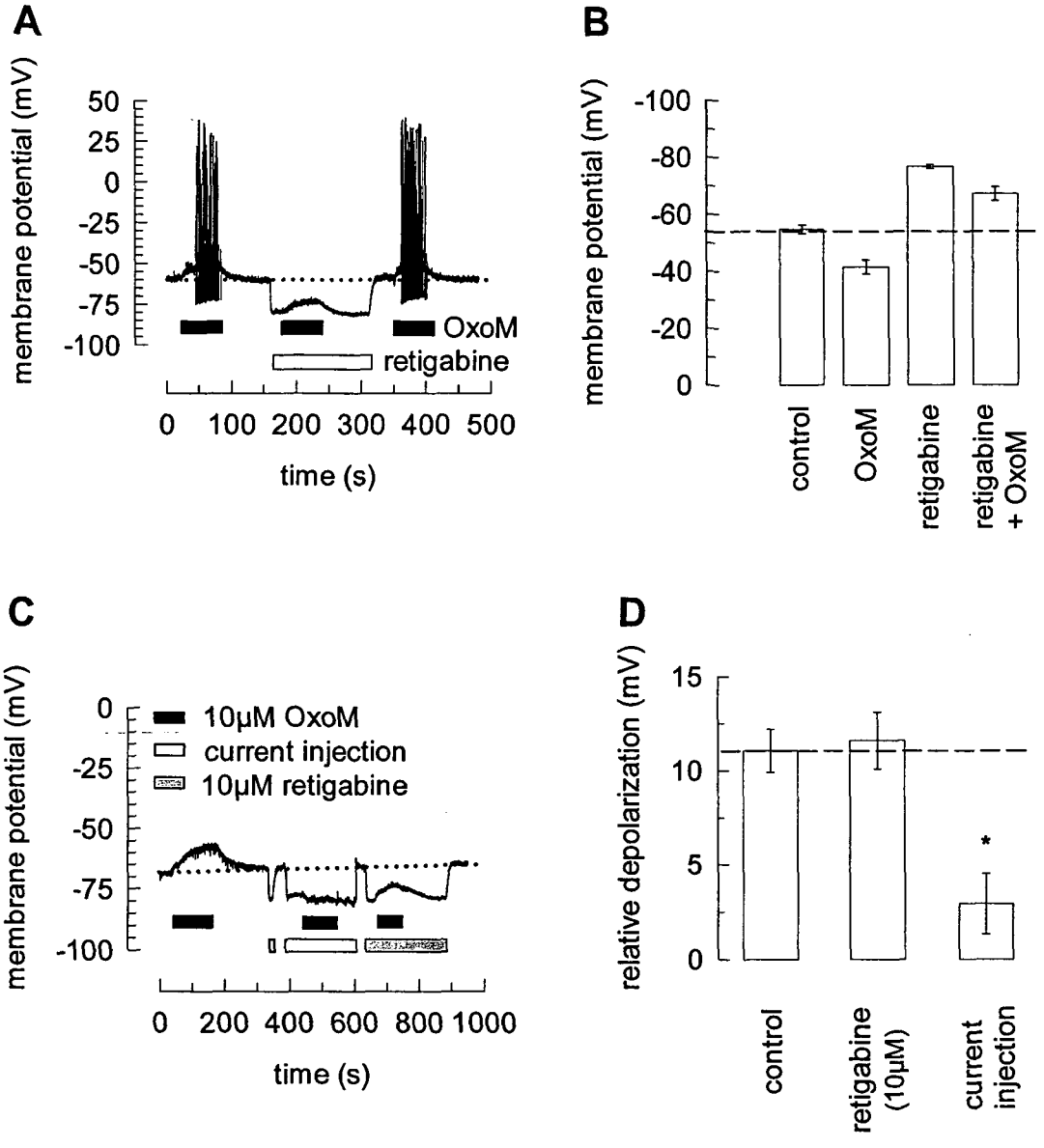

Figure 3-7. Effects of retigabine on the membrane potential.

*Membrane potentials of SCG neurons were recorded in the current-clamp mode using the amphotericin B-perforated patch technique. A shows the time course of the membrane potential of one SCG neuron. OxoM and retigabine (both at 10 µM) were applied as indicated by the bars. Note the firing of action potentials in the presence of OxoM. B summarizes the effects of 10 µM OxoM and 10 µM retigabine, applied either alone or together, on the membrane potentials of 6 to 8 SCG neurons; experiments were carried out as shown in (A). C shows the time course of the membrane potential. OxoM and retigabine (both at 10 µM) were applied as indicated by the bars. Subsequently to the application of retigabine, a hyperpolarizing current was injected to bring the membrane to the same potential as 10 µM retigabine did. Note that OxoM failed to cause a significant depolarisation only during current injection. D summarizes the depolarisations caused by 10 µM OxoM applied either alone (control), in the presence of 10 µM retigabine, or during the injection of hyperpolarizing currents, as described in C; n = 5-6; * $p < 0.05$ vs. the depolarisation und control conditions.*

3.1.8 *Retigabine does not alter action potential waveforms nor electrically evoked noradrenaline release.*

The fact that retigabine caused hyperpolarisations by about 20 mV, but failed to alter electrically evoked tritium overflow appears contradictory. To resolve this issue, 0.5 ms depolarising currents were injected and the arising action potentials were recorded in the absence and presence of 10 μM retigabine. The waveforms of elicited action potentials were not affected by retigabine, even though the neurons were hyperpolarized by about 20 mV (Figure 3-8A). However, in the presence of retigabine single SCG neurons were more reluctant to fire action potentials: at current strengths of 10 or 20 nA, more neurons fired action potentials in the absence than in the presence of retigabine (Figure 3-8B).

This result leads to the question whether retigabine might also cause a reduction of tritium outflow if electrical fields of different strengths are applied. Accordingly, the current intensities used to trigger transmitter release were reduced from 60 mA (which was routinely used in all other experiments) to 30 and subsequently 15 mA (Figure 3-8C). The amount of tritium overflow decreased in parallel with these decreased stimulation intensities, but 10 μM retigabine always failed to cause a significant inhibition of transmitter release (Figure 3-8D).

3.1.9 *Depletion of intracellular Ca^{2+} stores does not interfere with the stimulation of noradrenaline release by OxoM.*

Independently of its inhibitory effect on I_M, OxoM induces the formation of inositol triphophate and triggers Ca^{2+} release from intracellular stores in SCG neurons (del Rio et al, 1999). The resulting increase in intracellular Ca^{2+} might contribute to the stimulation of noradrenaline release by OxoM. To test for this hypothesis, radiotracer release experiments were performed in the absence and presence of 1 μM thapsigargin which prevents OxoM-dependent increases in intracellular Ca^{2+} in SCG neurons (del Rio et al, 1999). However, OxoM-induced tritium overflow in the presence of thapsigargin ($\pm$ 0. % of total radioactivity; n = 6) was not different from that in its absence ($\pm$ 0. % of total radioactivity; n = 6; p > 0.3). Likewise, electrically evoked tritium overflow was not affected by thapsigargin (not shown) which, however, did abolish the inhibition of I_M by bradykinin as a positive control (see Bofill-Cardona et al, 2000).

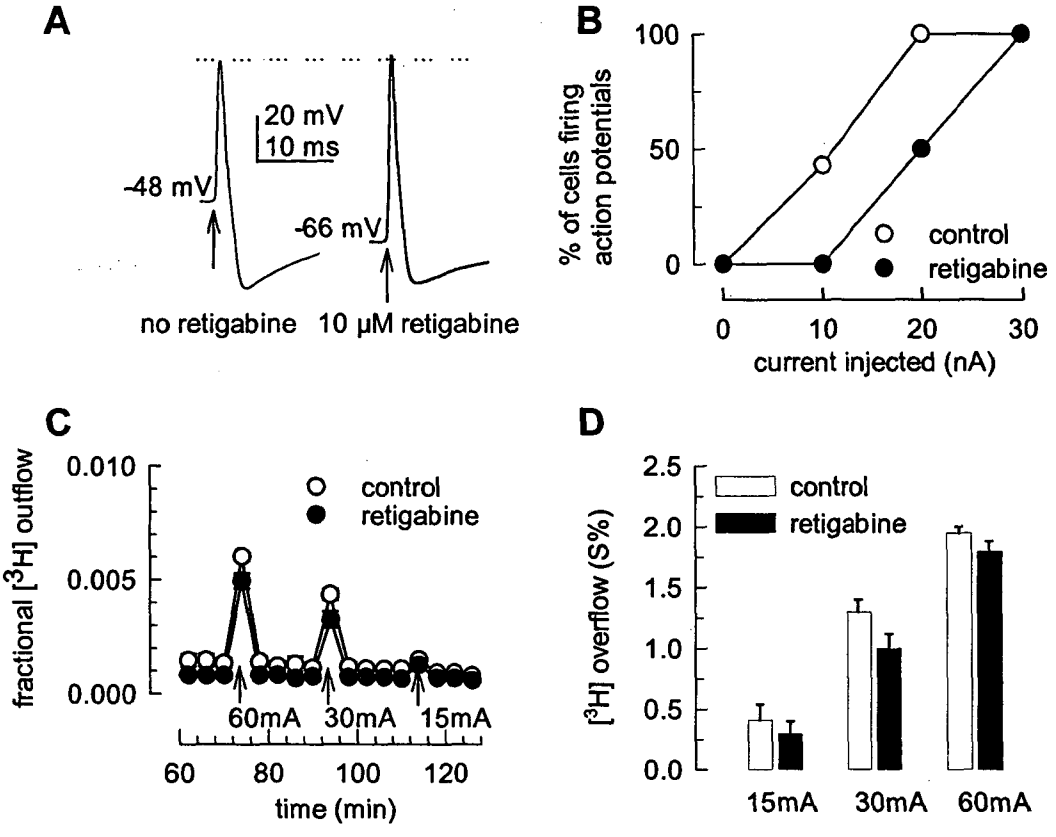

Figure 3-8. Retigabine does not alter action potentials waveforms nor electrically evoked noradrenaline release.

Membrane potentials of SCG neurons were recorded in the current-clamp mode using the amphotericin B-perforated patch technique. Action potentials were evoked by 0.5 ms depolarising current injection in the presence and in the absence of 500 nM TTX. To visualize the pure action potential, traces recorded in the presence of TTX were digitally subtracted from those recorded in its absence. A shows action potentials evoked by the injection of a depolarising 20 nA current in either the absence or presence of 10 µM retigabine. The values of the membrane potentials prior to the action potentials are indicated. B shows the relation between injected currents and the percentage of neurons firing action potentials in response to these currents in either the absence (○, control) or presence (●) of 10 µM retigabine. Experiments were performed as described above; n = 6-8. C, cultures were labelled with ^{3}[H]noradrenaline and superfused. Subsequent to a 60min washout period, 4min fractions of superfusate were collected. Tritium overflow was evoked three times by electrical field stimulation (EFS; 60 pulses, 1Hz) with decreasing strength starting at 72 min (60mA), 92 min (30mA) and 112 min (15mA) of superfusion. From minute 50 of superfusion onward, the buffer contained either no (control) or 10µM retibgabine. The graph shows the time course of fractional ^{3}[H] outflow per minute; n=3. D shows the effect of 10µM retigabine on tritium overflow (S%) induced by EFS with the indicated current strengths. Experiments were performed as described in C; n=6.

3.2 Part II – Metabotropic P2Y nucleotide receptors

To reveal whether the adenine nucleotide-dependent inhibition of both, adenylyl cyclase and I_{Ca}, in PC12 cells was mediated by one P2Y receptor subtype, the antithrombotic $P2Y_{12}$ receptor antagonists 2-MeSAMP (Jantzen et al, 1999; Hollopeter et al, 2001) and AR-C69931MX (Turner et al, 2001) were used in cyclic AMP accumulation assays, on one hand, and in whole-cell patch-clamp experiments, on the other hand.

3.2.1 Inhibition of adenylyl cyclase

After labelling of cellular purines of PC12 cells with [^{3}H]adenine, 272 $\pm$ 54 cpm (n = 18) were retrieved within the fraction of cyclic AMP, which corresponded to 0.041 $\pm$ 0.008 % of the total radioactivity in the cultures. These and all subsequent values were obtained in the presence of the phosphodiesterase inhibitor RO 20-1724. Thus, any drug-induced alteration will reflect changes in adenylyl cyclase activity rather than in cyclic AMP degradation (Unterberger et al, 2002). Exposure of PC12 cells to 1 μM of the A_{2A} adenosine receptor agonist CGS 21680 for 15 min caused a 25-fold increase in cellular cyclic AMP, and this effect was reduced in the presence of 0.01 to 100 μM ADP in a concentration-dependent manner. The inhibitory actions of the nucleotide were half maximal at 0.1 μM (figure 3-9A) and 0.17 μM (figure 3-9B), respectively, which closely corresponds to the values reported before (0.24 μM; Unterberger et al, 2002). In the presence of 30 μM 2-MeSAMP, the concentration response curve for the ADP-mediated inhibition of adenylyl cyclase was shifted to the right and the effect was half maximal at 3.9 μM. The maximum of inhibition achieved by ADP, however, remained unchanged (igure 3-9A). Assuming a competitive type of antagonism and thus applying the equation log (CR-1) = pA2 + log [B] (where CR is the ratio of equieffective agonist concentrations in the presence and absence of the antagonist concentration B, respectively; Arunlakshana and Schild, 1959) revealed an apparent affinity of 2-MeSAMP in the low micromolar range with a pA_2 value of 6.1.

To corroborate these data, we also used the more potent and selective antagonist AR-C69931MX. In the presence of 100 nM of this agent, the concentration response curve for the ADP-dependent inhibition of cyclic AMP accumulation was again shifted to the right without any significant change in the maximal effect exerted by ADP (figure 3-9B). The ratio of equieffective ADP concentrations in the presence and absence of AR-C69931MX was 29.6 which corresponds to a pA_2 value of 8.5. Thus, AR-C69931MX is more than 100-fold more potent an antagonist at the receptor mediating the inhibition of adenylyl cyclase than 2-MeSAMP.

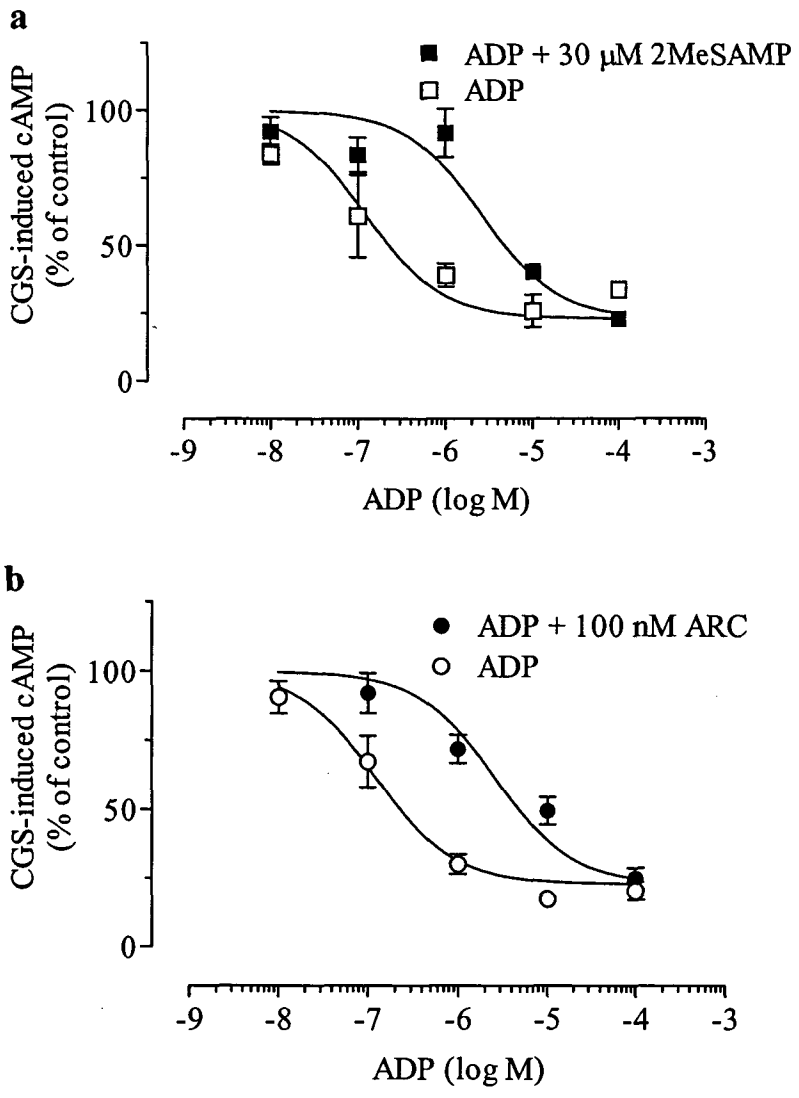

Figure 3-9, 2-MeSAMP and AR-C69931MX antagonize the inhibition of adenylyl cyclase by ADP.
*After loading with [^{3}H]adenine, PC12 cells were incubated in RO 20-1724 (100 μM) for 105 min. During the last 15 min of this incubation period, 1 μM CGS 21680 was present either alone or together with the indicated concentrations of ADP (open circles). The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell cultures, and values obtained in the presence of CGS 21680 were normalized to the data obtained in its absence (normalized to basal). Results obtained in the presence of ADP are then expressed as percentage of those obtained in its absence (% of control; see Methods). **A** In the cultures represented by filled squares, 30 μM 2-MeSAMP were present, whereas this antagonist lacked in the cultures represented by open symbols. n = 6 to 9. **B** In the cultures respresented by filled circles, 100 nM AR-C69931MX were present, whereas this antagonist lacked in the cultures represented by open symbols. n = 6 to 9.*

3.2.2 Inhibition of I_{Ca}

Depolarization of differentiated PC12 cells from –80 to 0 mV elicited rapidly activating I_{Ca} with amplitudes in the nanoampere range, as reported previously (Vartian and Boehm, 2001). In the presence of 100 μM ADP, activation kinetics were slowed and maximal current

amplitudes were reduced by about one half (figure 3-10A). To obtain preliminary evidence as to whether this effect might involve the same receptor as the inhibition of adenylyl cyclase, ADP (100 μM) was applied in the continuous presence of 2-MeSAMP (30 μM) which almost abolished the inhibitory action of ADP without causing *per se* an alteration in current amplitudes (figure 3-10A). Likewise, the ATP analogue AR-C69931MX (0.1 μM) did not alter I_{Ca}, but largely attenuated its inhibition by ADP (figure 3-10B). In contrast to these results, the selective $P2Y_1$ receptor antagonist MRS 2216 (1 μM; Nandanan et al, 1999) failed to attenuate the inhibition of I_{Ca} by 100 μM ADP (36.2 ± 8.8 % inhibition in the absence and 40.1 ± 11.9 % inhibition in the presence of MRS 2216, respectively; n = 3).

To further compare the actions of the two antithrombotic agents, we investigated their dissociation from the receptor mediating the inhibition of I_{Ca} by ADP (figure 3-10C). In the presence of 100 μM ADP, AR-C69931MX (10 μM) required more than 2 min to dissociate from the receptor, thus permitting maximal inhibition by ADP. 2-MeSAMP (300 μM), in contrast, was entirely replaced by ADP within less than 15 s, and I_{Ca} was immediately reduced to the same extent as without previous application of an antagonist. This indicates a large difference in dissociation constants of these two antagonists which should therefore display greatly varying affinities. Indeed, when determining full concentration-response curves for the antagonistic effects of 2-MeSAMP and AR-C69931MX versus the inhibition of I_{Ca} by 100 μM ADP, the half maximal concentrations were 6.7 μM for 2-MeSAMP and 0.06 μM for AR-C69931MX, respectively (figure 3-10D). Thus, AR-C69931MX is at least 100-fold more potent in attenuating the ADP-dependent inhibition of I_{Ca} than 2-MeSAMP.

The inhibition of I_{Ca} by ADP has been reported to be mimicked by ADPβS and 2-MeSADP, but not by uridine nucleotides; the two adenine nucleotides caused half maximal inhibition at 9 and 0.04 μM, respectively (Vartian and Boehm, 2001). To find out whether these agonists acted via the same receptor as ADP, they were applied first alone and then together with AR-C69931MX. As shown in figure 3-11, this antagonist attenuated the inhibitory actions of both of these agonists. When tested against maximally active concentrations of ADPβS (100 μM) and 2-MeSADP (1 μM), AR-C69931MX (1 nM to 10 μM) attenuated the inhibition of I_{Ca} within the same range of concentrations, namely with IC_{50} values of 0.06 and 0.07 μM, respectively.

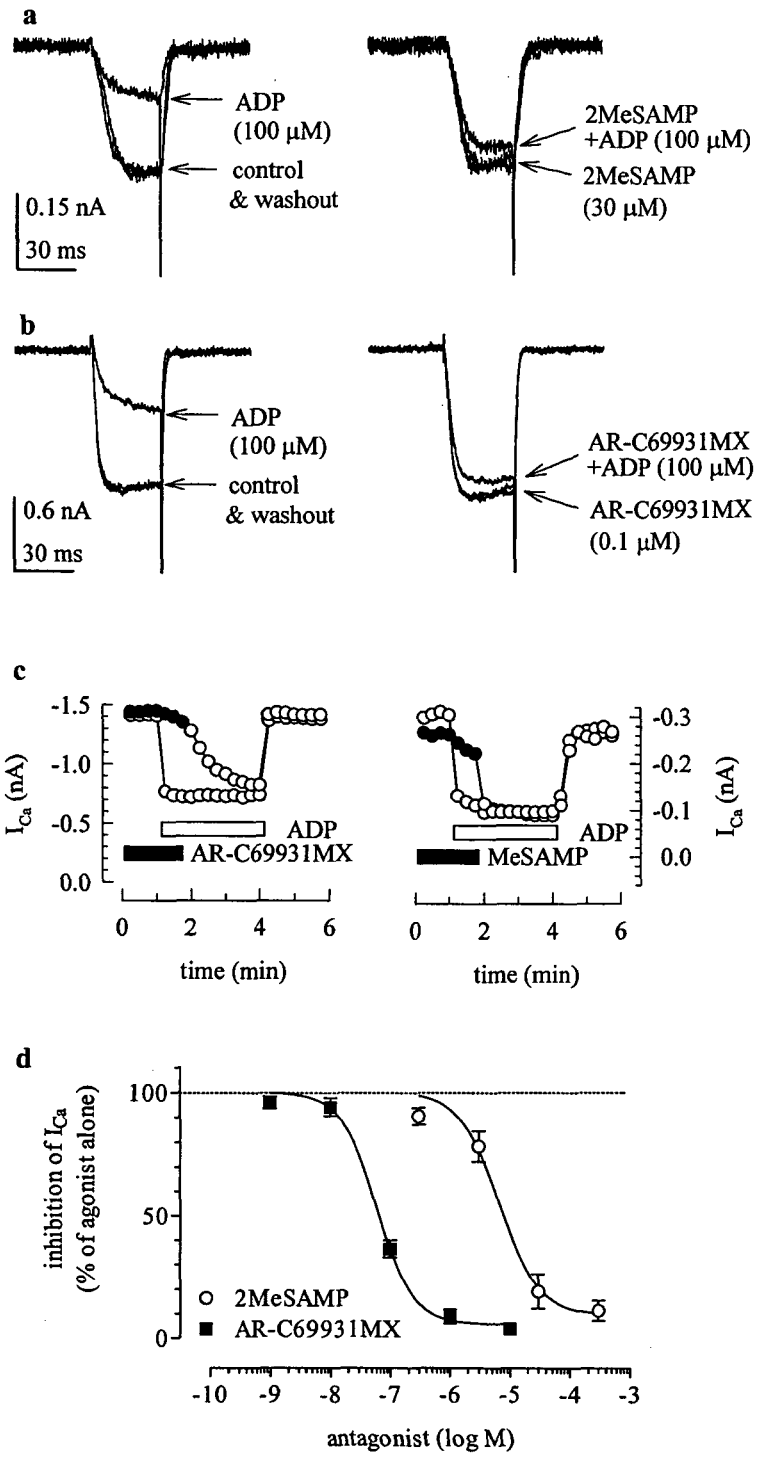

Figure 3-10, 2-MeSAMP and AR-C69931MX antagonize the inhibition of voltage-activated Ca^{2+} currents by ADP.

*Ca^{2+} currents were evoked in differentiated PC12 cells by 30 ms depolarizations from –80 to 0 mV applied once every 15 s. **A** The current traces shown left were obtained before (control), during and after (washout) the application of 100 µM ADP. Thereafter, 30 µM 2-MeSAMP were present, and ADP (100 µM) was applied again in the continuing presence of this antagonist; the traces shown right were obtained again before, during and after the application of ADP. **B** ADP (100 µM) and AR-C69931MX (0.1 µM) were applied to another differentiated PC12 cell in the manner described for 2-MeSAMP in A. **C** Time course of peak Ca^{2+} current amplitudes: ADP (100 µM, white bar), AR-C69931MX (10 µM, black bar, left), and 2-MeSAMP (300 µM, black bar, right) were present as indicated by the bars. Note that ADP achieved inhibition of I_{Ca} immediately when 2-MeSAMP was* removed, but required about 2 min to inhibit I_{Ca}, when AR-C69931MX was removed. **D** Concentration-dependence of the antagonism of 2-MeSAMP (0.3 to 300 µM) and AR-C69931MX (1 nM to 10 µM) versus the inhibition of I_{Ca} by ADP (100 µM). Currents were elicited and drugs were applied as described in A; n = 5 to 6.

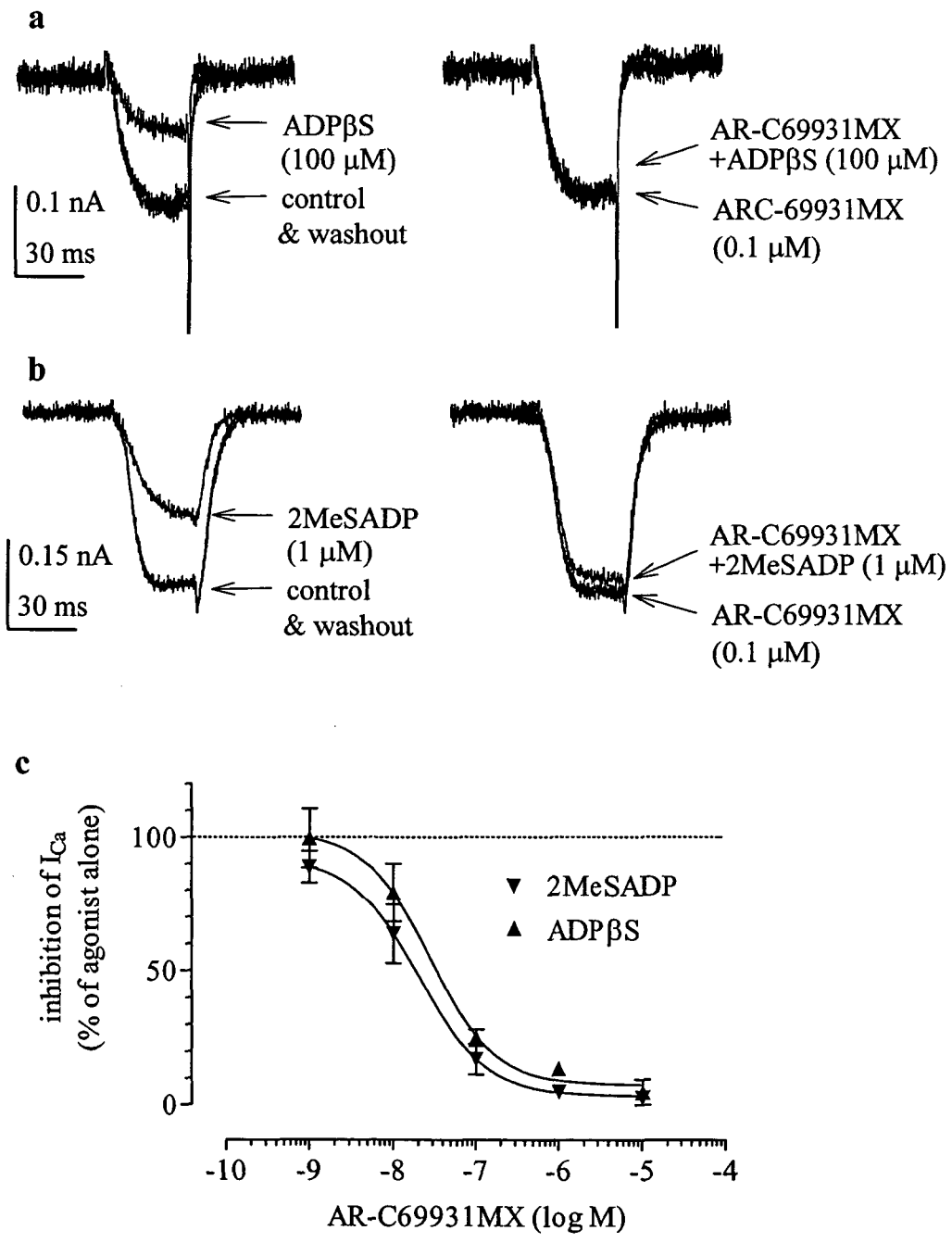

Figure 3-11, AR-C69931MX antagonizes the inhibition of voltage-activated Ca^{2+} currents by ADPβS and 2-MeSADP.

*Ca^{2+} currents were evoked in differentiated PC12 cells by 30 ms depolarizations from –80 to 0 mV applied once every 15 s. **A** The current traces shown left were obtained before (control), during and after (washout) the application of 100 μM ADPβS. Thereafter, 0.1 μM AR-C69931MX were present, and ADPβS (100 μM) was applied again in the continuing presence of this antagonist; the traces shown right were obtained again before, during and after the application of ADPβS. **B** 2-MeSADP (1 μM) and AR-C69931MX (0.1 μM) were applied to another differentiated PC12 cell in the manner described in A. **C** Concentration-dependence of the antagonism between AR-C69931MX (1 nM to 10 μM) and ADPβS (100 μM) or 2-MeSADP (1 μM). Currents were elicited and drugs were applied as described in A; n = 2 to 5.*

Thus, AR-C69931MX attenuated the inhibition of I_{Ca} irrespective of which agonistic nucleotide has been used.

To obtain more insight into the interactions between agonists and antagonists, ADP was applied at increasing concentrations in either the absence or presence of AR-C69931MX at 10, 30, or 100 nM. As shown in figure 3-12A, AR-C69931MX caused a rightward shift of the concentration response curve for the ADP-dependent inhibition of I_{Ca}, with no obvious change in the maximal effect achieved by ADP. The Schild analysis (Arunlakshana and Schild, 1959) of these data corroborated that AR-C69931MX behaved as a competitive antagonist (slope 1.09) with an apparent affinity in the low nanomolar range ($pK_B = 8.7$). The concentration response curve for the inhibition of I_{Ca} by ADPβS was also shifted to the right in the presence of 30 nM AR-C69931MX without any noticeable alteration in the maximal inhibition of I_{Ca} (figure 3-12B). The ratio of equieffective concentrations of ADPβS in the presence and absence of 30 nM AR-C69931MX, respectively, was 3.8, and the equivalent result for ADP was 4.9. As a consequence, the value log (CR-1) obtained with one concentration of AR-C69931MX versus ADPβS was almost superimposable on the linear regression of such values obtained with 3 concentrations of this antagonist versus ADP (figure 3-12C). Furthermore, the pA_2 value for AR-C69931MX versus ADPβS (8.1) shows considerable correlation with the pK_B value (8.7) obtained with ADP as an agonist.

Increasing concentrations of ADP were also applied in the absence and presence of 10 to 100 μM 2-MeSAMP, and the inhibition of I_{Ca} was determined. As observed for AR-C69931MX, these concentrations of 2-MeSAMP shifted the concentration response curve for the inhibition of I_{Ca} by ADP to the right (figure 3-13A). When these results were subjected to a Schild analysis (figure 3-13B), a slope of 0.95 indicated an underlying competitive mechanism, and a pK_B value of 5.4 corroborated an apparent affinity of 2-MeSAMP in the low micromolar range.

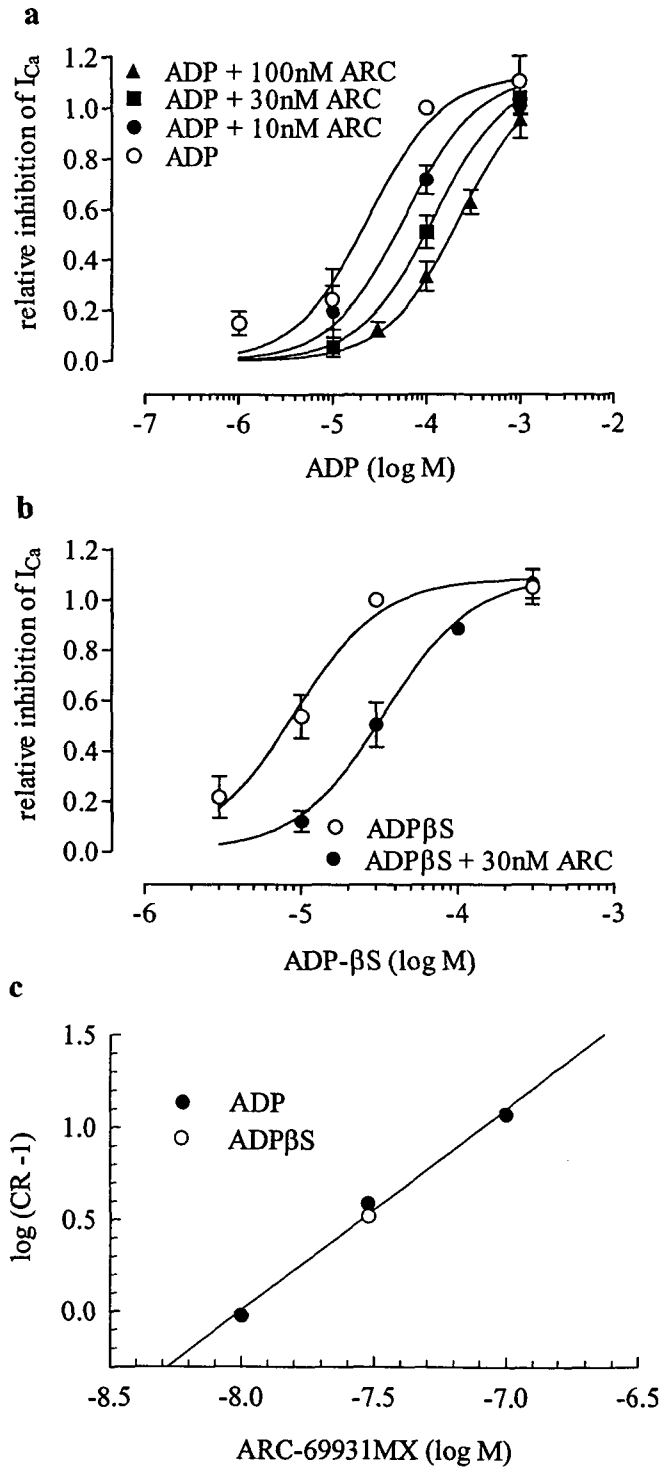

Figure 3-12, Antagonism between AR-C69931MX and ADP or ADPβS in the inhibition of voltage-activated Ca^{2+} currents.

Ca^{2+} currents were determined in, and drugs were applied to, differentiated PC12 cells as described in figures 3-10 and 3-11. A Concentration dependence for the inhibition of I_{Ca} by increasing concentrations of ADP applied either alone or in the continuing presence of 10, 30, or 100 nM AR-C69931MX; n = 4 to 6. B Concentration dependence for the inhibition of I_{Ca} by increasing concentrations of ADPβS applied either alone or in the continuing presence of 30 nM AR-C69931MX; n = 4 to 6. C Schild plot of the data shown in A and B. The slope of the linear regression was 1.09 and the estimated pK_B was 8.7.

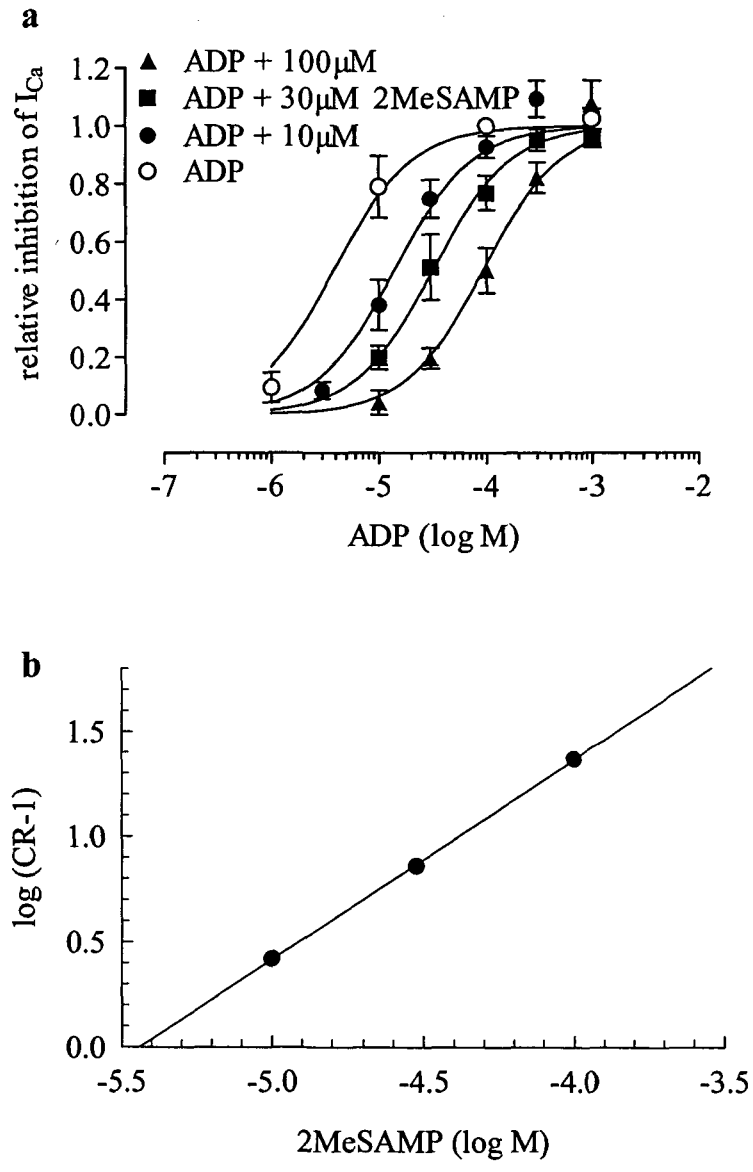

Figure 3-13, Antagonism between 2-MeSAMP and ADP in the inhibition of voltage-activated Ca^{2+} currents.

Ca^{2+} currents were determined in, and drugs were applied to, differentiated PC12 cells as described in figures 3-10 and 3-11. A Concentration dependence for the inhibition of I_{Ca} by increasing concentrations of ADP applied either alone or in the continuing presence of 10, 30, or 100 μM 2-MeSAMP; n = 5 to 6. B Schild plot of the data shown in A. The slope of the linear regression was of 0.95 and the estimated pK_B was 5.4.

3.2.3 *ADP inhibits stimulation-evoked noradrenaline release*

To evaluate whether ADP inhibits stimulation-evoked transmitter release, PC12 cells were loaded with [^{3}H]noradrenaline and superfused with physiological buffer. Noradrenaline release was evoked twice at 72min (S1) and 92min (S2) by depolarization with 30mM K^+ (figure 3-14A), and various ADP concentrations were added 4min before S2.. ADP significantly reduced transmitter release as indicated by a decrease in S2/S1 ratios. To investigate whether this inhibition was mediated by the same receptor that inhibits I_{Ca} and adenylyl cyclase ($P2Y_{12}$; see above), concentration response curves for the inhibition of stimulation-evoked transmitter release were obtained in the absence and presence of the $P2Y_{12}$ antagonist AR-C69931MX. ADP reduced noradrenaline release with an EC_{50} value of 0,08 ± 0,02 µM (figure 3-14B). In the presence of 100nM AR-C69931MX, the concentration-response curve was shifted to the right and the half maximal concentration amounted to 5,5 ± 3,4 µM (figure 3-14B). Assuming an underlying competitive antagonism, one can calculate a pA_2 value for AR-C69931MX for the inhibition of transmitter release by applying the equation $\log(CR - 1) = pA_2 + \log [B]$ (where CR is the ratio of equieffective agonist concentrations in the presence and in the absence of the antagonist concentration B; Arunlakshana & Schild, 1959). The calculated pA_2 value for the inhibition of stimulation-evoked transmitter release was 8,8, which shows that this effect is mediated by the same receptor that inhibits I_{Ca} ($pA_2 = 8,7$) and adenylyl cyclase ($pA_2 = 8,5$).

Calcium influx through voltage-gated calcium channels is known to link action potentials to transmitter release. Hence we tested, whether the inhibition of I_{Ca} is essential for the ADP induced inhibition of stimulation-evoked release or if any other mechanisms may also contribute. Therefore release was evoked either by depolarization with potassium or by application of 100µM ATP and the inhibitory effects of 10µM ADP and 100µM cadmium (Cd^{2+}) were compared. Depolarization with K^+ causes calcium influx through voltage-gated calcium channels (VACCs), whereas ATP activates ionotropic P2X receptors that mediate calcium entry, and thus triggers transmitter release independently of VACCs (Nakazawa & Inoue, 1992). ADP reduced transmitter release evoked by 30mM K^+ to 37,5 ± 7,2 % (figure 3-15E), whereas ATP induced release was not affected (figure 3-15A and E). Similar results were obtained by using cadmium (Cd^{2+}) instead of ADP. Cd^{2+} is known to be a potent blocker of voltage-gated calcium channels. As expected, 100µM Cd^{2+} reduced transmitter release evoked by 30mM K^+ to 5 ± 4 % (figure 3-15E), whereas ATP induced release was increased to 125,7 ± 6,5 % (figure 3-15A and E). Moreover, the effect of 100µM Cd^{2+} and 10µM ADP

on ATP induced currents through P2X receptors (I_{ATP}) and on voltage-gated calcium channels (I_{Ca}) were investigated. ADP reduced I_{Ca} to 58,1 ± 10,1 % (% of control) but caused no significant change in I_{ATP} (figure 3-15D). Cd^{2+} reduced I_{Ca} to 7,3 ± 3 % (% of control) and increased I_{ATP} to 233,5 ± 65,9% (figure 3-15D).

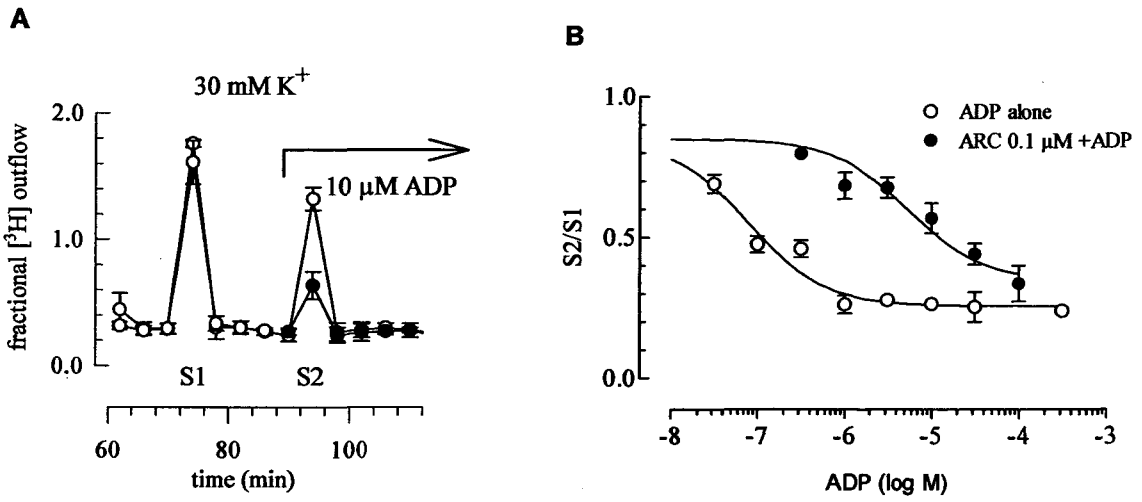

Figure 3-14. ADP inhibits stimulation-evoked transmitter release via activation of $P2Y_{12}$

PC12 cells were labelled with [³H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Transmitter release was evoked twice by 4 min application of 30mM K^+ starting at 72 min (S1) and at 92 min (S2). 10µM ADP (●) were present as indicated by the arrow. The graph shows the time course of fractional [³H] outflow per min (n = 3). (B), shows the concentration dependence of the ADP-induced inhibition of stimulation-evoked tritium overflow, either in the presence (●) or in the absence (○) of 100nM AR-C69931MX. Inhibition is depicted as the S2/S1 ratio in the presence of the indicated ADP concentrations added either alone (○) or together with 0,1µM ARC(●) (n = 5 - 9).

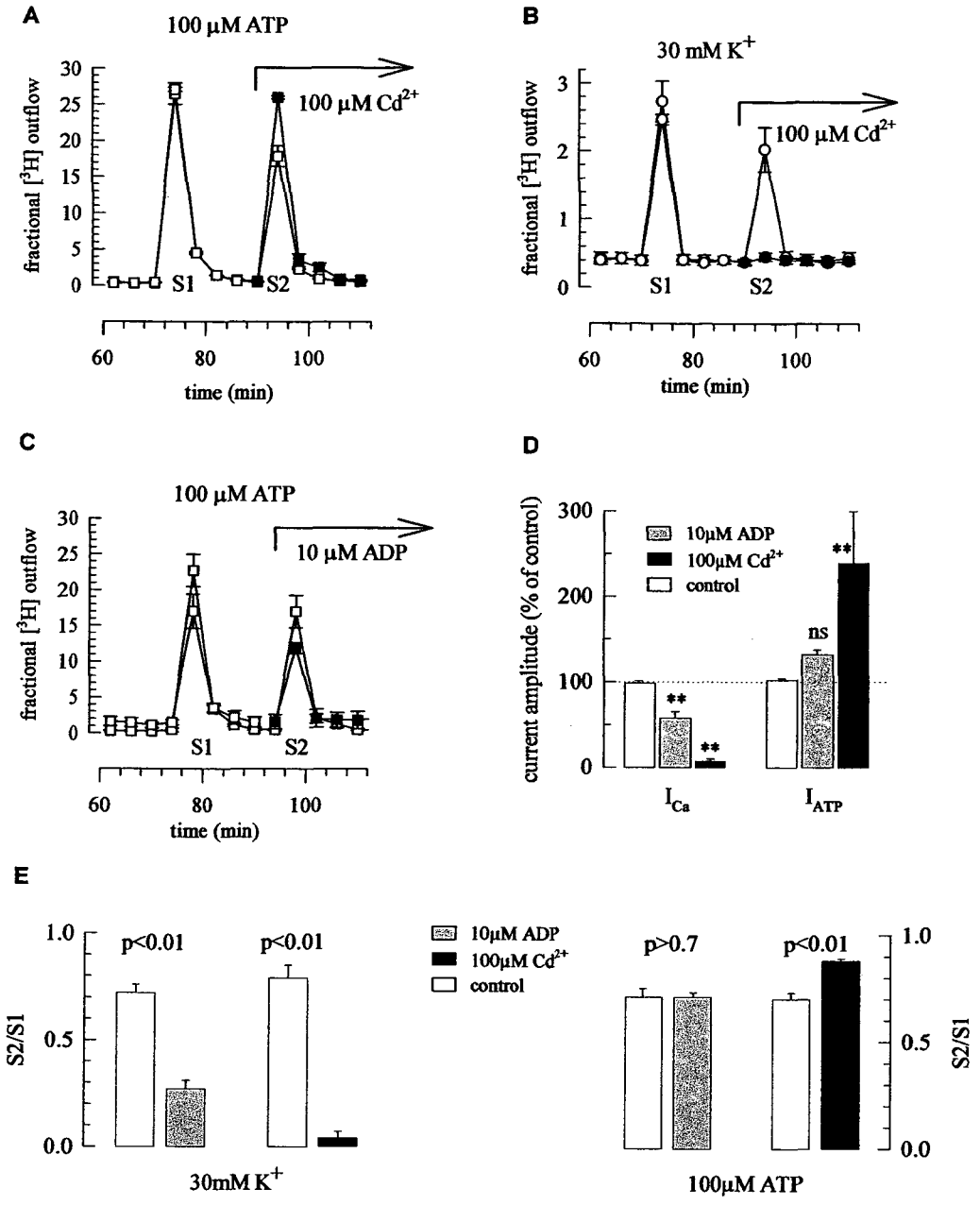

Figure 3-15, The inhibition of ICa is the crucial step

PC12 cells were labelled with [³H]noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. (A,C) Transmitter release was evoked twice by 1 min application of 100µM ATP starting at 72 min (S1) and at 92 min (S2). 10µM ADP and 100µM Cd^{2+} were present as indicated by the filled squares (■). The graphs show the time course of fractional [³H] outflow per min (n = 3). (B) Transmitter release was evoked twice by 4 min application of 30mM K^+ starting at 72 min (S1) and at 92 min (S2). 100µM Cd2+ were present as indicated by the filled circles (●).The graph shows the time course of fractional [³H] outflow per min (n = 3). (D); I_{Ca} was elicited and drugs were applied as described above (figure X). I_{ATP} was elicited by one second application of 100µM ATP to a cell clamped at -70mV. Current amplitudes were measured in the absence (control) or in the continuous presence of either 10µM ADP (grey bar) or 100µM Cd^{2+} (black bar). The graph shows the mean current amplitudes (n = 5) as percent of control (E), summarizes the effects of ADP and Cd^{2+} on transmitter release evoked either by application of 30mM K^+ or 100µM ATP. Inhibition is depicted as the S2/S1 ratio in the presence and absence of ADP or Cd2+ (n = 8).

3.2.4 *pCPT-cAMP reduces ADP induced inhibition of stimulation-evoked release*

The intracellular accumulation of cyclic AMP has consistently been shown to cause an enhancement of stimulation-evoked transmitter release from sympathetic neurons (reviewed in Boehm & Kubista, 2002). Since ADP not only suppresses Ca^{2+} currents, but also inhibits adenylyl cyclase, we investigated if this latter effect might contribute to the inhibition of stimulation-evoked release. Therefore noradrenaline release was evoked twice with 30mM K^+, either under normal conditions (S1) or in the continuous presence (S2) of either 1µM CGS21680, 10µM forskolin or 300µM pCPT-cAMP (8-(4-chlorophenylthio)adenosine 3':5'-cyclic monophosphate). CGS21680 elevates intracellular cAMP levels by activating A_{2A} adenosine receptors while forskolin directly activates adenylyl cyclase. pCPT-cAMP is a membrane permeable analogue of cAMP that is known to mimic the effects of cAMP. Figure 3-16A shows that stimulation-evoked release per se was not altered by any of these substances. In addition, the inhibitory effect of ADP in the presence of either of these substances was investigated. CGS21680 and forskolin did not alter the ADP induced inhibition of stimulation-evoked release (figure 3-16B). However, when cAMP levels were elevated independently of adenylyl cyclase activity by using pCPT-cAMP, ADP induced inhibition was reduced from 65,1 ± 3,3 % to 29,9 ± 4,8 % (figure 3-16B, cpt-cAMP). As shown above, inhibition of I_{Ca} is the crucial step for the ADP-induced inhibition of stimulation-evoked transmitter release. Hence, to reveal the mechanism by which pCPT-cAMP acts, we first investigated whether treatment with pCPT-cAMP has any effect on the ADP mediated inhibition of I_{Ca}. As already mentioned, G protein dependent inhibition of Ca^{2+} currents is accompanied by a slowing of activation kinetics (figure 3-16C left traces). Interestingly, when PC12 cells were treated with 300µM pCPT-cAMP for 30 minutes prior to recording, this kinetic effect of the inhibition was completely abolished (figure 3-16C right traces). The differences in activation kinetics are shown in figure 3-16D. During the early phase of the test pulse (4 – 5,5 ms) inhibition is reduced from 53,1 ± 6,77% in untreated cells to 22,9 ± 12,8 % in cells treated with pCPT-cAMP. By contrast, late inhibition (27-29 ms) remains largely unaltered; 47,4 ± 5,6 % in control cells and 45,2 ± 9,44 % after treatment with pCPT-cAMP.

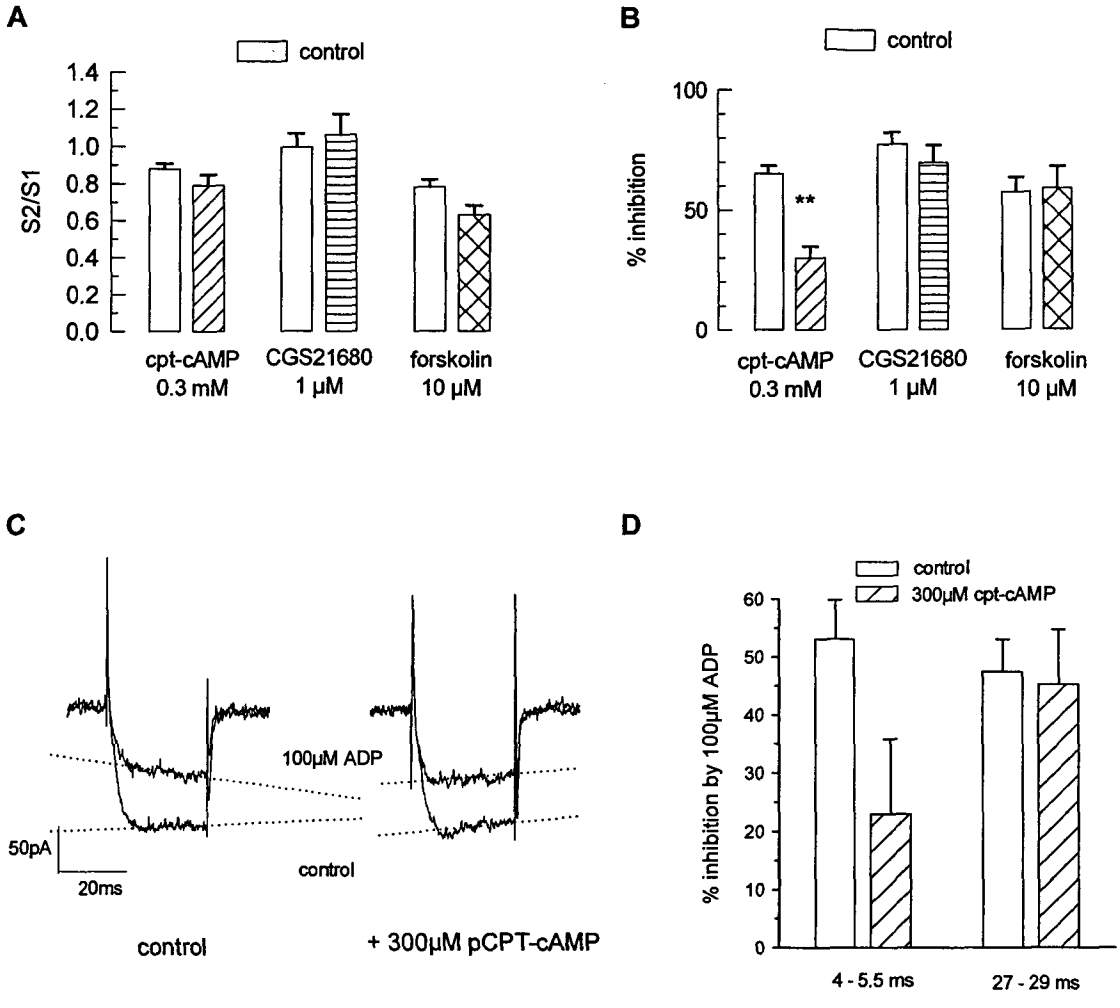

Figure 3-16, pCPT-cAMP reduces the ADP induced inhibition of stimulation-evoked release.

(A) [^{3}H] noradrenaline release was evoked twice at 72min (S1) and 92min (S2) by application of 30mM K^+ (see methods). The second stimulation (S2) was performed either in the absence (control) or in the continuous presence of modulatory agents (pCPT-cAMP, CGS21680 or forskolin) which were present from 76min onward. The graph shows the effect of modulatory agents on the S2/S1 ratio (n = 9 – 12). (B) Experiments were performed as described in (A). Additionally 10µM ADP was included in the buffer starting at 88 min. The graph compares the ADP (10µM) induced inhibition in the absence (control) and in the presence of modulatory agents (pCPT-cAMP, CGS21680 or forskolin; n = 9 - 12). (C) Ca^{2+} currenst were elicited as described above. 100µM ADP reduced I_{Ca} amplitudes and slowed activation kinetics (indicated with dotted lines, left trace). In cells pretreated with 300µM pCPT-cAMP for 30 minutes, 100µM ADP still reduced current amplitudes but the slowing of activation kinetics was not observed (right traces, indicated by dotted lines). (D) Uninhibited Ca^{2+} current usually reached a maximum after ~ 6ms and then slowly decayed due to inactivation. To determine the effect of pCPT-cAMP on ADP induced inhibition during channel activation, inhibition was calculated for the time interval from 4 - 5,5 ms (activation) and compared to the inhibition at 27 – 29 ms (channels are fully activated).

3.2.5 *The ADP induced inhibition of I_{Ca} involves two distinct $\beta\gamma$ mediated pathways*

The ADP induced inhibition of I_{Ca} has previously been reported to be pertussis toxin (PTX) sensitive and voltage-dependent, suggesting a membrane delimited pathway based on a direct interaction of Gβγ dimers with the calcium channels α_1-subunit (Vartian & Boehm, 2001). To corroborate these findings, the role of βγ-dimers was further investigated. A common approach to study the role of βγ-dimers in signal-transduction is by exogenous expression of so called βγ-scavengers, like α-transducin or βARK-ct (beta adrenergic receptor kinase C-tail). Such scavengers contain Gβγ binding domains and thus compete with possible target proteins for free βγ-dimers, thereby abolishing effects that are mediated by βγ subunits. PC12 cells were stably transfected with bovine retinal α-transducin, which has been shown to abolish the noradrenaline-mediated inhibition of Ca^{2+} currents in rat sympathetic neurons (Delmas et al, 1999). Overexpression of α-transducin reduced the ADP-mediated inhibition of I_{Ca} from 63,7 ± 16,2 % (wildtype PC12 cells; n = 7) to 19,1 ± 10,9 % (clone 5; n = 16) and 13,1 ± 12,2 % (clone 9; n = 7), respectively (figure 3-17A). This indicates a major contribution of Gβγ subunits to the ADP-mediated inhibition of I_{Ca}. To reveal whether the ADP-dependent inhibition of CGS-induced cAMP formation is also mediated by βγ subunits, appropriate concentration response curves were obtained for the two α-transducin expressing clones (clone5, clone9) and for untransfected PC12 cells (wt). Figure 3-17B shows that heterologously expressed α-transducin does not significantly affect the ADP induced inhibition of adenylyl cyclase (wt-EC50 = 20,1 ± 7,4 nM; clone5-EC50 = 26,1 ± 49,8 nM; clone9-EC50 = 31,5 ± 16,4 nM; n = 6).

To corroborate that βγ subunits are able to mediate an inhibition of I_{Ca} in PC12 cells, the cells were transfected with a construct containing DNA encoding for β_1 and γ_2 subunits. In sympathetic neurons, overexpression of G protein βγ subunits leads to an inhibition of Ca^{2+} currents that is characterized by a slowing of activation kinetics and which can be attenuated by a brief depolarizing prepulses preceding the actual test pulses, a phenomenon called facilitation (Ikeda, 1996; Herlitze et al, 1996). In untransfected PC12 cells, currents were rather insensitive to a prepulse facilitation, and amplitudes following the prepulse to +80 mV ($I_{Ca,pp}$) amounted to 106,5 ± 2.4 % of the currents recorded in the absence of a prepulse (I_{Ca}). In PC12 cells stably overexpressing $G\beta_1\gamma_2$ dimers, I_{Ca} exhibited slowed activation kinetics which were markedly accelerated by the facilitating prepulse. Moreover, due to the facilitation $I_{Ca,pp}$ amounted to 133,3 ± 6,5 % of I_{Ca} (figure 3-18B). In untransfected PC12

cells, ADP (10 μM) reduced I_{Ca} by 63,4 ± 1.2 % and $I_{Ca,pp}$ by 35,7 ± 6.6 % (n = 5; p < 0.01). In contrast, in $\beta_1\gamma_2$-overexpressing cells ADP reduced I_{Ca} and $I_{Ca,pp}$ by the same extent, by 47,9 ± 2,6 and 40,1 ± 3,6 % (n = 5; p > 0.1), respectively (figure 3-18D). Hence, in untransfected PC12 cells about one half of the ADP-induced inhibition of Ca^{2+} currents is voltage-dependent and thus removed by the facilitating prepulse. In $\beta_1\gamma_2$-overexpressing cells, however, there is no significant voltage-dependent inhibition caused by ADP as the facilitating prepulse failed to change the effects of the nucleotide. Nevertheless, the βγ-dimers did induce a voltage-dependent inhibition, as the Ca^{2+} currents were markedly facilitated by the prepulse even in the absence of ADP.

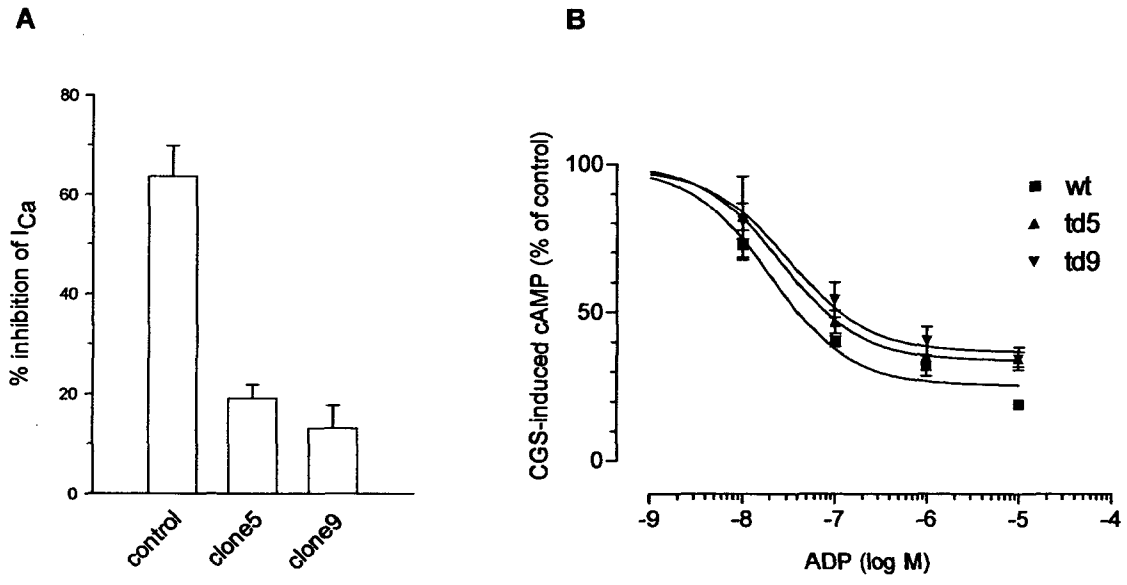

Figure 3-17; Effects of α-transducin overexpression on the ADP-induced inhibition of I_{Ca} and adenylyl cyclace. *(A), Ca^{2+} currents were recorded as described above (see figure X). In untransfected PC12 cells (control) 10μM ADP reduced I_{Ca} by 63,7 ± 16,2 % (n = 7), whereas in cells overexpressing α-transducin ADP reduced ICa only by 19,1 ± 10,9 % (clone 5; n = 16) and 13,1 ± 12,2 % (clone 9; n = 7). (B) After loading with [^{3}H]adenine, PC12 cells were incubated in RO 20-1724 (100 μM) for 105 min. During the last 15 min of this incubation period, 1 μM CGS 21680 was present either alone or together with the indicated concentrations of ADP. The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell cultures, and values obtained in the presence of CGS 21680 were normalized to the data obtained in its absence (normalized to basal). Results obtained in the presence of ADP are then expressed as percentage of those obtained in its absence (% of control; see Methods). Experiments were performed with untransfected PC12 cells (■), α-transducin clone 5 (▲) and clone 9 (▼).*

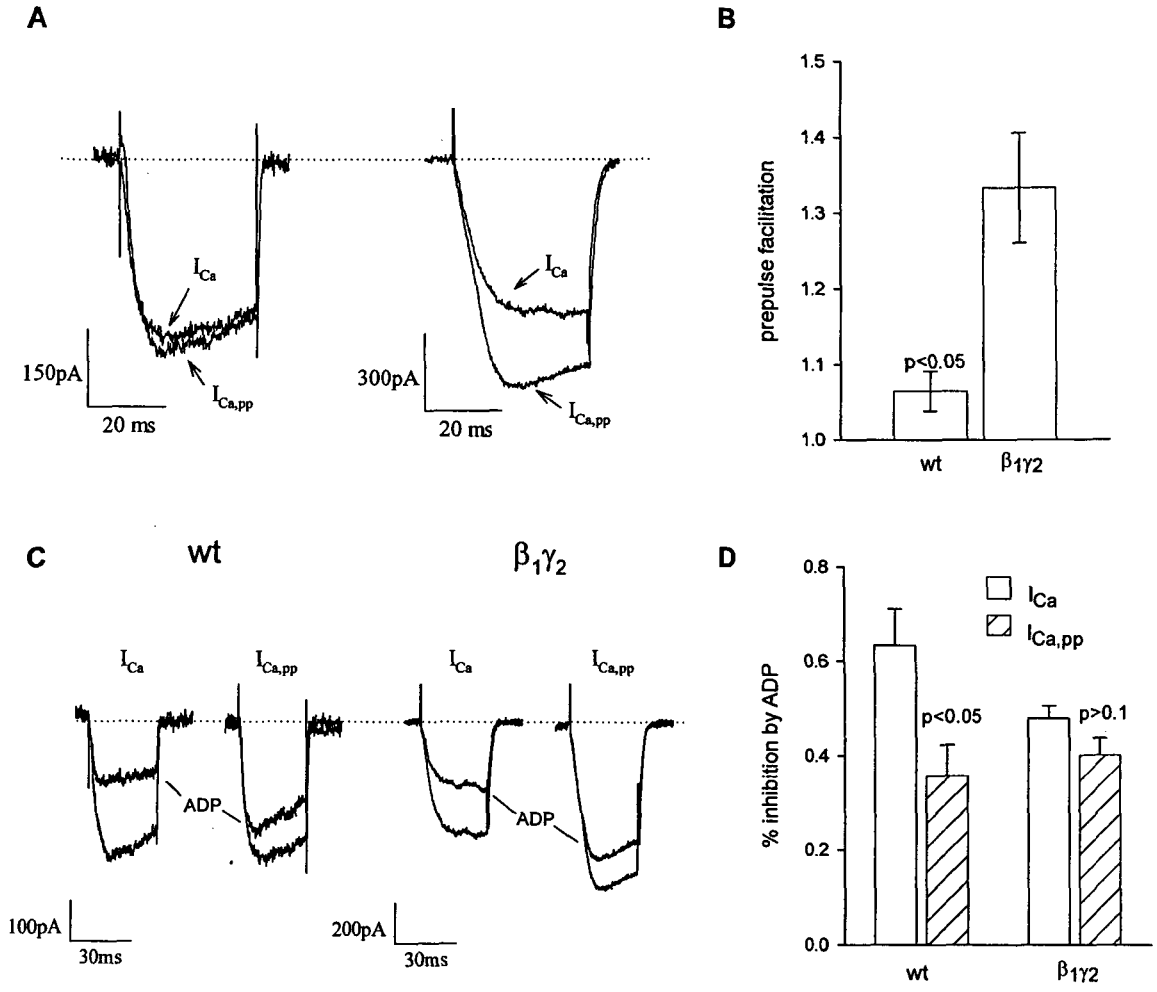

Figure 3-18, Effect of $\beta_1\gamma_2$ overexpression on ADP-induced inhibition of Ca^{2+} currents.
Ca^{2+} currents were recorded using the double pulse protocol (see methods). In A-D, currents elicited before and after the conditioning prepulse are referred to as I_{Ca} and $I_{Ca,pp}$, respectively. (A) In untransfected PC12 cells I_{Ca} was rather insensitive to prepulse facilitation (left traces, compare I_{Ca} and $I_{Ca,pp}$). In cells overexpressing $\beta_1\gamma_2$, I_{Ca} exhibited slowed activation kinetics (I_{Ca}, upper right trace), which were markedly accelerated by the prepulse ($I_{Ca,pp}$, lower right trace). (B) Prepulse facilitation was calculated as the ratio of peak current amplitudes $I_{Ca,pp}/I_{Ca}$. In wildtype PC12 cells (wt), facilitation amounted to 1,065 ± 0,024, while in cells stably overexpressing $\beta_1\gamma_2$-dimers facilitation was 1,333 ± 0,065 ($n = 5$; $p < 0,05$). (C), shows representative current traces recorded from wildtype PC12 cells (wt) and from cells overexpressing $\beta_1\gamma_2$, in the presence and absence of 10μM ADP (as indicated), before (I_{Ca}) and after ($I_{Ca,pp}$) the conditioning prepulse. (D), summarizes the effects of 10μM ADP on Ca^{2+} currents seen in (C) for a set of five cells. In wildtype PC12 cells ADP (10μM) reduced I_{Ca} by 63,4 ± 1.2 % and $I_{Ca,pp}$ by 35,7 ± 6.6 %, respectively ($n = 5$; $p < 0.01$). In cells transfected with $G\beta_1\gamma_2$, I_{Ca} and $I_{Ca,pp}$ were reduced by the same extent, 47,9 ± 2,6 and 40,1 ± 3,6 % ($n = 5$; $p > 0.1$), respectively.

4 Discussion

Acetylcholine and ATP are the major transmitters in the sympathetic nervous system. Both exert their actions by activating ionotropic receptors and/or G protein coupled receptors. G protein-coupled receptors in general are known to modulate ion channel activity, and so do muscarinic acetylcholine receptors (mAChRs) and P2Y nucleotide receptors. In the present work I investigated, how the modulation of voltage-gated ion channels, in particular Ca^{2+} channels and M-type potassium channels, by P2Y receptors and muscarinic actetylcholine receptors, respectively, affects the release of neurotransmitters from sympathetic neurons.

4.1 Part I

Acetylcholine has long been known to excite sympathetic neurons via muscarinic receptors by causing a slowly developing depolarisation. This effect is mediated by the closure of K_M channels (Brown, 1983). The present results show that this very mechanism is involved in noradrenaline release from rat SCG neurons triggered by the muscarinic agonist OxoM.

4.1.1 Identification of the receptor mediating the secretagogue action of OxoM.

OxoM is generally considered a pure muscarinic receptor agonist with some preference for the M_2 subtype (Caulfield, 1993). Nevertheless, evidence has been presented that OxoM may also activate nAChRs in either Xenopus myocytes (Reitstetter et al, 1994) or guinea pig neurons (Xian et al, 1994). In rat SCG neurons, as shown here, OxoM was able to induce inward currents at negative membrane potentials at concentrations $\geq$ 100 μM, and these currents were abolished by the nAChR antagonist mecamylamine. Hence, OxoM did activate nAChRs, but this effect did not contribute to its release stimulating effect since OxoM-induced noradrenaline release reached its maximum at 10 to 30 μM and was not significantly reduced by mecamylamine. Thus, OxoM triggered transmitter release exclusively via one or more subtypes of muscarinic receptors.

Amongst the five known subtypes of mAChRs, two (M_2 and M_4) are linked to PTX-sensitive G proteins, whereas M_1, M_3, and M_5 are coupled to toxin-insensitive G proteins (Caulfield and Birdsall, 1998; Eglen et al, 2001). PTX enhanced rather than prevented the secretagogue action of OxoM. Therefore, the release stimulating effect cannot be mediated by M_2 or M_4 receptors. This conclusion is supported by the fact that bethanechol failed to trigger transmitter release, but only inhibited electrically evoked release. In rat SCG neurons, this mAChR agonist selectively activates M_2 receptors (Liu and Rittenhouse, 2003). This result

corroborates the idea that M_2 and/or M_4 mAChRs are located at postganglionic sympathetic axon terminals and mediate an inhibition of noradrenaline release (Trendelenburg et al, 2003; Boehm and Kubista, 2002). Accordingly, the inactivation of their associated signalling cascades by PTX is likely to be the reason for the enhancement of OxoM-induced transmitter release by PTX as observed in the present study.

As outlined above, the secretagogue action of OxoM was mediated by M_1, M_3, and/or M_5 receptors. One can differentiate between these receptor subtypes by using, for instance, pirenzepine which displays an affinity of about 10 nM at M_1 receptors and at least tenfold lower affinities at M_3 and M_5 receptors (Caulfield, 1993; Caulfield and Birdsall, 1998). Pirenzepine caused a rightward shift in the concentration response curve for OxoM-induced noradrenaline release which indicates an underlying competitive mechanism, and the calculated pA_2 value (Arunlakshana and Schild, 1959) of 7.4 suggests an antagonist affinity of about 40 nM. To verify whether the secretagogue effect was indeed mediated by M_1 mAChRs, cultures were incubated in MT-7 which selectively and for up to 8 h irreversibly blocks M_1 receptors (Olianas et al, 2000; Caulfield and Birdsall, 1998). In MT-7 treated cultures, the secretagogue action of OxoM was entirely lost. We therefore conclude that OxoM triggered transmitter release from rat SCG neurons via M_1 mAChRs.

4.1.2 *The M_1 mAChR mediates both, the inhibition of I_M and the stimulation of noradrenaline release.*

It is well established that the muscarinic inhibition of I_M in SCG neurons is mediated by the M_1 receptor subtype (Marrion et al, 1989; Bernheim et al, 1992; Shapiro et al, 2001). This is the same receptor subtype as the one that was found to mediate the secretagogue action of OxoM. Therefore, one might expect that OxoM evokes noradrenaline release and reduces I_M in the same range of concentrations. In contrast to this expectation, more than tenfold higher concentrations of OxoM were required to trigger transmitter release than to elicit an inhibition of I_M (compare figure 1B and D). However, as soon as the release inhibiting mechanisms of mAChRs were inactivated by PTX (see above), these two effects displayed similar values for half-maximal concentrations of OxoM: 1 μM for the inhibition of I_M and 3 μM for the stimulation of transmitter release. Furthermore, both effects were antagonized by pirenzepine in an apparently competitive manner. The calculation of pA_2 values (Arunlakshana and Shild, 1959) from the rightward shifts of the two concentration response curves in the presence of pirenzepine yielded 7.4 for the stimulation of release and 7.6 for the inhibition of I_M. Thus, pirenzepine antagonized both effects with apparently identical receptor affinity, which

indicates that only one receptor subtype was involved. Previously, a pA_2 value of 7.5 has been reported for the antagonism between pirenzepine and muscarine in the inhibition of I_M in rat SCG neurons (Marrion et al, 1989). Furthermore, both effects were entirely abolished by the M_1 receptor-selective toxin MT-7. Thus, the M_1 receptor mediates both, the inhibition of I_M and the stimulation of transmitter release.

4.1.3 *The inhibition of I_M is involved in the stimulation of noradrenaline release by OxoM*

The above results suggest that the inhibition of I_M might be involved in OxoM-induced noradrenaline release. This idea is also supported by the previous observation that agents which block I_M in SCG neurons independently of neurotransmitter receptors also trigger transmitter release: the K_M channel blockers linopirdine and Ba^{2+} evoked noradrenaline release in an entirely Cd^{2+}- and TTX-sensitive manner (Kristufek et al, 1999), as shown in the present study for the mAChR agonist OxoM (Figure 3-12C). Thus, M_1 receptor activation as well as direct K_M channel blockade elicits action potentials with ensuing transmembrane Ca^{2+} entry and concomitant vesicle exocytosis.

To verify whether K_M channels are involved in the secretagogue action of OxoM, the effects of the anticonvulsant retigabine were investigated here. Retigabine has been shown to enhance currents through channels formed by KCNQ2 and -3 heteromultimers (Main et al, 2000; Wickenden et al, 2000) and to selectively enhance I_M in rat SCG neurons (Tatulian et al, 2001). Accordingly, in the presence of retigabine, non-inactivating outward currents at –30 mV were enhanced, but I_M relaxation amplitudes caused by hyperpolarisations to –55 mV were reduced. These complex changes arose due to a shift in the voltage-dependence of K_M channels by about –20 mV, as reported before (Main et al, 2000; Tatulian et al, 2001). In parallel with these changes in the voltage-dependence of I_M, retigabine hyperpolarized the membrane potential of SCG neurons also by 20 mV. Similar retigabine-induced changes in membrane potential have been observed in Xenopus oocytes expressing only KCNQ2/3 channels (Main et al, 2000). The congruence of these results suggests that I_M is a major determinant of the membrane potential in SCG neurons.

This conclusion is also supported by the finding that OxoM depolarised the neurons in parallel with the blockade of K_M channels. In at least one third of the neurons, OxoM also triggered action potential discharge which finally led to transmitter release. In the presence of retigabine, OxoM still depolarised the neurons, but always failed to trigger action potentials. Thus, retigabine did not interfere with the activation of M_1 receptor-associated signalling cascades by OxoM, but only neutralized its effect on outward currents and on the membrane

potential (Figures 6E and 7B). Nevertheless, retigabine did abolish noradrenaline release evoked by the muscarinic agonist in a concentration-dependent manner. Electrically evoked release, in contrast, remained largely unchanged in the presence of retigabine. This lack of effect of retigabine might have been attributable to supramaximal electrical field stimulation intensities used in radiotracer release experiments, since the K_M channel opener rendered single SCG neurons less likely to fire action potentials in response to depolarising current injections. Nevertheless, when the strengths of electrical fields were reduced, [^{3}H]noradrenaline release declined, but retigabine still failed to cause a significant reduction. Furthermore, retigabine left action potential waveforms unaltered. Thus, retigabine did not interfere with some general mechanism of action potential-dependent vesicle exocytosis. Accordingly, the OxoM-induced noradrenaline release must have been abolished by retigabine because of the change in the voltage-dependence of K_M channels which indicate that the inhibition of K_M channels is involved in the stimulation of transmitter release.

Noradrenaline release from dissociated sympathetic neurons in primary cell culture occurs only at axons or axon terminals and not at neuronal somata (Boehm, 1999; Boehm and Huck, 1997). The fact that OxoM-induced release was entirely abolished by TTX indicates that the receptors involved were remote from the release sites. Thus, the release stimulating M_1 receptors are not presynaptic ones, but rather located at the somatodendritic region of SCG neurons. In addition to the inhibition of K_M channels, M_1 receptors of SCG neurons are linked to other intracellular signalling cascades, in particular to the inositol triphosphate-dependent liberation of Ca^{2+} from intracellular stores (del Rio et al, 1999). Considering that the release stimulating M_1 receptors are not directly located at axon terminals, it appears unlikely that OxoM-induced increases in intracellular Ca^{2+} might contribute to the stimulation of noradrenaline release. This assumption was corroborated by the finding that depletion of intracellular Ca^{2+} stores by thapsigargin did not alter OxoM-induced transmitter release. Previously, this Ca^{2+}-ATPase inhibitor was found not to affect the inhibition of K_M channels via M_1 receptors (del Rio et al, 1999; Bofill-Cardona et al, 2000). Thus, stimulation of transmitter release and inhibition of I_M by OxoM are both independent of Ca^{2+} release from intracellular stores.

In conclusion, the present results demonstrate that activation of M_1 mAChRs triggers action potential discharges and concomitant transmitter release from SCG neurons. The link between receptor activation and action potentials is most likely provided by the inhibition of K_M channels.

4.2 Part II

The fact that P2X receptors mediate and/or modulate synaptic transmission in central and peripheral neurons is quite well established (reviewed in Khakh, 2001). By contrast, much less is known about the role of P2Y receptors, in particular inhibitory P2Y receptors. During the last 15 years, several studies provided evidence for the existence of presynaptic nucleotide receptors that mediate a feedback inhibition of sympathetic transmitter release. Originally these inhibitory effects were thought to be mediated by adenosine, a degradation product of ATP, but with the use of selective P2 receptor antagonists, it became apparent that it were rather P2 receptors, most likely metabotropic P2Y receptors, that were responsible for the presynaptic inhibition of transmitter release, but the particular receptor subtype has never been identified.

Recently we found that adenine nucleotides inhibit voltage-gated calcium channels in PC12 cells, which are ontogentically related to sympathetic neurons, and the rank order of agonist potency was the following: 2-MeSADP = 2MeSATP > ADPβS > ADP = ATP. Uridine nucleotides, in contrast, were ineffective, and the inhibitiory action of ADP was antagonized only by reactive blue 2, but not by any other prototypical P2 receptor antagonist, such as A3P5P, PPADS, or suramin (Vartian and Boehm, 2001). This pharmacological profile was similar to those reported for inhibitory presynaptic P2Y receptors, and similar to those of recombinant $P2Y_{12}$ receptors (Hollopeter et al, 2001; Zhang et al, 2001; Takasaki et al, 2001). However, reactive blue 2 blocks many P2 receptor subtypes, including $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_{12}$ (Hansmann et al, 1997; Bogdanov et al, 1998; Takasaki et al, 2001) and thus it remained unclear which P2Y receptor subtype mediated the observed inhibition of I_{Ca}. To establish whether $P2Y_{12}$ receptors might be involved in the regulation of VACCs by adenine nucleotides, we now used 2-MeSAMP and AR-C69931MX, antagonists at recombinant $P2Y_{12}$ receptors (Hollopeter et al, 2001; Takasaki et al, 2001).

4.2.1 $P2Y_{12}$ receptors mediate both, the inhibition of I_{Ca} and of adenylyl cyclase

In analogy to platelets (Hollopeter et al, 2001; Foster et al, 2001) and glioma cells (Jin et al, 2001), the ADP-dependent inhibition of adenylyl cyclase activity in PC12 cells was suggested to be mediated by $P2Y_{12}$ receptors (Unterberger et al, 2002). In support of this conclusion, both 2-MeSAMP (30 μM) and AR-C69931MX (100 nM) caused a rightward shift of the concentration response curve for the ADP-dependent reduction of the cyclic AMP

accumulation elicited by the A_{2A} receptor agonist CGS 21680. As the maximal inhibition by ADP was not altered, one may infer a competitive mechanism. The pA_2 values (Arunlakshana and Schild, 1959) calculated on the basis of this assumption were 6.1 for 2-MeSAMP and 8.4 for AR-C69931MX. Thus, these two antagonists block the P2Y receptors mediating the inhibition of adenylyl cyclase in PC12 cells with apparent affinities in the low micromolar and nanomolar range, respectively.

When whole-cell I_{Ca} of PC12 cells was reduced by 100 μM ADP, this effect was attenuated by 2-MeSAMP at 3 to 300 μM and by AR-C69931MX at 0.01 to 10 μM. Moreover, 2-MeSAMP required less than 15 s to diffuse from the receptor in order to permit reinhibition of I_{Ca} by ADP, whereas AR-C69931MX required about 2 min to do so. These results indicated that both agents blocked the receptor mediating the ADP-dependent modulation of VACCs and suggested that AR-C69931MX is equipped with a considerably higher affinity. To investigate the interaction between ADP and the two antagonists with respect to the inhibition of I_{Ca} in further detail, increasing concentrations of the agonistic nucleotide were applied in the absence and presence of several concentrations of these antagonists. As with the ADP-induced inhibition of cyclic AMP synthesis, there was no change in the maximal effect of ADP, but the concentration response curves were shifted to the right. Application of the analysis introduced by Arunlakshana and Schild (1959) indicated underlying competitive mechanisms with pK_B values for the two antagonists of 5.4 (2-MeSAMP) and 8.7 (AR-C69931MX), respectively. Thus, the two antagonists interfered with the ADP-dependent inhibition of I_{Ca} in the same range of concentrations as with the P2Y receptor-mediated inhibition of adenylyl cyclase. These results indicate that in PC12 cells, one single P2Y receptor subtype mediates not only an inhibition of adenylyl cyclase, but also of VACCs.

AR-C69931MX displayed an about 1000-fold higher affinity for the receptor mediating the inhibition of I_{Ca} in PC12 cells than 2-MeSAMP. At recombinant $P2Y_{12}$ receptors, AR-C69931MX is also about 1000-fold more potent an antagonist than 2-MeSAMP (Takasaki et al, 2001). Moreover, the apparent affinities of AR-C69931MX and 2-MeSAMP for the receptor mediating the modulation of VACCs in PC12 cells (K_B values of 2 nM and 4 μM, respectively) correlate well with their reported potencies in blocking heterologously expressed $P2Y_{12}$ receptors (half maximal inhibition at 2 nM and 5 μM, respectively; Takasaki et al, 2001, Hollopeter et al, 2001). Taken together, these data suggest that it was $P2Y_{12}$ receptors that mediated the inhibition of both, adenylyl cyclase and VACCs.

The observed rank order of agonist potency (2-MeSADP > ADPβS > ADP) may also be indicative of a role of $P2Y_1$ receptors. In addition, heterologously expressed $P2Y_1$ receptors

were shown to mediate an adenine nucleotide-dependent inhibition of VACCs in sympathetic neurons (Filippov et al, 2000). Thus, $P2Y_1$ receptors might also be involved in the adenine nucleotide-dependent reduction of I_{Ca} in PC12 cells. However, the inhibition of I_{Ca} by ADPβS and 2-MeSADP was also antagonized by nanomolar concentrations of AR-C69931MX, and the reduction of I_{Ca} by ADP is not attenuated by $P2Y_1$ receptor antagonists (present results and Vartian and Boehm, 2001). Most importantly, PC12 cells were reported not to express $P2Y_1$ receptors (Arslan et al, 2000; Unterberger et al, 2002). Thus, $P2Y_1$ receptors did not contribute to the inhibiton of VACCs by ADP in PC12 cells.

A receptor that displays pharmacological characteristics similar to those of $P2Y_{12}$ is the $P2Y_{13}$ receptor (Communi et al, 2001; Zhang et al, 2002). This receptor subtype is also capable of mediating an inhibition of adenylyl cyclase and might thus be involved in the effects described above. At both, the human and murine variants of the $P2Y_{13}$ receptor, 2-MeSADP, ADPβS, and ADP are more or less equipotent agonists (Communi et al, 2001; Zhang et al, 2002), whereas at recombinant $P2Y_{12}$ receptors (Takasaki et al, 2001) and at the receptor mediating the inhibition of VACCs in PC12 cells (Vartian and Boehm, 2001), 2-MeSADP is about 1000-fold more potent than ADP. Moreover it was shown, that AR-C69931MX acts as a non-competitive antagonist at human $P2Y_{13}$ receptors, while it is a competitive antagonist at human and murine variants of $P2Y_{12}$ (Marteau et al, 2003) and at the receptor mediating the inhibition of VACCs in PC12 cells (see figure 3-12). These discrepancies suggest that $P2Y_{13}$ receptors do not contribute to the inhibition of I_{Ca} in PC12 cells.

4.2.2 *$P2Y_{12}$ receptors mediate an inhibition of transmitter release*

As mentioned before, P2Y receptors with similar pharmacological characteristics to those observed for the $P2Y_{12}$ receptor, were shown to mediate a presynaptic feedback inhibition of sympathetic transmitter release. Hence we tested, whether activation of $P2Y_{12}$ receptors may also mediate an inhibition of transmitter release from PC12 cells. ADP reduced ^{3}[H]noradrenaline release, evoked by depolarization with high potassium concentrations (30mM), in a concentration dependent manner (EC_{50} = 0,08 μM). In the presence of AR-C69931MX the concentration dependence of this inhibition was shifted towards higher concentrations (EC_{50} = 5,5μM). Since the maximal effect of ADP was not altered by the presence of AR-C69931MX, one can assume an underlying competitive mechanism and calculate a pA_2 value by applying the equation $\log(CR-1) = pA_2 + \log [B]$ (Arunlakshana & Schild, 1959). The resulting pA_2 value (8,8) shows considerable correlation with the affinity values obtained for the inhibition of I_{Ca} (pK_B = 8,7) and adenylyl cyclase (pA_2 = 8,5), which

strongly suggests that all three effects are mediated by the very same receptor, namely $P2Y_{12}$ (as discussed above).

4.2.3 *The inhibition of I_{Ca} is involved in the inhibition of transmitter release*

Besides the inhibition of VACCs, other mechanisms that act downstream of calcium entry have also been reported to modulate transmitter release. Hence it is not self-evident that the $P2Y_{12}$ mediated inhibition of I_{Ca} is responsible for the ADP-induced inhibition of noradrenaline release. To clarify this, we further investigated the mechanisms underlying the inhibitory action of ADP on noradrenaline release. Application of 100µM Cd^{2+}, which is known to be a potent blocker of voltage-gated calcium channels, completely abolished noradrenaline release evoked by depolarization with 30mM K^+ (figure 3-15). Although this clearly demonstrates that calcium influx through VACCs is required for depolarization-evoked (30mM K^+) transmitter release and that principally an inhibition of these channels by ADP may also be sufficient to reduce noradrenaline release, it does not rule out the contribution of additional mechanisms that act downstream of calcium entry. Hence, noradrenaline release was alternatively evoked by application of ATP. ATP activates ionotropic P2X receptors that mediate calcium entry, and thus triggers transmitter release independently of VACCs (Nakazawa & Inoue, 1992). As expected, Cd^{2+} did not reduce ATP-induced noradrenaline release, which supports the assumption that this type of stimulation does not require calcium influx through VACCs. Moreover, ATP-induced noradrenaline release was not affected by the application of ADP. Hence, the ADP-induced inhibition of stimulation-evoked transmitter release is based on an inhibition of voltage-gated calcium channels that is mediated by $P2Y_{12}$ receptors.

4.2.4 *Increased cAMP attenuates the inhibition via $P2Y_{12}$ receptors*

The intracellular accumulation of cyclic AMP has consistently been shown to cause an enhancement of stimulation-evoked transmitter release from sympathetic neurons (reviewed in Boehm & Kubista, 2002). Since ADP not only suppresses Ca^{2+} currents, but also inhibits adenylyl cyclase, we were interested whether this latter effect may be involved in the inhibition of noradrenaline release. Therefore cAMP levels were elevated by application of either CGS21680, forskolin or pCPT-cAMP. CGS21680 elevates intracellular cAMP levels by activating A_{2A} adenosine receptors while forskolin directly activates adenylyl cyclase. pCPT-cAMP is a membrane permeant analogue of cAMP that is known to mimic the effects of cAMP. However, neither of these substances enhanced stimulation-evoked noradrenaline

release per se (figure 3-16A). Moreover, ADP-induced inhibition of noradrenaline release was not altered in the presence of CGS21680 or forskolin, which is not too surprising, since ADP has been shown to markedly reduce cAMP accumulation induced by these substances (see figure 3-9; Unterberger et al, 2002) and thus no major change in cAMP levels can be expected. Interestingly, when cAMP levels were elevated independently of adenylyl cyclase activity by using pCPT-cAMP, the ADP-induced inhibition of release was reduced by more than 50% (figure 3-16B). Since pCPT-cAMP did not alter stimulation-evoked release per se, it was straightforward to assume that it might interfere with the G-protein dependent inhibition of I_{Ca} but does not affect channel activity itself or any events downstream of calcium entry. In accordance with this idea, we found that the ADP mediated inhibition of I_{Ca} was modified in cells pretreated with pCPT-cAMP. Although the reduction of peak current amplitudes was hardly affected, the slowing of activation kinetics, which is characteristic for the G-protein dependent inhibition of Ca^{2+} currents, was completely aboslished (figure 3-16C) after treatment with pCPT-cAMP. Hence, the inhibition of Ca^{2+} current amplitudes by ADP was significantly reduced by pCPT-cAMP when measured ~5ms after the beginning of the depolarization, but not when measured at the end of the 30 ms test pulse (figure 3-16D). Under physiological conditions as well as in the release experiments, calcium channels are not activated by 30 ms test pulses, but rather open in response to action potential discharges that last only a few milliseconds. Thus the effect of pCPT-cAMP on the kinetic component of Ca^{2+} current inhibition (early phase of the test pulse) appears more relevant and provides an explanation for the reduction of the ADP-induced inhibition observed in the ^{3}[H]noradrenaline release experiments (figure 3-16B).

4.2.5 *The inhibition of I_{Ca} via $P2Y_{12}$ receptors involves more than one signal cascade*

The ADP induced inhibition of I_{Ca} has previously been shown to be pertussis toxin (PTX)-sensitive but was only partly relieved by application of a conditioning prepulse (Vartian & Boehm, 2001). A possible explanation for this incomplete reversal of inhibition may be the fact that the ADP-induced inhibition of I_{Ca} is mediated by two distinct pathways. To test this hypothesis we further investigated the mechanisms underlying the inhibition of I_{Ca}. PTX-sensitive voltage-dependent inhibition is thought to be primarily mediated by a direct interaction of $G\beta\gamma$-dimers derived from G_o proteins with the calcium channel α_1-subunit (Caulfield et al, 1994). In accordance with this, overexpression of the $\beta\gamma$-sequestering agent α-transducin, markedly reduced ADP-induced inhibition of I_{Ca} (>80%). Although

overexpression of βγ-sequestering agents is a common method to investigate effects of βγ-dimers, there are some concerns about their use, since they may not bind all βγ-dimers and may interfere with the receptor-G-protein interaction. The latter problem can be excluded, since α-transducin did not affect the $P2Y_{12}$ mediated inhibition of adenylyl cyclase. Besides ruling out a possible transducin-receptor interaction, this experiment also suggests, that the inhibition of adenylyl cyclase involves G protein α subunits rather than βγ-dimers. However, α-transducin abolished almost all of the ADP-induce inhibition of I_{Ca}, but since it binds various combinations of β and γ isoforms (Delmas et al, 1999), it still remained unclear whether multiple pathways are involved in the ADP-induced inhibition or not.

To clarify this, PC12 cell were alternatively transfected with a construct encoding for β_1 and γ_2 subunits. Overexpression of $\beta_1\gamma_2$-dimers has previously been reported to mimic the voltage-dependent inhibition of N-type calcium channels (Ikeda, 1996; Herlitze, 1996). As expected, Ca^{2+} currents in cells stably overexpressing $G\beta_1\gamma_2$ exhibited slowed activation kinetics due to a tonic inhibition by free βγ-dimers that could be relieved by a depolarizing prepulse prior to the test pulse (figure 3-18C). Hence $\beta_1\gamma_2$ mediated inhibition was voltage-dependent (VD). Interestingly ADP still further reduced I_{Ca} by 47,9% in these clones. This additional inhibition seemed to be voltage-independent (VI), since it was not changed by the conditioning prepulse (figure 3-18C). The total inhibition of I_{Ca} ($\beta_1\gamma_2$ + ADP) amounted to 66,1 %, with ~30 % being contributed by $\beta_1\gamma_2$ and the residual ~36 % being contributed by some other, voltage-independent mechanism. This correlated well with the results obtained in untransfected PC12 cells. There ADP reduced I_{Ca} by 63,7 %, with ~29 % (VD) being relieved by the prepulse and the residual ~35 % being presumably voltage-independent (VI).

Taken together, the results suggest that the ADP-induced inhibition of voltage-gated calcium channels involves two distinct mechanisms: a voltage-dependent action of $\beta_1\gamma_2$-dimers and a voltage-independent action that is probably mediated by a different βγ pair. The idea that different combinations of Gβ and Gγ subunits may exert different effects on Ca^{2+} channels is supported by the observation that voltage-dependent inhibition is replicated by exogenous expression of $G\beta_1$ and $G\beta_2$ subunits but not $G\beta_3$ and $G\beta_4$ (Garcia D.E. et al, 1998). Moreover, it was shown that the PTX-sensitive adrenergic inhibition of N-type Ca^{2+} currents in chick sensory neurons (Diversé-Pierluissi et al, 1995, 1997) and in rat sympathetic neurons (Delmas et al, 1999) that has previously been thought to be primarily mediated by βγ-dimers derived from G_o proteins also involves different βγ-dimers derived from G_i proteins that appear to

contribute a voltage-independent component to the inhibition. However, whether the different βγ-dimers are released from the same receptor ($P2Y_{12}$) that couples to multiple G-protein trimers or from a different yet unidentified receptor subtype still remains to be established.

In conclusion the data demonstrate that activation of $P2Y_{12}$ receptors mediates both, the inhibition of adenylyl cyclase and of voltage-gated calcium channels. Moreover, the inhibition of Ca^{2+} channels causes a reduction of stimulation-evoked noradrenaline release. Since $P2Y_{12}$ receptors are also expressed in sympathetic neurons (unpublished observation) one may infer that at least some of the previously described inhibitory presynaptic nucleotide receptors belong to the $P2Y_{12}$ subtype.

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