

On the Autonomic Control of Blood Flow and Secretion in Salivary Glands

**Functional and morphological aspects on muscarinic
receptor subtypes in different species**

By

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*Till Pappa och Ninni,
och till minnet av dem
som inte fick följa mig hela vägen*

Abstract

Parasympathetic nervous activity is the principal stimulus for evoking fluid responses within salivary glands. Concomitantly to the onset of this response, the blood flow increases. The responses, in particular the vasodilatation, consist of an atropine-sensitive acetylcholine-mediated part and an atropine-resistant part mediated via non-adrenergic, non-cholinergic (NANC) transmitters. It has been generally agreed that the cholinergic effects are mediated by muscarinic M3 receptors. However, this view has been questioned, since most muscarinic receptors are expressed and muscarinic M1 receptors elicit functional effects in salivary glands. The distribution and function of the muscarinic receptors is not unravelled, neither according to secretion nor vasodilatation. The aim of this thesis has been to investigate the roles of different muscarinic subtypes in the control of blood flow and secretion in salivary glands.

In the thesis, the expression of muscarinic receptors in salivary glands and related blood vessels was investigated using immunoblotting and/or immunohistochemistry. Furthermore the effects of muscarinic stimulation and blockade were investigated on isolated vessels, on the secretion of saliva, on glandular blood flow and vessel permeability. The thesis includes observations on rats, sheep and man.

It is shown that M1 receptors contribute considerably, in addition to the functionally most significant M3 receptor, to the fluid secretory responses of rats and sheep. The M1 receptor is particularly apparent in seromucous and mucous glands, and of particular functional significance at low intense stimulation. Since the occurrence pattern was the same in human salivary glands, M1 receptors may be of significance in man also. Notably, in the human glands, inflammation increased the expression of muscarinic M5 receptors. In the arterial blood vessels muscarinic M1 receptors generally occurred in the endothelium, and muscarinic M5 receptors, and possibly M3 also, were detected in the smooth muscle. In venous endothelium muscarinic M1 and M4 receptors occurred, while M1 and/or M3 were expressed in the smooth muscle layer. Cholinergic stimulation generally caused arterial vasodilatation, which was mainly dependent on nitric oxide. The response was mediated by muscarinic M1 and possibly M5 receptors, in addition to the M3 receptor. The venous response included a contractile

M1 mediated component that may preserve perfusion pressure during the secretory process. In tissue in close vicinity to the parenchymal tissues, M1 and in particular M4 receptors occurred. In the sheep, the increase of submandibular secretory and vasodilator responses to electrical stimulation of the parasympathetic nerve in the presence of muscarinic antagonists were explained by neuronal muscarinic M4 receptors. These receptors inhibited the release of transmitters as was shown for the NANC transmitter VIP. The role of muscarinic M5 receptors is unclear but may affect on the vascular response or more likely to be correlated to inflammation.

In general, the expression pattern and functions of the muscarinic receptors subtypes showed resemblance in the examined species. All muscarinic receptors occur in the salivary glands. In seromucous/mucous glands, muscarinic M1 receptors contribute substantially to the secretory response. In the vasculature, the muscarinic receptor subtypes interact, possibly via autocrine mechanisms, for preserving the hemodynamics in the glands.

Keywords: Muscarinic receptor, salivary gland, vasoactive intestinal peptide, blood flow, human, rat sheep

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals

Paper I

Expression of muscarinic receptor subtypes in salivary glands of rats, sheep and man.

Anders T Ryberg, Gunnar Warfvinge, Louise Axelsson, Ondrej Soukup, Bengt Götrick and Gunnar Tobin

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Paper II

In vitro cholinergic effects and muscarinic receptor expression in blood vessels of the rat

Anders T Ryberg, Hanna Selberg, Ondrej Soukup, Kathryn Gradin and Gunnar Tobin

Submitted

Paper III

Distribution and function of muscarinic receptor subtypes in the ovine submandibular gland

Gunnar Tobin, Anders T Ryberg, Scott Gentle and the late Anthony V Edwards

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Paper IV

In vivo effects of muscarinic receptor antagonists on the release of VIP in the ovine submandibular gland

Anders T Ryberg, Ondrej Soukup, Gunnar Tobin

Submitted

Reprint of the papers has been approved by the respective publisher.

Contents

Abstract	4
List of abbreviations	8
Introduction	9
Background.....	9
<u>Aims</u>	16
Materials and methods.....	17
Immunoblotting.....	17
Immunohistochemistry	18
In vitro experiments on blood vessels.....	18
In vivo experiments.....	19
Drugs.....	21
Calculations and statistics.....	21
Results and discussion.....	23
I. Expression of muscarinic receptors.....	23
Glandular tissue (papers I and III).....	26
Stromal tissue (papers I and III)	30
II. Functional characterization.....	32
In vitro effects on blood vessel contraction (paper II)	33
In vivo effects on blood flow (papers I, III and IV)	39
In vivo effects on vascular permeability (paper II)	44
Secretory effects of muscarinic receptors	47
In vivo effects on secretion of fluid (papers I, III and IV).....	47
In vivo effects on secretion of protein (papers I, III and IV).....	51
III. Neuronal release of transmitter (paper IV).....	57
General discussion.....	61
Concluding comments	65
Acknowledgements	68

List of abbreviations

BSA	bovine serum albumin
4-DAMP	diphenylacetoxy- <i>N</i> -methylpiperidine methiodide
L-NNA	<i>N</i> - ω -nitro- <i>L</i> -arginine
NANC	non-adrenergic, non-cholinergic
NO	nitric oxide
p-F-HHSiD	p-fluoro-hexahydro-sila-diphenidol hydrochloride
SVR	submandibular vascular resistance
VIP	vasoactive intestinal peptide

Introduction

Background

The blood flow in salivary glands is largely controlled by the autonomic innervation, likewise to the secretory process (Proctor & Carpenter, 2007). The two functions are due to activity in different sets of nerve fibres (Emmelin & Engstrom, 1960), but any direct nerve evoked change may indirectly influence responses regulated by the other type. So may, in due course, a total vasoconstriction cause the secretory fluid response to cease (Lung, 1998; Thakor *et al.*, 2003). The glandular blood flow is the effect of perfusion pressure and the resistance within the glandular vasculature. This is either increased or decreased by the activity within the autonomic innervation, while the activity in any of the autonomic nervous divisions increases secretion (Emmelin, 1981). At rest, the vascular resistance is largely under the influence of the tone of sympathetic innervation. However, concomitantly to parasympathetic nerve-evoked flow of saliva the parasympathetic activity causes vasodilatation (Edwards, 1998). Since the plasma fluid is a pre-requisite for a persisting secretory response, any change in the perfusion pressure will have impact on the secretory response, unless compensated for (Thakor *et al.*, 2003). A short-lasting flow of saliva in response to electrical stimulation of the parasympathetic innervation of salivary glands is possible to achieve without any increase in the blood flow to the salivary glands (Lung, 1998). However, an ongoing flow of blood is crucial for maintaining the sustained response (Thakor *et al.*, 2003).

The chemical transmission of parasympathetic nerve signals, involves, in addition to the classical transmitter acetylcholine, non-adrenergic, non-cholinergic peptidergic (NANC) transmitters, such as vasoactive intestinal peptide (VIP) and substance P, which regulate secretory as well as vasodilator responses (see Ekström, 1999). At intense electrical stimulation, the parasympathetically nerve-evoked vasodilatation shows a conspicuous resistance to atropine. VIP has been shown to be an important transmitter in this part, perhaps the most important (Edwards, 1998). However, at less intense stimulation of the parasympathetic innervation of salivary glands, atropine inhibits or even abolishes the vasodilator response as has been shown in the feline, rat and ovine submandibular glands (Emmelin *et al.*, 1968; Lundberg *et al.*, 1981b;

Edwards *et al.*, 2003). Thus, the parasympathetic nerve may via acetylcholine cause vasodilatation in salivary glands. Generally, acetylcholine represents a vasodilator of most vascular beds in the orofacial region (Kummer & Haberberger, 1999). Even so, acetylcholine seems to be of less importance for the vasodilator response, at least the sustained vasodilatation at intense parasympathetic nerve activity, while it is the principal mediator of secretory stimulator signals. The transient, immediate part of the parasympathetic nerve-evoked increase in salivary gland blood flow is more sensitive towards atropine than the sustained phase (Lundberg *et al.*, 1981b). Also, the vasodilatation evoked by exogenous acetylcholine mimics the phasic response of the nerve-evoked vasodilatation (Lundberg *et al.*, 1982). Furthermore, one part of the acetylcholine-evoked vasodilatation is dependent on the synthesis of nitric oxide (NO), and one is not. Also, one part of the response seems dependent on an intact endothelium (Anderson & Garrett, 2004).

Generally, salivary glands are densely innervated by cholinergic fibres, which occur close to acini and ducts as well as myoepithelial cells (Garrett, 1999). However, cholinergic innervation of blood vessels is a matter of debate since no cholinergic nerves have been clearly visualized in the vicinity of blood vessels, at least not reaching the intimal vessel parts (van Zwieten *et al.*, 1995). As mentioned above, electrical stimulation of the glandular parasympathetic nerves induces an atropine-sensitive vasodilatation (via acetylcholine) in a number of different species. Needless to say, it does not necessarily mean that the effect is directly evoked. It should be noted that there exist non-neuronal sources for the release of acetylcholine also, such as endothelial cells. Kummer and Haberberger (1999) put forward the hypothesis that an intimal cholinergic system regulates basal vascular tone responding to local stimuli, while the perivascular nerve fibres act on top of this by providing fine tuning in response to reflex activation due to systemic demands. This means that acetylcholine may have an autoregulatory function, *e.g.* release by shear stress (Ayer *et al.*, 2007). According to the extrinsic system, it could be expected that resistance vessels are the major target of cholinergic innervation. And conformingly, in the vasculatures being cholinergically innervated, *i.e.* in the lung and tongue, axons preferentially occur at large arteries, and the frequency of their occurrence decrease towards the periphery (Haberberger *et al.*, 1997; Henrich *et al.*, 2003). The same pattern has

been shown in salivary glands where cholinergic perivascular nerves seem to principally occur at glandular arteries in rat submandibular glands (Jones, 1979).

Theoretically, the hydrostatic blood pressure in the glandular venous outflow would be lowered by a profound secretory response in parallel with an increase of the oncotic pressure along the flow of blood through the salivary gland vasculature. This could be hazardous in cases of profuse secretion. However, during the parasympathetic secretory process, the hilar venous pressure increases in the canine submandibular gland (Lung, 1998). The effect was frequency-dependent and occurred even under controlled blood flow, *i.e.* it did not depend on a passive flooding effect. Therefore, the opening of arteriovenous anastomoses was tentatively put forward as a plausible explanation. However, all salivary glands do not express arteriovenous anastomoses, such as those in the rat and the rabbit (Fraser & Smaje, 1977; Ohtani *et al.*, 1983). Some mechanism overriding the decrease in hydrostatic pressure and increase in oncotic pressure is likely to occur. Thus, the preservation of the transmitted pressures must be exerted by some other mechanism in the latter species.

The acetylcholine-evoked vasodilatation was for long considered to be more or less dependent on NO synthesis. This idea was largely based on the much publicised findings by Furchgott and Zawadski (1980) in helical strip preparations of the rabbit descending thoracic aorta. Here, acetylcholine was considered to cause contraction until Furchgott and Zawadski reported that acetylcholine could induce relaxation at a low concentration (Furchgott, 1999). However, if the intimal surface was rubbed off, the cholinergic relaxation was changed into contraction. The factor in the phenomenon was in due course identified as nitric oxide. In salivary glands, NO has been shown to be of importance for cholinergic vasodilator responses, but also for VIPergic (Edwards & Garrett, 1993; Edwards *et al.*, 1996; Tobin *et al.*, 1997; Anderson & Garrett, 1998; Hanna & Edwards, 1998; Tobin *et al.*, 2002). These findings indicate the complexity of factors being involved in the regulation of blood flow.

The effects of acetylcholine released from postganglionic nerves are mediated by muscarinic receptors located on glandular, vascular and neuronal tissues. Orthodoxy, peripheral muscarinic receptors have been

regarded as a homogenous receptor group evoking either smooth muscle contraction or glandular secretion. However, in the beginning of the 80's, the controversial idea was presented that the muscarinic receptors may be of two subtypes – M1 and M2 receptors, however, nowadays an outdated nomenclature. As more refined pharmacological and molecular methods became available, more subtypes could be distinguished. Today the muscarinic receptors are considered to comprise five subtypes - muscarinic M1, M2, M3, M4 and M5 receptors (Caulfield & Birdsall, 1998; Eglén, 2006). Out of these, the muscarinic M1, M3 and M5 receptors are excitatory, while the muscarinic M2 and M4 receptors are inhibitory. The intronless genes encoding the receptor subtypes have been cloned from several species and show a high sequence homology of the subtypes in all species so far examined (Kubo *et al.*, 1986; Hulme, 1990; Hulme *et al.*, 1990). Originally, the muscarinic receptors mediating the metabotropic effects of acetylcholine at non-neuronal effector cells was thought to be of the M3 receptor subtype, first recognised as the M2 subtype (Goyal, 1988; Caulfield, 1993). It has been well recognised for a long period of time that other subtypes of the receptor can be found on glandular as well as on smooth muscle cells when examined morphologically. However, the functional significance of the different receptor subtypes has not been fully unravelled. Data have accrued indicating that the heterogeneity of the receptor population has functional implications according to distinct pre- and postjunctional effects as well as to interactive mechanisms at its respective location (Somogyi & de Groat, 1999; Unno *et al.*, 2006).

Thus, the increase in salivary flow evoked by cholinergic stimulation has for long been attributed mainly to the activation of muscarinic receptors solely of the M3 subtype (Caulfield, 1993; Baum & Wellner, 1999). However, contradictory results have been found depending on which kind of salivary gland being examined. Binding and molecular experiments have shown the expression of all five muscarinic receptors in salivary glands (Hammer *et al.*, 1980; Buckley & Burnstock, 1986; Martos *et al.*, 1987; Vilaro *et al.*, 1990; Flynn *et al.*, 1997) and functional roles for the muscarinic M1 and possibly M5 receptors, in addition to those of the muscarinic M3 receptors, have also been demonstrated in these glands (Tobin, 1995; Culp *et al.*, 1996; Eglén & Nahorski, 2000; Meloy *et al.*, 2001; Tobin *et al.*, 2002). The general view still is that muscarinic M3 receptors are the main mediators of responses to acetylcholine in salivary glands. Other muscarinic

receptors are also thought to be involved, at least by neuronally modulating the transmission, but to have minor or no significance for neither secretory nor vascular responses (Tobin, 1998, 2002; Tobin *et al.*, 2002; Nakamura *et al.*, 2004). However, little is known about the muscarinic receptors participating in blood flow regulation. In rat salivary glands, muscarinic M3 receptors have been suggested to mediate the cholinergic-induced vasodilatation (Tobin *et al.*, 2002). In human blood vessels, acetylcholine may induce relaxation and contraction and these effects involve muscarinic receptors located on endothelial and smooth muscle cells. In human blood vessels, muscarinic receptors seem to be prevalent on endothelial cells as well as on smooth muscle cells (Walch *et al.*, 2001). In contrast, in rabbit aortic preparations the muscarinic M3 receptors mediate contractions if the preparations are endothelium-denuded (Watson & Eglén, 1994). The M3 receptor is not the only muscarinic receptor put forward as a candidate for mediating vasodilatation. In certain vascular beds, M1 receptors have been suggested to evoke arterial vasodilatation (Walch *et al.*, 1999) but in veins to induce contractile responses (Watson *et al.*, 1995).

Transmission in the parasympathetic innervation of salivary glands may be modulated by prejunctional muscarinic receptors (Tobin, 1995, 1998, 2002). In rat salivary glands, muscarinic M1 receptors normally facilitate transmitter release during short, intense nerve activity. At low frequencies, on the other hand, muscarinic M2, or possibly, M4 receptors, inhibit cholinergic as well as peptidergic transmission, but only after some delay. These effects, in addition to the fact that the release of neuropeptides preferentially occurs at intense nervous activity (Bloom & Edwards, 1979; Andersson *et al.*, 1982a), may explain that stimulation of the parasympathetic nerve in bursts is more efficient than a continuous pattern of stimulation (Bloom & Edwards, 1979; Andersson *et al.*, 1982b). Thus, a short-lasting stimulation activating facilitator and not inhibitory receptor mechanisms are likely to contribute to the effectiveness of the burst stimulation pattern (Tobin, 1998, 2002).

In the parasympathetic glandular neurons, the neuropeptide VIP may be co-localised with acetylcholine (Lundberg *et al.*, 1981b). In the submandibular gland of the sheep, VIP is present in nerve terminals adjacent to both small blood vessels and acini (Wathuta, 1986). In this

gland, as well as in the ovine parotid gland, VIP mediates secretion of protein-rich submandibular saliva, in addition the vasodilator effects (Reid & Heywood, 1988; Hanna & Edwards, 1998; Edwards *et al.*, 2003). Importantly, unselective muscarinic receptor blockade of prejunctional receptors has been shown in a number of species to increase VIPergic responses together with the release of VIP upon electrical stimulation of the parasympathetic glandular innervation (Tobin *et al.*, 1991; Tobin *et al.*, 1994; Edwards *et al.*, 2003). The effect of the blockade of the prejunctional receptors seems to be unspecific and affect both the release of the neuropeptide VIP and the classical parasympathetic transmitter acetylcholine (Tobin, 1998). However, the muscarinic receptor subtype mediating the effect has not been characterized.

The classical view that muscarinic receptors mediate only the acetylcholine-evoked secretory (Baum & Wellner, 1999), smooth muscle contractile and relaxatory (Eglen *et al.*, 1994) and autoreceptor effects, has been challenged lately. Muscarinic receptors have also been suggested to be implicated in the control of inflammation, cell growth and proliferation (Ventura *et al.*, 2002; Ukegawa *et al.*, 2003; Kawashima & Fujii, 2004; Profita *et al.*, 2005; Casanova & Trippe, 2006; Racke *et al.*, 2006). Sjögren's syndrome is an autoimmune disease that affects salivary and lacrimal glands, in which the parenchyma of the affected glands is progressively destroyed and replaced by a lymphoreticular cell infiltrate, thereby causing salivary gland hypofunction and xerostomia (Tyldesley & Field, 1995). The initial steps seem to involve changes in the susceptibility of the muscarinic receptors. Although no specific autoantibodies have been identified, autoantibodies against muscarinic receptors have been suggested (Dawson *et al.*, 2005; Fox, 2005). In the state of Sjögren's syndrome, the acinar expression of M3 receptors has been shown to be increased (Beroukas *et al.*, 2002). However, little is known about other subtypes of muscarinic receptors being involved in inflammatory and proliferatory responses. In knockout mice, neither the M1 nor M3 receptor seems to have any effect on parenchymal structure (Nakamura *et al.*, 2004). The muscarinic M5 receptor seems to be coupled to hypertrophic effects in an animal model of interstitial cystitis (Giglio *et al.*, 2005), a condition that may be related to Sjögren's syndrome. In view of this, the M5 subtype is of interest in pathological glandular conditions in salivary glands also.

At the time of planning the studies dealt with in the first section of this thesis, the complete expression pattern of the five muscarinic receptors had not been fully described in salivary glands, neither regarding glandular parenchymal tissue nor glandular blood vessels. Furthermore, the expression in human salivary glands was largely unknown, in particular the variation of expression pattern caused by diseases such as Sjögren's syndrome. The cellular location of the receptors was also largely unknown but could provide interesting insights into their functional roles. Thus, this section includes immunoblotting and/or immunohistochemistry findings in the major salivary glands of the rat, in ovine submandibular and parotid glands and in minor labial salivary glands of man. This first section focuses on describing the expression pattern in different species in order to find general features of the expression.

The muscarinic receptor subtypes mediating vascular effects in salivary glands were thus mainly unknown by the start of this thesis project. The second section of the thesis deals with functional cholinergic effects mediated by different muscarinic receptor subtypes. In the first part of this section, effects on blood flow within salivary glands and the characterization of subtypes mediating the effects both *in vitro* and *in vivo*. In the section, *in vitro* findings are related to *in vivo* blood flow findings, both with respect to flow and glandular perfusion as reflected by capillary permeability. The blood vessels to submandibular glands are easily identified and therefore, the effects of muscarinic agonists were studied on the vasculature of submandibular glands of rats and sheep. Comparisons were made with larger vessels more distally to the rat submandibular vasculature (carotid and jugular veins).

In the second part in the functional section, secretory effects are discussed. The overall secretory effects of muscarinic receptor stimulation have been known for more than a century. Even though a number of binding and molecular studies, as well as studies on salivary gland cell lines exist, the distinct contribution of the respective muscarinic receptor subtypes is fairly unidentified. In the third section, cholinergic secretory effects, on flow as well as on protein output, are discussed in relation to subtype determination. The functional findings are described in the rat parotid and submandibular glands and in ovine submandibular glands.

The postsynaptic effect of muscarinic receptors is of course in the *in vivo* situation affected by the amount of acetylcholine being released from the parasympathetic nerve. The amount is firstly the effect of the intensity, *i.e.* the firing frequency, of the nerve signals. However, facilitator and inhibitory muscarinic receptors on the nerve terminals also affect the amount being released (Powis & Bunn, 1995). In studies on muscarinic autoreceptor function, it has either been examined by using unselective antagonist (Lundberg *et al.*, 1982; Tobin *et al.*, 1991; Tobin *et al.*, 1994) or by examining selective blocking effects indirectly; *i.e.* on the responses and not on the actual transmitter release (Tobin, 1998). In the last section of the thesis, the muscarinic receptor subtypes modulating VIP release into the venous drainage of the ovine submandibular is characterized. Since VIP is co-stored in the parasympathetic glandular nerve fibres, VIP may be considered as a biomarker for any transmitter being released from the same neuron. Consequently, the impact of muscarinic autoreceptors is discussed.

Aims

The general aim of the thesis was to conclude how the different subtypes of the heterogeneous muscarinic receptor population of salivary glands principally interact. In order to provide data for such a conclusion, the muscarinic receptor expression and their functional effects were characterized in salivary glands of different species.

The specific aims were

- To establish the occurrence of specific receptor subtypes and their cellular location.
- To functionally characterize the muscarinic receptor subtypes according to vascular effects
- To functionally characterize the muscarinic receptor subtypes according to secretory effects
- To functionally characterize the muscarinic receptor subtypes according to neuronal transmission

Materials and methods

Adult rats and sheep were used in the morphological and functional experiments, whereas tissues from humans were included in the morphological examination. The ethical committees either of Göteborg University or Cambridge University approved the animal experiments in which Sprague Dawley rats and ewes of different breeds were used. All animals were killed at the end of the experiments (during which the animals were deeply anaesthetized) or directly if the experiment was performed on isolated tissues, either by an overdose of anaesthesia or by carbon dioxide. Anesthesia was induced and maintained with sodium pentobarbitone in both the rats (Natriumpentobarbital, APL, Göteborg, Sweden) and the sheep (Sagatal, Rhône Mérieux Ltd., Harlow, U.K.). At the end of each experiment the animal was given a lethal dose of barbiturate (sheep; Pentoject, Animalcare Ltd., York, U.K.; *ca* 15 ml 20% w/v) or pentobarbitone (rats; Natriumpentobarbital, APL, Göteborg, Sweden; 180 mg/kg I.V.). The ethical committee of human trials of the MAS University Hospital, Malmö, approved the procedures of the examination of human tissue. The tissue was obtained from routine biopsies for the assessment of Sjögren's syndrome. The patients had either histologically normal labial glands, or had labial glands with autoimmune sialadenitis (*i.e.* a focus score of >1 lymphocyte focus/4 mm²) compatible with Sjögren's syndrome.

Immunoblotting

The tissues were homogenized. The lysate was heated in a reducing sample buffer and the proteins were fractured on NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, US) and electroblotted onto PDVF membranes (Invitrogen). Phosphate-buffered saline containing Tween 20 and I-Block was used to block non-specific binding. The membranes were incubated overnight with polyclonal anti-muscarinic subtype specific antibodies (Research and Diagnostic Antibodies, Berkley, US). The binding was visualized with the Flour-S system (BioRad, Hercules, US) and analyzed using the QuantityOne software (BioRad). For negative controls, primary antibodies were omitted in the procedure described above. As an additional control the antibodies were occasionally pre-absorbed with the appropriate peptide immunogen as well, before proceeding as described above.

Immunohistochemistry

Preparation

The specimens were fixed in phosphate buffered paraformaldehyde, and then embedded in paraffin. From humans, the labial glandular tissues were dissected out under local anaesthesia and sent for ordinary pathological examination fixed in buffered paraformaldehyde.

Immunohistochemistry

Specimens, prepared in 4 μm sections, were de-paraffinized and then micro waved in 10 mM citrate buffer. Endogenous peroxidase was blocked with hydrogen peroxidase and non-specific protein binding with bovine serum albumin (BSA). The sections were incubated with polyclonal rabbit muscarinic receptor subtype specific antibodies (Research and Diagnostic Antibodies, Berkley, US) overnight at room temperature. Two techniques were used to reveal the presence of staining for the muscarinic receptors, either by using an avidin-biotin-complex immunoperoxidase method (ABC Staining System, Santa Cruz Biotechnology, Santa Cruz, US) or by using a Radiance 2000 Confocal Imaging System (Bio-Rad, Hercules, US) and the LaserSharp2000 software (Bio-Rad). The sections analyzed by the ABC method were counterstained with Mayer's haematoxylin, while in the confocal system, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Eugene, US) was used. As a negative control, duplicate sections were immunostained without exposure to the primary antibody, which resulted in no brown or fluorescent staining of the tissue. Occasionally the binding of the antibodies was blocked by preincubation with its specific antigen.

In vitro experiments on blood vessels

Preparations

Contractions and relaxations of isolated rat carotid and jugular vessels were examined in 25-mL organ baths. Two thin metal hooks were inserted through the lumen of each vessel segment. The segments were mounted between a fixed and an adjustable steel rod immersed in organ baths containing Krebs bicarbonate solution (pH=7.25) of the following composition (mM): NaCl 118, KCl 4.6, CaCl₂ 1.25, KH₂PO₄ 1.15, MgSO₄ 1.15, NaHCO₃ 25, and glucose 5.5, which was gassed with 5% CO₂ in O₂. The temperature was kept at 37°C by a thermostat. The segments were pre-stretched and allowed to equilibrate to a stable

tension of about 5 mN. In order to assess the viability of the preparations, KCl (100 mM) was administered at the start of each experiment.

Large vessels

In order to examine relaxatory effects of muscarinic stimulation on the vessels, a muscarinic agonist was administered to pre-stretched or phenylephrine pre-contracted segments. In the latter case, the effects in the absence and presence of muscarinic antagonists and NO synthase inhibitor were examined. All drugs were administered to the organ baths in a volume of 125 μ l, and the antagonists were administered 10 min prior to addition of the agonist.

Small vessels

Contractions and relaxations of rat submandibular arteries and veins were examined in 5-mL microvascular baths. The segments were threaded onto two stainless wires in myograph baths. Otherwise the experimental procedures and conditions were mainly the same as above. The internal circumference of the vessels was determined automatically by the computer software (Myodaq 2.01, Myonic Software, Aarhus, Denmark). The relation between resting wall tension and internal circumference was determined and from this the internal circumference L_{100} corresponding to transmural pressure of 100 mmHg for a relaxed vessel in situ was calculated. The vessels were set to the internal circumference L_1 , given by $L_1=0.9L_{100}$ (circumference 455 ± 10 μ m).

The contractile responses of arteries were examined on noradrenaline precontracted vessels. When a stable plateau was obtained after noradrenaline administration, increasing concentrations of methacholine were added. In experiments on veins, potassium (50 mM) was used to provide tone in each vein segment. When a stable plateau was obtained, increasing concentrations of noradrenaline or methacholine were examined.

In vivo experiments

Preparations

After induction of anaesthesia, the trachea was cannulated and the body temperature was maintained at about 38°C. The blood pressure was measured continuously via a catheter placed into the femoral artery. The ducts of the salivary glands (submandibulars and parotids)

were cannulated. In the rats, blood flow changes were measured by using a laser Doppler flowmeter (PeriFlux PF3; Perimed; Järfälla, Sweden). The flowmeter probe was placed against the gland in order to measure changes in glandular blood flow. The probe was fixated against the gland by using a round, plastic disc adapter (10 mm diameter) with a centre hole through which the probe was placed close to the glandular surface. The disc was attached to the skin surrounding the exposed gland. In the sheep, each of the tributaries of the ipsilateral linguofacial vein, except that draining the submandibular gland, was ligated. The animal was heparinized and the linguofacial vein cannulated. The submandibular venous effluent was thereby diverted through a second photoelectric drop-counter and returned to the animal by a pump, *via* the ipsilateral jugular vein, in such a way as to match input to output.

Secretory responses

Vascular and secretory responses were provoked either by administration of a muscarinic agonist into the blood stream or by electrical stimulation of the parasympathetic chorda-lingual nerve at varying frequencies and stimulation patterns. The latter procedure was performed with a bipolar platinum stimulating electrode placed under the duct and chorda tympani close to the hilum of the gland. All saliva secreted in response to stimulation was collected and weighed. A cannula placed in the femoral vein was used for all drug administrations. The protein content of the fluid responses was analyzed for its protein content by the method of Lowry (Lowry et al., 1951).

Blood flow and VIP output

The rates of flow of submandibular blood (and of saliva) were recorded photometrically drop by drop and also estimated gravimetrically. After the samples of blood had been collected for gravimetric estimation of blood flow, the blood was returned to the animal to preserve the circulating blood volume, except for that volume of submandibular venous effluent blood kept for VIP estimations. Arterial blood samples were collected at intervals for calculations of the glandular release of VIP into the circulation; difference between arterial and venous VIP concentration. The samples were collected into chilled pre-weighed tubes containing aprotinin (2500 KIU ml blood⁻¹). They were then centrifuged at 4°C as soon as possible and the

plasma sequestered at -20°C . Plasma VIP concentrations were measured by an enzyme immunoassay by (EIA for VIP, Peninsula Laboratories Inc., US). The minimum detectable concentration for VIP was 0.02 pmol ml^{-1} (range $0 - 7.6\text{ pmol ml}^{-1}$; linear range $0.03 - 0.61\text{ pmol ml}^{-1}$).

Permeability

In one set of rat experiments, Evans blue was slowly infused intravenously over a period of 1 min at a dose of 20 mg/kg . After one-hour exposure time period, the salivary glands were excised. In control rats, no procedure was undertaken during this period, whereas methacholine was infused at $1.5\text{ mg kg}^{-1}\text{ min}^{-1}$ in the absence or presence of muscarinic receptor antagonist. If performed in the presence of any antagonist, this was administered immediately prior to the start of the infusion of Evans blue. At the end of the experiment, the animals were killed with an overdose of pentobarbitone and the animal was perfused with 100 ml of cold saline. The submandibular glands were removed and put in preweighed tubes, which were then weighed. The tissues were transferred to tubes containing 2 ml of formamide, and the Evans blue was extracted by incubation at 50°C for 20 h . Evans blue was quantified by determining the optical density of the formamide extract at 620 nm . The absorbance was compared with a standard curve.

Drugs

The drugs employed were Pentobarbitone (Sagatal, Rhône Mérieux Ltd., Harlow, U.K.); Mutiparin (CP Pharmaceuticals Ltd., Wrexham, U. K.); pirenzepine dihydrochloride (Sigma, St Louis, US); methocramine tetrahydrochloride (Sigma); *p*-fluoro-hexahydro-sila-diphenidol hydrochloride (p-F-HHSiD; Sigma); atropine sulphate (Sigma); diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP; Sigma); phentolamine methansulphate (Sigma); propranolol hydrochloride (Sigma); acetyl-b-methylcholine chloride (methacholine; Sigma); carbamylcholine chloride (carbachol; Sigma); *N*- ω -nitro-*L*-arginine (L-NNA; Sigma); noradrenaline (Sigma); phenylephrine hydrochloride (Sigma) and Evans blue (Sigma).

Calculations and statistics

Statistical significance was determined by Student's t-test for paired or unpaired data or by repeated measures ANOVA, followed by a

Bonferroni test, if appropriate. P-values of 0.05 or less were regarded as statistically significant. Data are presented in the form of means±S.E.M. Graphs were generated and parameters computed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, US). Results are expressed as mean values ± S.E.M.

Submandibular vascular resistance (SVR) was estimated by dividing the perfusion (arterial blood) pressure (mm Hg) by the submandibular blood flow ($\mu\text{l min}^{-1} [\text{g gland}]^{-1}$) and expressed as the % changes from experimental time=0. P values less than 0.05 are considered to be statistically significant. All flows and outputs are expressed per unit weight of the contralateral gland.

Results and discussion

I. Expression of muscarinic receptors

Generally, muscarinic receptors appeared in all tissues examined. The location on different cell types showed variations. One has to notice that the methods used do not allow for exact comparisons according to the degree of expression of the different receptor subtypes. Each antibody has its specific antigen and the affinity for its binding may vary from one antibody to another. Furthermore, variations of binding affinity may also occur between species. This applies both to the immunoblotting and the immunohistochemistry. Anyhow, the examination gives indications for the occurrence of receptor subtypes in a gland or vessel. In the examinations, polyclonal antibodies were used, which caused non-specific bands in immunoblotting. Nevertheless, the bands corresponding to the predicted molecular masses of the muscarinic receptors were identified. The molecular mass estimates for muscarinic M1-M4 receptors in the present studies are in agreement with reports from other tissues and the mass estimate for the M5 receptor is in agreement with the predicted mass (McLeskey & Wojcik, 1990; Ndoye *et al.*, 1998; Preiksaitis *et al.*, 2000; Giglio *et al.*, 2005). As there was always a good correlation between the immunoblotting and the immunohistochemistry, the immunohistochemical antibody binding may be considered as specific as well. As a general observation, it can be noted that immunoblotting for the muscarinic M3 receptors always produced weak bands. It thus seems reasonable to believe, in the view of the established presence and role for the subtype, both by binding and molecular studies and by functional studies (Martos *et al.*, 1987; Maeda *et al.*, 1988; Mei *et al.*, 1990; Meloy *et al.*, 2001; Nelson *et al.*, 2004) as well as in knockout studies (Nakamura *et al.*, 2004), that the signal for the M3 receptor has been underestimated in comparison with that of the other subtypes of muscarinic receptors.

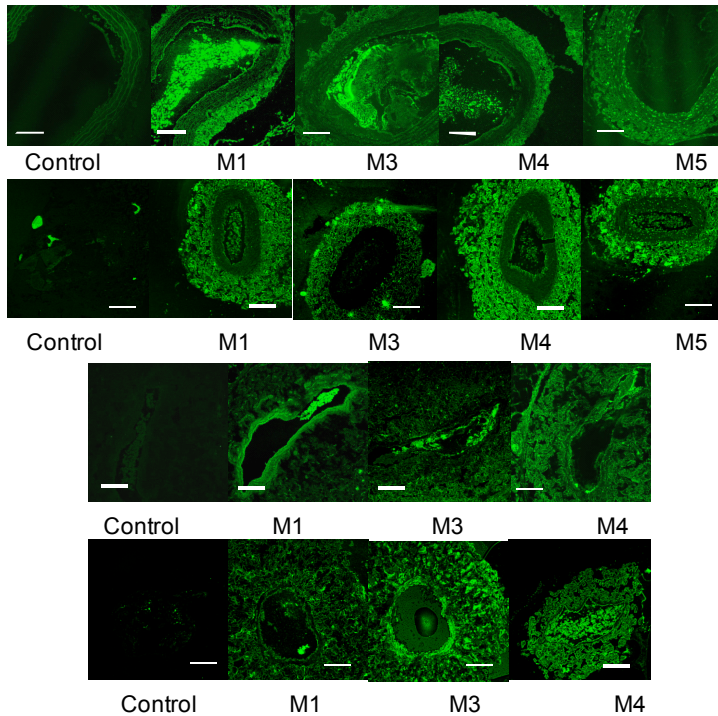


Figure 1. Immunohistochemical labeling of arteries (panel 1 carotid and panel 2 submandibular artery) and veins (panel 3 jugular and panel 4 submandibular vein). Images demonstrate staining in absence of antibody (control); staining in the presence of muscarinic M1, M3, M4 and M5 receptor. Bar in panels 1 and 3 indicates 100 μm and in panels 2 and 4 10 μm .

Blood vessels (papers I, II, III and IV)

Endothelial cells commonly possess a functional non-neuronal cholinergic system (see Wessler et al., 2001 for review) and choline acetyltransferase immunoreactivity has been demonstrated in vascular cells (Kirkpatrick et al., 2003). This provides compelling evidence for cholinergic effects in vasculature. Immunohistochemistry on rat intraglandular vessels indicated expression of muscarinic M1 receptors in both arteries and veins in submandibular glands. Muscarinic M3 receptors were expressed in all submandibular and parotid vessels, whereas muscarinic M2 and M5 receptors were expressed occasionally. The expression was examined in rat extraglandular vessels also; in the

submandibular artery and vein, in the carotid artery, and in the jugular vein. In all these vessels, muscarinic M1 receptors could be detected. The receptor constantly appeared in the endothelium, but in the veins in the smooth muscle also, particularly in the jugular vein. Regarding the M3 receptor, at least a vague signal may have occurred in the smooth muscle of all the vessels, being very pronounced in the submandibular vein. Regarding the M4 receptor, it occurred in the endothelium, but not in the smooth muscle layer of the vessels. A non-ubiquitously distributed signal for M5 receptors occurred in the smooth muscle layer in the arteries. In the sheep, on the other hand, all subtypes of the muscarinic receptor except the muscarinic M2 receptor were detected in the submandibular arterial and venous endothelium. While muscarinic M3, M4 and M5 receptors appeared in the smooth muscle layer in the artery, M1 and M4 receptors could be detected in the vein (Figure 1).

Thus, the expression of muscarinic receptors on the vasculature of the submandibular glands of the two species shows pronounced resemblance. In arteries in both species, the muscarinic M3 receptors are expressed on endothelial and smooth muscle cells. Furthermore, muscarinic M1 receptors seem to be the most prominent in the endothelium, and notably, the excitatory muscarinic M1 receptor occurs in the venous smooth muscle layer also. The non-ubiquitously distribution of the muscarinic M5 receptor, could tentatively be a consequence of that this particular receptor has been associated with modulatory effects on inflammatory cells (see Kawashima and Fujii, 2004). When studied in animals, the expression of the muscarinic M1, M2 and M3 receptors has been identified in aortic endothelial cells (Tracey & Peach, 1992), while all muscarinic receptors except the M4 receptor have been detected in the basilar and mesenteric arteries (Phillips *et al.*, 1996, 1997). In the human pulmonary vasculature, M1 receptors have been described in the endothelium (Walch *et al.*, 2001). And further, while M3 receptors are prevalent on the human pulmonary endothelial cells as well as on smooth muscle cells, the M4 subtype has not been described in human vessels (Walch *et al.*, 2001). In the brain microvasculature, however, the endothelial cells express both M2 and M5 receptors, while the vascular smooth muscle cells express all subtypes except M4 (Elhousseiny & Hamel, 2000). In general the endothelium seems to prevalently express muscarinic M1, M3 and possibly M5 receptors, while muscarinic M3 receptors are expressed in the arterial and muscarinic M1 receptors in the venous smooth muscle.

In venous preparations, the muscarinic M1 receptor has been associated with an endothelium-independent vasoconstriction in the canine saphenous vein (O'Rourke & Vanhoutte, 1987) and in the human umbilical vein (Pujol Lereis et al., 2006). These observations support the present morphological findings describing the expression of muscarinic M1 receptor in the venous smooth muscle layers.

Glandular tissue (papers I and III)

Salivary glands have been extensively studied according to the expression of muscarinic receptors (see Caulfield, 1993; Caulfield and Birdsall, 1998; Baum and Wellner, 1999). However, in many of early ligand binding studies, the salivary gland tissue was used as reference material for a tissue exclusively expressing muscarinic M3 receptors. During the previous decades, data from expression studies accrued showing a heterogeneous muscarinic receptor population (Maeda *et al.*, 1988; Dorje *et al.*, 1991; Levey, 1993; Watson & Culp, 1994; Culp *et al.*, 1996; Watson *et al.*, 1996; Flynn *et al.*, 1997; Khosravani *et al.*, 2007). These studies indicated that more or less all subtypes could be detected in salivary glands, but to varying degrees depending on the type of gland and on the species examined.

In the immunoblotting, glandular tissues from rats and sheep were investigated, while in the immunohistochemistry human glands were included as well. Immunoreactivity for the same muscarinic receptors was detected whether using immunoblotting or immunohistochemistry, even though the signal in some cases varied in the immunoblotting compared to the immunohistochemistry. Needless to say, the immunoblotting represents the total occurrence of a subtype in the investigated tissue, irrespective of which kind of structure. The immunohistochemistry, on the other hand, shows the actual localisation of the receptor.

Generally, the most intense immunoreactivity was detected in the outer parts of acini and/or the demilunar and myoepithelial cells. In the rat, except for in the sublingual gland, immunoreactivity for all of the muscarinic subtypes was detected in acini. The current observations on the rat parotid gland indicated that this gland differed from the submandibular and sublingual glands. In the latter glands, the

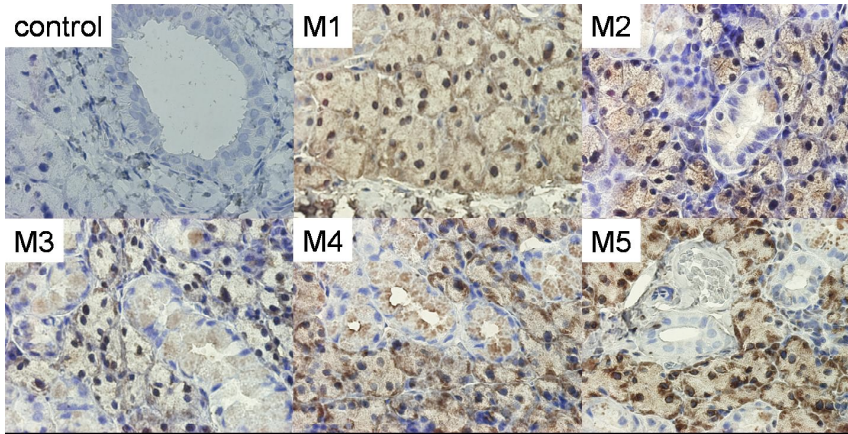


Figure 2. Immunohistochemical labeling of rat submandibular gland. Panels demonstrate staining in absence of antibody (control); staining in the presence of muscarinic M1, M2, M3, M4 and M5 receptor antibodies (M1, M2, M3, M4 and M5, respectively). All sections are counterstained with haematoxylin. Bar indicates 50 μ m.

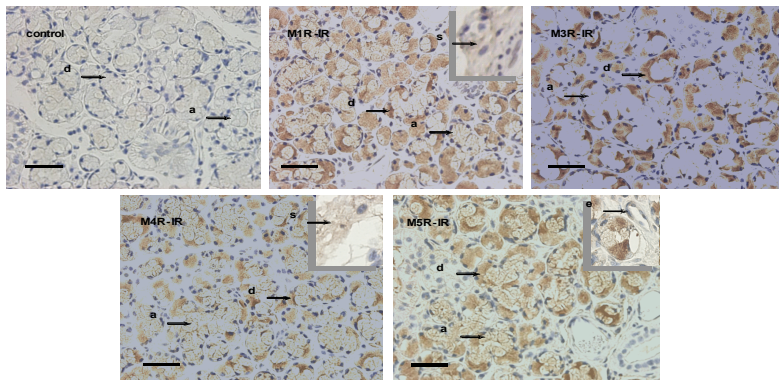


Figure 3. Immunohistochemical labeling of ovine submandibular glands. Panels demonstrate staining in absence of antibody (control); staining in the presence of muscarinic M1, M3, M4 and M5 receptor antibodies (M1R-IR, M3R-IR, M4R-IR, M5R-IR, respectively; inserts in M1R-IR, M4R-IR and M5R-IR for demonstration of appearances in stroma and endothelium). All sections are counterstained with haematoxyline. Bar indicates 50 μ m and the arrow close to the letters a, d, e and s indicate acinar cells, demilunar cells, endothelial cells and stroma, respectively.

muscarinic M1 receptor was particularly evident. Occasional immunoreactivity for different muscarinic subtypes was detected in the ducts of different glands as well. However, this immunoreactivity was not as strong as the immunoreactivity in the acini. The phenomenon seemed particularly evident for the M5 receptors. In the ovine parotid gland, clear signals for the M2, M3 and M4 receptors occurred, while in the sheep submandibular glands, clear signals for the M1, M3, M4 and M5 receptors occurred in and around the acini (Figure 2 & 3). Even though a negative finding should be interpreted with caution, the absent signal for muscarinic M2 receptors in the ovine submandibular gland is supported by the functional findings discussed in section II. In the human labial glands, muscarinic M1 and M3 receptors occurred evenly distributed over the whole specimen, and M5 receptors could be detected as well. M2 and M4 receptors seemed to appear on the outer parts of the acini, or on tissue in close vicinity to these. In the specimens from patients with Sjögren-like symptoms, the staining for M3, M4 and M5 appeared to be stronger than in the healthy glands and staining for the M4 receptor could be observed in ducts (Figure 4). Sjögren's syndrome is a syndrome causing salivary gland hypofunction, xerostomia and severe effects on the oral health (Tyldesley & Field, 1995). A general agreement has been that the hypofunction is a direct consequence of immune-mediated destruction of the secretory parenchyma. However, the pathology involves changes in the susceptibility of the muscarinic receptors also (Dawson *et al.*, 2005; Fox, 2005). The innervation is not affected in Sjögren's syndrome, while the acinar expression of M3 receptors has been shown to be increased in Sjögren's syndrome (Beroukas *et al.*, 2002), sometimes resulting in glandular hyperfunction (Dawson *et al.*, 2005). Noteworthy, the current studies showed the expression pattern of muscarinic M5 receptors differed in comparison with that of the other subtypes in all species examined – it was markedly patchy. In relation to these present observations, some other reports on inflammation and acetylcholine are worth considering. First, acetylcholine does not only mediate the classical autonomic effects, but has also been shown to influence inflammation within different organs (Pavlov & Tracey, 2006; Ohama *et al.*, 2007). Also, the induction of muscarinic M3 and M5 receptors has been shown to be associated with differentiation of cultured inflammatory cells into monocytic/macrophagic cells (Mita *et al.*, 1996). Secondly, as mentioned introductory, increase in the expression of muscarinic M5 and possibly M1 receptors, have been

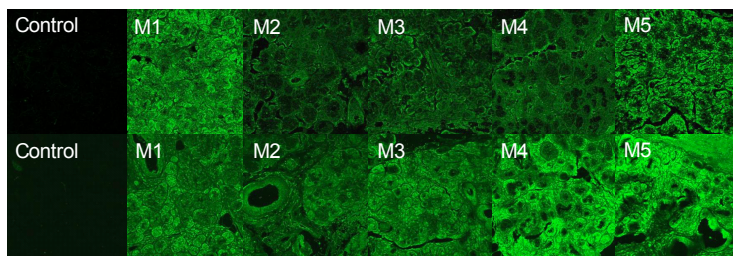


Figure 4. Immunohistochemical labeling of human labial glands. Upper panel: From patients with normal glands. Lower panel: From patients with Sjögren-like symptoms. Panels demonstrate staining in absence of antibody (control); staining in the presence of muscarinic M1, M2, M3, M4 and M5 receptor antibodies (M1, M2, M3, M4 and M5, respectively). Bar indicates 50 μ m.

coupled to inflammatory and hypertrophic effects in the urinary bladder (Giglio *et al.*, 2005), and lastly, muscarinic receptors seem to participate in remodelling processes known to occur in chronic inflammatory diseases (Gosens *et al.*, 2005), and mechanisms by muscarinic M3 receptors have been linked to cellular proliferation in cancer cells (Frucht *et al.*, 1999; Yang & Frucht, 2000). Altogether, the expression appearance and the prominent increase in labial glands of patients suffering from adenitis (Sjögren's syndrome), may favour the idea that muscarinic M5 receptors may mediate cross-talk between the autonomic and the immune system. Considering the suggestions of muscarinic M5 receptors having hypertrophic effects, the increase observed in the Sjögren patients may reflect a compensatory mechanism for the immune-mediated destruction of the secretory parenchyma.

The current studies show the presence of most muscarinic receptors in salivary glands, regardless of species or gland, which is in agreement with the current view of a heterogeneous muscarinic receptor population. They also show that the common view that all salivary glands are the same irrespective of which kind or from which species is erroneous. Great variations occur both when examined by functional or by morphological methods. These studies also show that there exists no archetypical gland, even though the submandibular glands from various species showed some resemblance; significant levels of muscarinic M1

and M5 receptors could be detected, and vaguer signals for muscarinic M3 and, in particular for, M4 receptors.

In conclusion, besides muscarinic M3 receptors, the M1 receptor seems to be commonly expressed in salivary glands, particularly in seromucous/mucous glands, as judged by the findings in rat, ovine and human salivary glands. It should be noted that the ovine parotid gland might differ from the rat parotid, since the former has been suggested not to be a pure serous gland (Shackleford & Wilborn, 1968; van Lennep *et al.*, 1977; Pinkstaff, 1993).

Stromal tissue (papers I and III)

Prejunctional muscarinic receptors have been recognized for long (Sharma & Banerjee, 1978; Buckley & Burnstock, 1984). In recent years, characterization by employing immunohistochemistry has demonstrated presynaptic muscarinic receptor expression of the subtypes M1-M4 in the rat neuromuscular junction (Garcia *et al.*, 2005) and in the enteric nervous system of different species, including man, muscarinic neuronal M1, M2 and M4 receptors have likewise been visualized (Takeuchi *et al.*, 2005; Harrington *et al.*, 2007). In salivary glands, functional modulator effects by prejunctional muscarinic receptors have been demonstrated in the rat, ferret, cat and rabbit (Lundberg *et al.*, 1984; Tobin *et al.*, 1991; Tobin, 1995, 1998, 2002). Even though the muscarinic receptors have been characterized into facilitatory or inhibitory out of functional effects, no subtype characterization has been performed in salivary glands. In this thesis, a functional characterization is reported. The immunohistochemistry experiments in the current studies do not establish the expression of any particular neuronal muscarinic receptor subtype. However, some observations may be discussed in the context of prejunctional receptor expression.

The immunohistochemical studies on rat, ovine and human glands in the current thesis describe expression of muscarinic M1, M4 and M5 receptors in vicinity to glandular acini of more or less all kinds of glands. The data indicate generally more marked staining in the peripheral region of the acini. This could mean that cells surrounding the acini, *e.g.* demilunar and myoepithelial cells, and nerve fibres, express muscarinic receptors. Since the staining of the peripheral part

of the cells seemed to vary between the different antibodies, it may indicate different receptor expression on surrounding cells. Since also myoepithelial cells receive cholinergic innervation (Emmelin *et al.*, 1968), the observations could reflect expression of myoepithelial muscarinic receptors as well, which in that case would mean any of the excitatory subtypes (M1, M3 or M5). If the expression reflects nerve terminal expression instead, an inhibitory subtype is also possible. In view of that facilitatory effects by muscarinic M1 receptors have been described functionally (Tobin, 1998), muscarinic M1 receptor expression may occur on the neurons as well. However, the same receptor subtypes were occasionally found in the stromal parts of the glands. The localisation of these latter receptors could represent cells of the immune system (Kawashima & Fujii, 2004), but the expression of the same subtypes of muscarinic receptor both in the stroma and close to acini, indicates a neuronal localisation. Thus, the immunohistochemistry seems to support the functional findings from the ovine submandibular gland (see below; muscarinic M4 receptor antagonism inhibits VIP release).

The functional data, discussed later, give no evidence for muscarinic M4 receptor involvement in the postjunctional responses. As the muscarinic M4 receptor was the only inhibitory muscarinic receptor found in the ovine submandibular gland, and it was found in the rat and ovine parotid glands as well, in stromal parts, where parasympathetic nerve fibres may occur, this may indicate an autoreceptor role in these glands. The muscarinic M4 receptor has been shown to play this role in other organs as well (D'Agostino *et al.*, 2000; Zhang *et al.*, 2002). However, the occurrence of other stromal muscarinic receptor subtypes, such as muscarinic M1 receptors, may favour the idea that the parasympathetic innervation exhibits such receptors, possibly facilitating transmitter release.

II. Functional characterization

The functional characterization of muscarinic receptor subtypes is hampered by the lack of pharmacological tools exhibiting pronounced selectivity for the subtypes. Three subtypes of muscarinic receptors (M1, M2 and M3) may be distinguished pharmacologically relatively well. A number of subtype selective antimuscarinic agents exists that exhibit at least 10-fold selectivity for each of the M1–M3 subtypes, but pirenzepine, methoctramine, 4-DAMP and p-F-HHSiD are most frequently used when exploring muscarinic receptor populations pharmacologically (Caulfield, 1993; Caulfield & Birdsall, 1998; Eglen & Nahorski, 2000; Jerusalinsky *et al.*, 2000). The most selective non-peptidergic muscarinic receptor antagonist is pirenzepine, which until recently has been regarded more or less as “M1-selective”. Even though this antagonist shows selectivity towards M2 and M3 receptors, it discriminates less markedly between M1 and M4 receptors (Eglen & Nahorski, 2000). Nevertheless, muscarinic M1 receptors have a high affinity for pirenzepine, a low affinity for methoctramine and an intermediate affinity for p-F-HHSiD. 4-DAMP discriminates only between the excitatory and the inhibitory groups of muscarinic receptors, and shows almost identical affinities for M1, M3 and M5 receptors. While M2 receptors have a high affinity for methoctramine and a low affinity for pirenzepine and p-F-HHSiD, M3 receptors have a high affinity for p-F-HHSiD (and 4-DAMP), an intermediate affinity for pirenzepine and a low affinity for methoctramine (Caulfield, 1993). The affinity of an antagonist thus represents the composite affinity at multiple receptor subtypes that may occur at unknown levels in a tissue expressing several subtypes (Caulfield & Birdsall, 1998). Since this is the case in salivary glands, this is probably one explanation for the often-bewildering array of data describing the receptor mediation of acetylcholine functional effects. Also, the functional antagonism *per se*, and in particular that *in vivo*, of the substances may often diverge from the out of binding experiments estimated receptor subtype affinities (Tobin & Sjogren, 1995; Eglen & Nahorski, 2000; Meloy *et al.*, 2001). Of the antagonists used in this thesis, methoctramine may show this feature. Therefore, the antagonism has to be validated in both *in vitro* and *in vivo* functional studies.

In *in vitro* studies, exact concentration response curves can be constructed, experimental conditions can be well controlled and

usually a large number of drug administrations can be performed. In view of the lack of highly selective antagonists and agonists, the advantages with *in vitro* studies are evident. This is at least usually valid for smooth muscle contractile studies, while, however, vessel smooth muscle does not always allow for experiments over long periods of time. When it comes to studies of secretion, the situation is different. Even though *in vitro* experiments can be performed (Larsson *et al.*, 1990), a marker for fluid secretion has to be assessed, and consequently, *in vivo* experiments may be more reliable. Anyhow, in both glandular blood vessels and in the glands, as described by the immunohistochemistry, multiple muscarinic receptor subtypes exist. In addition to the *in vitro* functional characterization, the findings have been tried to be confirmed in *in vivo* experiments.

In vitro effects on blood vessel contraction (paper II)

In the vasculature, acetylcholine may evoke contractile as well as relaxatory responses. The classical experiments performed by Furchgott and Zawadzki (1980) showed that in endothelium-denuded rabbit aortic preparations, acetylcholine-evoked relaxations were changed into contractions. And further, as already mentioned, in intact vessel preparations, relaxations occurred at low concentrations of muscarinic agonists, but were changed into contractions when the preparations were challenged by large concentrations (Furchgott & Zawadzki, 1980). The different effects by acetylcholine are also apparent when comparing arterial and venous preparations. In general, the acetylcholine relaxation effect is the principal arterial response, whereas a contractile effect has been described on veins in the vasculature of some organs of animals as well as in man (Krausz, 1977; Walch *et al.*, 2001; Pujol Lereis *et al.*, 2006; Wang & Lung, 2006; Ding & Murray, 2007). In the canine nasal venous system, acetylcholine may, likewise to the observations made by Furchgott and Zawadzki (1980), induce NO-dependent relaxations of outflow veins at low concentrations followed by NO-independent contractions at larger (Wang & Lung, 2006). However, a dual response seemed not to occur in the collecting veins, indicating different physiological mechanisms at varying levels of the venous drainage.

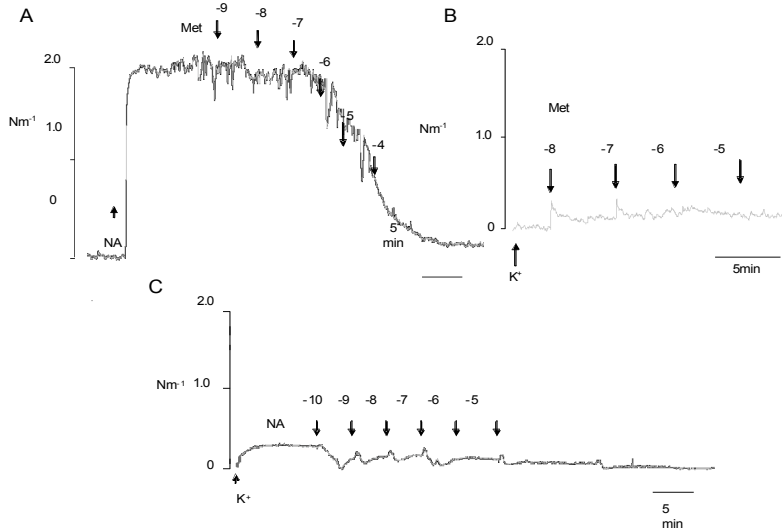


Figure 5. Original trace recordings of A) responses to methacholine (10^{-9} – 10^{-4} M) on noradrenaline precontracted submandibular artery, B) responses to methacholine (10^{-8} – 10^{-5} M) on potassium precontracted submandibular vein and C) responses to noradrenaline (10^{-10} – 10^{-5} M) on potassium precontracted submandibular vein. Horizontal bars represent 5 minutes.

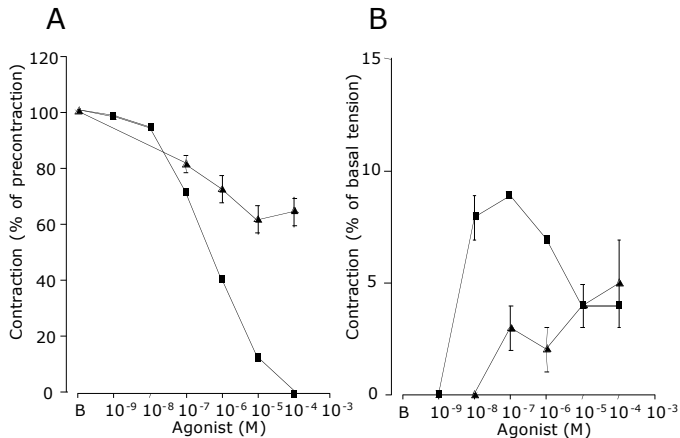


Figure 6. A) Responses of carotid arteries to carbachol (▲; n=25) and of submandibular arteries to methacholine (■; n=6) expressed in percentages of precontraction tension. B) Responses of jugular veins to carbachol (▲; n=14) and of submandibular veins to methacholine (■; n=5) expressed in percentages of basal tension level. Each symbol represents mean ± SEM.

In the current studies, the vessels were examined in two different experimental methods. The larger vessels were examined in the more insensitive organ bath set-up, whereas the smaller had to be examined in the myograph, which is time-consuming and therefore does not allow long experimental protocols. Nevertheless, independently of the type of protocol, the responses to cholinergic stimulation of rat isolated arteries and veins were opposite. While the arteries showed pronounced relaxations in response to muscarinic agonists, the veins showed contractions (Figure 5). The reactions from carotid and submandibular arteries and jugular and submandibular veins, respectively, were similar (Figure 6). Cholinergic-induced relaxations of carotid arteries have been reported previously (Sendao Oliveira & Bendhack, 2004), likewise to cholinergic contractile responses of isolated veins (Pujol Lereis *et al.*, 2006; Ding & Murray, 2007). Also, adrenoceptor stimulation had disparate effects on the arteries and the veins. It induced a possible short-lasting contraction, if any, followed by a tendency towards relaxation in the veins, while it caused pronounced contractions in the arteries. Contractile and dilator adrenoceptor effects have previously been shown in carotid arteries and jugular veins, respectively (Cohen & Wiley, 1978; Deighan *et al.*, 2005). The cholinergic responses from small vessels were larger than the corresponding responses from the carotid artery and the jugular vein. Tentatively, this could mean that acetylcholine has a greater impact in the smaller vessels, which in that case could mean that parasympathetic stimulation of this region would benefit the maintenance of salivation.

The relaxation to muscarinic agonists of precontracted carotid arteries was significantly reduced, but not completely abolished by the nitric oxide synthase inhibitor L-NNA, indicating a NO-dependent component. 4-DAMP and pirenzepine also abolished the relaxation, while pirenzepine at 10^{-5} M caused carbachol at 10^{-4} M to evoke significant contractions, *i.e.*, a dual response was indicated (Figure 7). The observations are in line with previous reports in the nasal vasculature showing relaxatory effects at low concentrations of

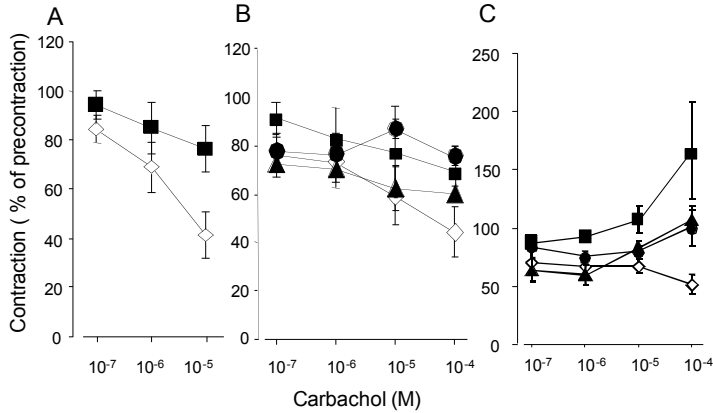


Figure 7. Responses of carotid arterial preparations to carbachol expressed in percentages of precontraction tension. A) Responses before (\diamond) and after L-NNA (\blacksquare ; 10^{-4} M; n=7), B) before (\diamond) and after 4-DAMP (\blacktriangle : 10^{-8} M; \bullet : 10^{-7} M; \blacksquare : 10^{-6} ; n= 6) and C) before (\diamond) and after pirenzepine (\blacktriangle : 10^{-7} M; \bullet : 10^{-6} M; \blacksquare : 10^{-5} ; n=6).

muscarinic agonists and contractile at large (Wang & Lung, 2006). However, since 4-DAMP, and in particular, pirenzepine have a selectivity for muscarinic M1 receptors (Flynn *et al.*, 1997), the relaxatory response seems to involve the M1 receptor subtype. Furthermore, since the relaxation is dependent on nitric oxide synthesis, an indirect effect is probable. The interpretation of the observations could very well be that there exist two muscarinic pathways in the artery, one dominantly being NO-dependent and another appearing during blockade of the former and causing contraction. Since the contraction did not occur in the presence of 4-DAMP, the contractile effects may be exerted via M3 or M5 receptors. A similar muscarinic mechanism has been demonstrated in the mouse stomach fundus, where a M1-mediated relaxatory component is masked by a M3-mediated contractile component (Stengel & Cohen, 2003). The affinity of pirenzepine is about three times and 4-DAMP two times larger on M5 than on M3 receptors (Eglen & Nahorski, 2000). Even though both antagonists have larger affinity for muscarinic M1 than for M5 receptors, the results do not contradict a muscarinic M5 receptor involvement in the control of glandular blood flow control, in particular since staining for muscarinic M5 receptors was observed. The effects of methoctramine on muscarinic receptor vascular responses, on the other

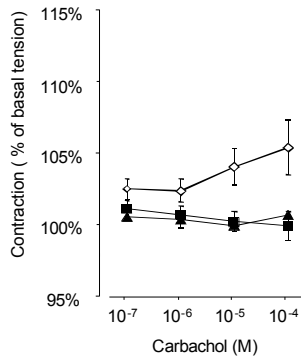


Figure 8. Responses of jugular veins to carbachol expressed in percentages of basal tension in the absence of antagonist (◇; n=14), and in the presence of pirenzepine (■; 10⁻⁵ M; n=5) and 4-DAMP (▲; 10⁻⁶ n=4). Each symbol represents mean±SEM.

hand, were ambiguous. At low concentrations it had no significant effect on the relaxations, and at increasing concentrations unspecific adrenergic inhibition seemed to occur affecting the precontraction. However, it is well-known that many of the “muscarinic M2 receptor” antagonists, including methoctramine, may have composite mechanisms of action sometimes resulting in unspecific effects (Howell et al., 1994).

The jugular vein contains little smooth muscle in comparison with the carotid artery, and any stimulation evokes only small and fatigable responses. However, in comparison with the submandibular vein, the responses were well defined and allowed further examinations with antagonists. In these preparations, 4-DAMP, pirenzepine and methoctramine inhibited the carbachol induced contractions (Figure 8). However, since 4-DAMP did only inhibit the responses at a ten-fold lower concentration than pirenzepine did, and further, since the effect of the muscarinic agonist was excitation (contraction), a muscarinic M1 receptor response is plausible. Further, since methoctramine also had some effect, this could indicate that M2/M4 receptors participate as well. Furthermore, since M1 and M4 are clearly detected in the immunohistochemistry, M4 seems to be the likely candidate as a companion to M1. Presently, however, the role of the M4 receptors is ambiguous. They could of course cause a direct relaxatory effect, but

perhaps more likely, they could inhibit the synthesis of nitric oxide, particularly since the M4 receptors were exclusively detected in the endothelium. Notably, the observations on venous preparations had to be made over relatively short periods of time, and neither could observations be made on pre-contracted veins. This makes it hard to exclude a relaxatory component in the cholinergic venous response in addition to the contractile.

The contractile effect of muscarinic agonists on vein preparations could mean, in light of the occurrence of M1 receptors in the jugular vein, that a cholinergic contractile mechanism is involved in the blood flow regulation. Support for this hypothesis is found in the fact that pirenzepine completely abolished the carbachol response and the same mechanism has been demonstrated in other venous preparations (Pujol Lereis et al., 2006). However, as mentioned above, the role of the M4 receptors is ambiguous but may speculatively contribute to contraction by NO-inhibiting effects. Anyhow, a venous contractile muscarinic mechanism could mean that the hydrostatical pressure is preserved and makes more fluid available for secretion, as been indicated by studies on the canine submandibular gland (Lung, 1998). Even though vascular conductance primarily depends on the tone of the resistance vessels rather than on that of the venous vessels, the tone of the latter may tentatively also act just to prevent blood pooling in low resistance vessels.

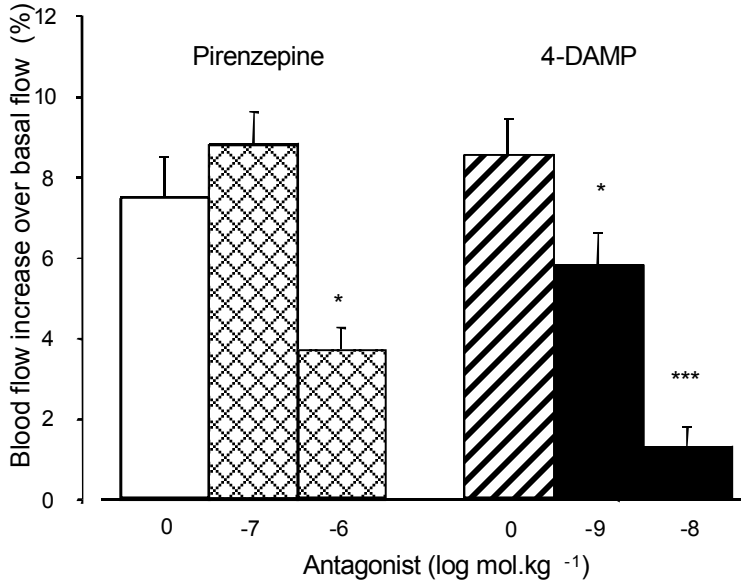


Figure 9. Submandibular blood flow changes as indicated by the laser Doppler flowmeter signal. Changes to methacholine $1 \mu\text{g.kg}^{-1}$ *i.v.* before (\square ; n=6) and after pirenzepine (\boxtimes ; n=6) and before (\boxdot ; n=5) and after 4-DAMP (\blacksquare ; n=5) are expressed as means of the percentage increase over basal blood flow. Vertical bars represent S.E.M. * p=0.05; ** p=0.01; *** p=0.001.

In vivo effects on blood flow (papers I, III and IV)

In vivo studies characterizing the muscarinic receptor subtypes that regulate blood flow are in general few (Walch *et al.*, 2001; Eglén, 2006). The vasodilatation in salivary glands evoked by muscarinic receptor stimulation is well recognised and thoroughly examined in *in vivo* experiments (Morley *et al.*, 1966; Lundberg *et al.*, 1981b, 1982; Emmelin, 1987). However, when the parasympathetic nerve is stimulated electrically, a large part of the vasodilatation is resistant towards atropine (Heidenhain, 1872; Edwards, 1998). Even though this part is dominating, the increase of the vasodilatation is slower in the presence of atropine (Lundberg *et al.*, 1981b). As mentioned above, at low intense parasympathetic activity, a substantially greater part is

sensitive towards atropine and thus includes a significant muscarinic part (Emmelin *et al.*, 1968).

In the current experiments, the muscarinic agonist methacholine caused significant increases in blood flow in rat submandibular glands as assessed by a Doppler flowmeter. Both pirenzepine and 4-DAMP inhibited these increases, but pirenzepine at about a 100–1000-fold higher dose (Figure 9). This indicates that the cholinergic-evoked vasodilatation is at least mainly mediated via another muscarinic receptor than M1. In the rat parotid gland, it has been suggested to be M3-mediated (Tobin, 1998), and this seems to be the case in the submandibular gland as well. Since 4-DAMP is “M1/3/5-selective”, involvement of the muscarinic M5 receptor is also of course possible. The cholinergic dilatation in cerebral blood vessels is largely mediated by muscarinic M5 receptors (Yamada *et al.*, 2001), and, in view of the expression of muscarinic M5 receptors in the rat submandibular artery, a M5 receptor involvement may occur in salivary glands as well. Contradicting this hypothesis are the findings in muscarinic M5 receptor knockout-mice, in which no changes in extracerebral vascular responses have been detected (Yamada *et al.*, 2001).

The ovine submandibular vascular responses were evoked by electrical stimulation of the chorda tympani. The size of the sheep makes gravimetric estimations of the blood flow possible. One advantage with evoking the responses by nerve stimulation is that you may study the changes in glandular vascular resistance (reflecting glandular vascular responses) without affecting the blood pressure or heart rate. The frequency-dependent responses were well maintained for the duration of stimulation. Stimulation in bursts tended to increase blood flow more than continuous stimulation (Figure 10). High frequency stimulation given intermittently, resulting in the same total number of impulses as a low frequency continuous stimulation, enhances the release of VIP and results in greater decrease of glandular vascular resistance (Bloom & Edwards, 1980; Andersson *et al.*, 1982b; Tobin *et al.*, 1990a; Edwards *et al.*, 2003). However, Edwards *et al.* (2003) has shown that continuous low frequency stimulation (<4 Hz) evokes acetylcholine-dependent vasodilator responses in the sheep as well. Also in the current experiments, pre-treatment with atropine did not affect the fall in vascular resistance at 8 Hz given continuously,

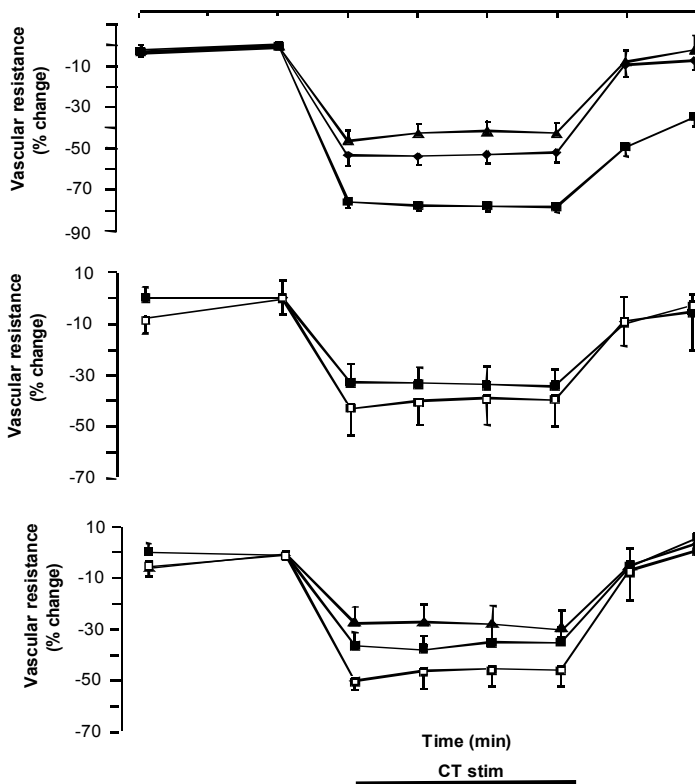


Figure 10. Upper panel: Changes in submandibular vascular resistance in response to chorda tympani stimulation at 2 Hz continuously (\blacktriangle), 20 Hz in bursts (1:10 s; \bullet) and 8 Hz continuously (\blacksquare) for 10 min in 13 anaesthetized sheep.

Middle panel: Changes in submandibular vascular resistance in response to chorda tympani stimulation at 2 Hz continuously for 10 min in the absence (\square) and in the presence of methoctramine ($100 \mu\text{g kg}^{-1}$ I.V.; \blacksquare) in five anaesthetized sheep.

Lower panel: Changes in submandibular vascular resistance in response to chorda tympani stimulation at 2 Hz continuously for 10 min in the absence of antagonists (\square) and in the presence of pirenzepine ($40 \mu\text{g kg}^{-1}$ I.V.; \blacksquare) before and after administration of p-F-HHSiD ($4 \mu\text{g kg}^{-1}$ I.V.; \blacktriangle) in five anaesthetized sheep.

Vertical bars: S.E. M. value. Horizontal bar: duration of stimulation (CT stim).

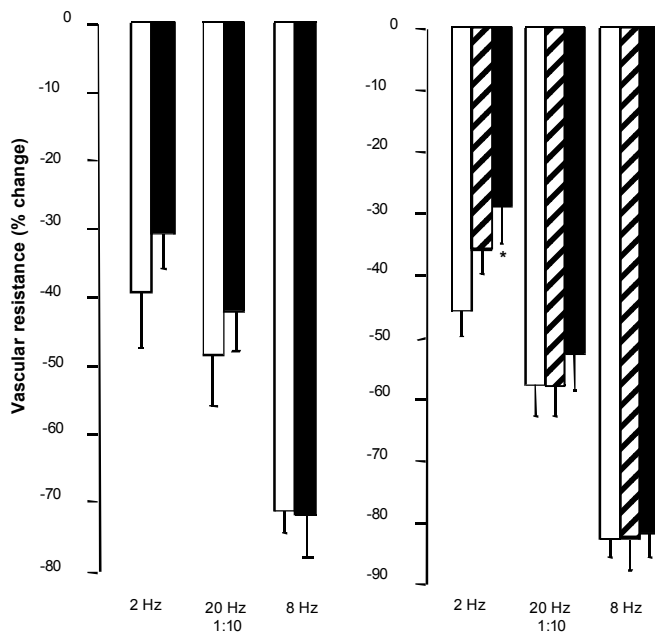


Figure 11. Changes in mean vascular resistance over the 10 min stimulation period. The column doublets (left panel) and triplets (right panel) in each panel show from left to right the mean responses to chorda tympani stimulation at 2 Hz continuously, 20 Hz in bursts and 8 Hz continuously. The left panel show the mean responses in the absence (□) and presence (■) of methoctramine ($100 \mu\text{g kg}^{-1}$ I.V.) in five anaesthetized sheep and the right panel show the mean responses in the absence (□) and presence of pirenzepine ($40 \mu\text{g kg}^{-1}$ I.V.) before (▨) and after administration p-F-HHSiD (■; $4 \mu\text{g kg}^{-1}$ I.V.) in five other anaesthetized sheep. Vertical bars: S.E. M. * $P < 0.05$, ** $P < 0.01$.

whereas the fall in the submandibular vascular resistance during stimulation at 2 Hz continuously was more than halved.

Pirenzepine did not significantly affect the vascular responses to parasympathetic stimulation in the ovine submandibular gland, and therefore, muscarinic M1 receptors are unlikely to substantially contribute to the vasodilatation in sheep, at least the nerve evoked. Since a low dose of p-F-HHSiD in the presence of pirenzepine, significantly reduced the vasodilatation, the cholinergic vascular

response is mediated by some other muscarinic receptor subtype(s) (Figure 11). In view of the affinity profile of p-F-HHSiD, and further, its pronounced effect on the concomitantly occurring salivation compared to the vascular effect, the cholinergic vasodilator response may include muscarinic M5 receptors in addition to the M3 receptor mediation. Since both muscarinic M3 and M5 receptors belong to the excitatory group, the vasodilator responses are likely to be linked to NO. Both receptors have, as well as the muscarinic M1 receptor, been shown to exert effects via NO in several studies (Elhousseiny & Hamel, 2000; Giglio *et al.*, 2005).

The doses of the antagonists have previously been validated in *in vivo* experiments to enabling “selective” discrimination of subtype specific responses (Tobin, 1995; Tobin & Sjogren, 1995; Tobin, 1998; Tobin *et al.*, 2002). Also, the current observations on the heart rate support the idea that the antagonists have been acting in semi-selective ways. Namely, methoctramine significantly and consistently increased the heart rate without affecting the flow of saliva. Pirenzepine and p-F-HHSiD, on the other hand, both tended to decrease the heart rate, if anything.

It is concluded that muscarinic M1 receptor effects are of less importance than the effects via other excitatory muscarinic receptors for the cholinergic vasodilator response in the rat and the ovine submandibular glands. Doubtlessly, acetylcholine exerts modulator effects on the vasculature, but the origin of the transmitter is under debate. As mentioned earlier, Kummer and Haberberger (1999) suggested, based on immunohistochemical, biochemical and functional studies, two separate cholinergic systems in the arterial vascular wall. One, an intrinsic, intimal cholinergic system serves as a regulator of basal vascular tone responding to local, luminal stimuli, whereas the other, the perivascular nerve fibres, *i.e.* the extrinsic, adventitial cholinergic system act on top of this basal tone by providing fine tuning in response to reflex activation due to systemic demands. In the current *in vivo* experiments, pirenzepine seemed to be more effective in the rat submandibular than in the ovine gland. One reason could in such a case have been that in the rat, the intrinsic system was preferentially activated (intravenous methacholine injections); whereas the extrinsic system was activated in the sheep (nerve stimulation).

In vivo effects on vascular permeability (paper II)

The differences in hydrostatic pressures and osmotic pressures along and across the walls of the exchange vessels have been widely accepted to balancing the filtration and absorption of fluid (see Michels *et al.*, 1984). According to the Starling hypothesis (Starling, 1896), absorption of fluid would be expected to occur at venule side of the capillary bed because of the successive increase in oncotic plasma pressure and decrease in hydrostatic pressure. But in contrast to the Starling hypothesis, filtration coefficient increases with decreasing pressure in both capillaries and venules (Fraser *et al.*, 1978). Another disparity, which has puzzled vascular experimenters for long, is that in the intact circulation, the transcapillary gradients of hydrostatic and osmotic pressure are themselves determined by the transcapillary filtration rates and the capillary permeability of osmotic-acting molecules (Krogh *et al.*, 1932; Hughes *et al.*, 1958; Guyton, 1963; Lunde & Waaler, 1969). However, the Starling hypothesis has recently been re-evaluated considering endothelial glycocalyx functions, which partly explains the paradox (see (Levick, 2004)). Anyhow a large part of the fluid filtration in the microcirculation occurs in postcapillary venules (Fraser *et al.*, 1978; Qiao & Bhattacharya, 1991). Normally, there would be little net extravasation of fluid and colloids despite large pores in venules, because of small differences in hydrostatic and oncotic pressure between intra- and extravascular spaces (Jacob *et al.*, 2007). Therefore, any change in the balancing forces at the venule level could be expected to have impact on filtration.

Likewise to filtration of fluid at postcapillary venules, extravasation of macromolecules is considered to occur at this level in the vasculature and this site thus provides physiological access of large molecules to the tissue (Grega & Adamski, 1988; Qiao & Bhattacharya, 1991; Figini *et al.*, 1997; Jacob *et al.*, 2007). Evans blue binds to plasma proteins and may therefore serve as a marker for extravasation (LeVeen & Fishman, 1947). Evans blue has previously been used when examining vessel permeability in rat salivary glands, and in the submandibular gland, the muscarinic receptor agonist pilocarpine was shown to increase the extravasation (Asztely *et al.*, 1998). Also in the present examination, methacholine induced conspicuous increases in the efflux of Evans blue into the glandular tissue. The antagonistic effect of

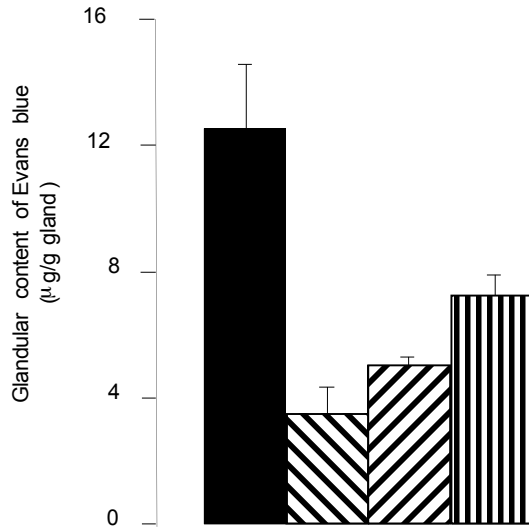


Figure 12. Vascular protein efflux in the submandibular glands of anaesthetized rats indicated by tissue content of Evans blue one hour after its administration (20 mg/kg i.v. infusion over 1 min). Glandular content was measured in rats infused with methacholine (1.5 µg/kg/min i.v. for one hour) in the absence (■; n=6) and in the presence of pirenzepine (50 nmol/kg i.v.; ▨; n=5), 4-DAMP (0.3 nmol/kg i.v.; ▩; n=5) and methoctramine (20 nmol/kg i.v.; ▧; n=5). Each column represents mean and vertical bars ±SEM.

pirenzepine and 4-DAMP were examined at doses well below the threshold for affecting methacholine induced vasodilatation (Figure 12). Since pirenzepine and 4-DAMP at these low doses both potently inhibited the methacholine-induced efflux and methoctramine with conspicuously lesser potency, the *in vivo* findings could be interpreted in support for the idea that the effect of muscarinic M1 receptors may influence vascular filtration in the rat submandibular gland.

During the secretory process in the rat submandibular gland there is a substantial decrease in fluid interstitial pressure, which favours fluid filtration and preserves salivation (Berggreen & Wiig, 2006). In the perspective of the low compliance of the gland, the mechanism has been suggested to be exerted by myoepithelial cells. When the cells are

stimulated, which may be induced by muscarinic stimulation (Garrett, 1987), the tissue surrounding the cells undergoes conformation, which in turn reduces interstitial fluid pressure (Berggreen & Wiig, 2006). The muscarinic receptors on the myoepithelial cells have not been characterized. And if the muscarinic receptor would be of the M1 subtype, the results on the blockade of extravasation of Evans blue could very well had been an effect caused by blockade of muscarinic M1 receptors on the myoepithelial cells. Since it is not possible to exclude the occurrence of muscarinic M1 receptors on myoepithelial cells, as the staining gives inconclusive results, an effect of muscarinic M1 receptors acting directly on the myoepithelial cells cannot be excluded as a partial explanation of the decreased permeability.

Secretory effects of muscarinic receptors
In vivo effects on secretion of fluid (papers I, III and IV)

Parasympathetic impulses releasing acetylcholine that acts on glandular muscarinic receptors, predominantly of the M3 subtype, are by far the most important stimuli for production of voluminous fluid salivary gland responses (Baum & Wellner, 1999). In contrast to the sympathetic glandular activation, the cholinergic response is usually poor in protein (Martinez *et al.*, 1975). However, in the cholinergic neuron, NANC transmitters such as VIP may be co-stored (Lundberg *et al.*, 1980). VIP may *per se* evoke a sparse and protein rich secretion (Tobin *et al.*, 1990b). In spite of the rather early morphological characterization of a heterogeneous muscarinic receptor population on parenchymal secretory structures in salivary glands, studies on the functional significance of other muscarinic receptors than those of the M3 subtype are few. Most functional studies are performed on knock-out mice and in these whole saliva responses are measured (Matsui *et al.*, 2000; Takeuchi *et al.*, 2002; Bymaster *et al.*, 2003; Gautam *et al.*, 2004). Before this thesis, the only functional *in vivo* studies measuring the actual secretory responses according to the muscarinic subtype distribution from the individual glands have been performed in the rat and the rabbit (Tobin, 1995, 1998; Tobin *et al.*, 2002).

The observations in the thesis agree with the previous findings in that methacholine caused a profuse secretion from the rat parotid and submandibular glands. And likewise to the general conception, administration of 4-DAMP exerted pronounced inhibitory effects on the salivation. Furthermore, it equipotently inhibited the secretion in both glands. In contrast, pirenzepine caused potent inhibition in the submandibular glands, but in the parotids only at larger doses (Figure 13). In the ovine submandibular gland, electrical stimulation of the parasympathetic chorda tympani nerve induced profuse and markedly atropine-sensitive fluid responses. However, at the highest frequency stimulation employed (8 Hz), a small secretion still occurred in the presence of a large atropine dose (6 % of that in the absence of atropine), which indicate that other transmitters than acetylcholine are released (Figure 14). One NANC transmitter being released in the ovine submandibular gland has been shown to be VIP (Edwards *et al.*, 2003). Even so, NANC transmitters had relatively little influence on the parasympathetic fluid response, since stimulation in bursts, which is

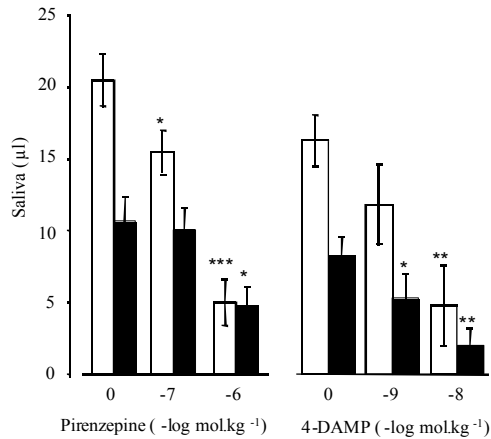


Figure 13. Protein output from rat submandibular (□; n=8) and parotid (■; n=8) glands. represent mean protein output responses to methacholine at 1 µg.kg⁻¹ i.v. in the absence and presence of pirenzepine (left) and in the absence and presence of 4-DAMP (right). Vertical bars represent S.E.M. * p=0.05; ** p=0.01; *** p=0.001.

known to enhance peptidergic transmitter release (Edwards *et al.*, 1984), did not improve the secretory response in comparison to that of a continuous stimulation. Anyhow, the nerve-evoked response was well-maintained during stimulation and well reproducible. Methoctramine did not affect the flow of saliva at any of the stimulation patterns and frequencies when assessing the changes over the whole 10 min stimulation period. Pirenzepine, on the other hand, significantly reduced the flow of saliva at all frequencies examined, most conspicuously at low frequency stimulation. At stimulation at 2 Hz given continuously, the flow was reduced by 69%. In addition to the reduction of the flow of saliva, pirenzepine consistently delayed the onset of the flow of saliva, especially at low frequencies. The addition of an extremely low dose of p-F-HHSiD had no or little effect on the pirenzepine-inhibited fluid response to the intensive stimulations, but almost abolished the fluid response at 2 Hz. The fact that this low dose affected the response supports the assumption of selective antagonism, both for pirenzepine and p-F-HHSiD (Figure 15).

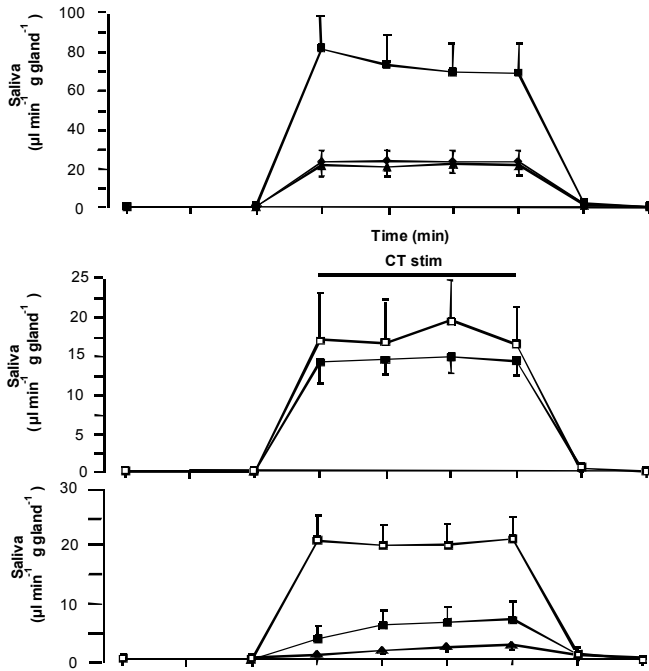


Figure 14. Upper panel: Changes in submandibular salivary flow in response to chorda tympani stimulation at 2 Hz continuously (▲), 20 Hz in bursts (1:10 s; ●) and 8 Hz continuously (■) for 10 min in 13 anaesthetized sheep.

Middle panel: Changes in submandibular salivary flow in response to chorda tympani stimulation at 2 Hz continuously for 10 min in the absence (□) and in the presence of methoctramine (100 µg kg⁻¹ I.V.; ■) in five anaesthetized sheep.

Lower panel: Changes in submandibular salivary flow in response to chorda tympani stimulation at 2 Hz continuously for 10 min in the absence of antagonists (□) and in the presence of pirenzepine (40 µg kg⁻¹ I.V.; ■) before and after administration of p-F-HHSiD (4 µg kg⁻¹ I.V.; ▲) in five anaesthetized sheep.

Vertical bars: S.E. M. value. Horizontal bar: duration of stimulation (CT stim)

The thesis thus includes the ovine and rat submandibular glands in the row of glands in which muscarinic M1 receptors evoke flow of saliva. In previous studies, muscarinic M1 receptor-activated salivary flow has

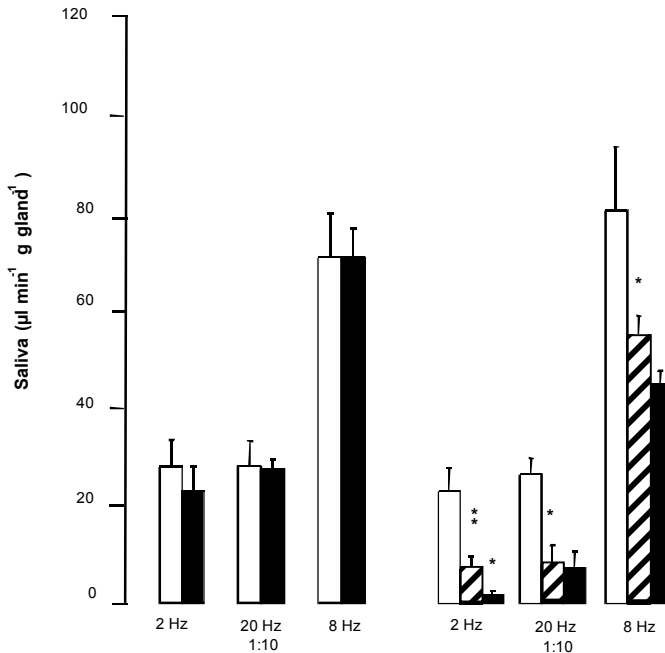


Figure 15. Changes in mean salivary flow over the 10 min stimulation period. The column doublets (left panel) and triplets (right panel) in each panel show from left to right the mean responses to chorda tympani stimulation at 2 Hz continuously, 20 Hz in bursts and 8 Hz continuously. The left panel show the mean responses in the absence (□) and presence (■) of methoctramine ($100 \mu\text{g kg}^{-1}$ I.V.) in five anaesthetized sheep and the right panel show the mean responses in the absence (□) and presence of pirenzepine ($40 \mu\text{g kg}^{-1}$ I.V.) before (▨) and after administration p-F-HHSiD (■; $4 \mu\text{g kg}^{-1}$ I.V.) in five other anaesthetized sheep. Vertical bars: S.E. M. * $P < 0.05$, ** $P < 0.01$.

been demonstrated in the rabbit submandibular gland (Tobin, 1995) and in the rat sublingual gland (Culp *et al.*, 1996; Tobin *et al.*, 2002). In these glands, as well as the murine parotid gland (Watson *et al.*, 1996), a co-expression of muscarinic M1 and M3 receptors occurs, and in knock-out mice, as well as in the sublingual gland of the rat, a simultaneous activation of both subtypes seems to be a prerequisite for evoking a maximal fluid response (Luo *et al.*, 2001; Gautam *et al.*, 2004). Because of the number of different ways positive interactions

could be exerted regarding the fluid response, it is hard to make any absolute estimation of the relative contribution to the fluid response of muscarinic M1 and M3 receptors in the current studies. The ovine submandibular and parotid glands share the characteristics of salivary glands of several other species in that stimulation of the parasympathetic innervation at relatively high frequencies produces an atropine-resistant fluid secretion mediated by peptidergic transmitters (Reid & Titchen, 1988; Ekström, 1999). But the absence of an overt secretion at the low intense stimulations does not guarantee a pure cholinergic response. In the ferret and the cat, it has been shown that, even though an overt atropine-resistant response is absent, peptidergic transmitters may anyway act in concert with the classical transmitter, *i.e.* acetylcholine, and enlarge the amount or alter the quality of the saliva (Lundberg *et al.*, 1982; Ekström & Tobin, 1990). Nevertheless, at low frequencies muscarinic M1 receptors account for a large part, if not nearly the whole secretory response. Furthermore, in all cases a maximal secretory response was not elicited under muscarinic M1 receptor blockade. Thus, muscarinic M1 receptors account for a significant proportion of the cholinergic parasympathetic secretory response of the rat and ovine submandibular glands, and, at low intensity of nerve activity, their contribution may be larger than that of muscarinic M3 receptors.

In vivo effects on secretion of protein (papers I, III and IV)

When administered into the bloodstream, cholinergic agonists evoke a profuse salivary secretion, which is poor in proteins. VIP stimulates, in most species examined so far, a conspicuous glandular output of proteins, in some of these species accompanied by a sparse fluid response as well (Ekstrom *et al.*, 1983; Ekstrom & Tobin, 1989, 1990; Edwards & Titchen, 2002). Accordingly, electrical stimulation produces a secretory response with higher protein content than that evoked by cholinergic stimulation, but lower than that produced by VIP (Ekstrom & Tobin, 1990). Another fact, which further contributes to enhance the nerve-evoked protein output, is that simultaneous administration of VIP and methacholine potentiates the output far more than would be expected by just an additive effect (Ekstrom & Tobin, 1990). Also in this thesis the same observations were made according to nerve stimulation and stimulation by muscarinic agonist. In the rat salivary glands methacholine was used, and in the sheep, the

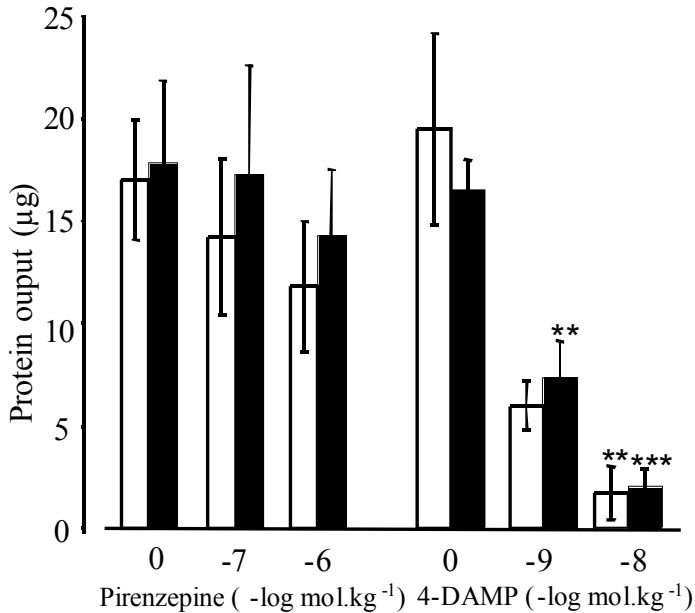


Figure 16. Protein output from rat submandibular (□; n=8) and parotid (■; n=8) glands. Columns represent mean protein output responses to methacholine at 1 µg.kg⁻¹ i.v. in the absence and presence of pirenzepine (left) and in the absence and presence of 4-DAMP (right). Vertical bars represent S.E.M. * p=0.05; ** p=0.01; *** p=0.001.

parasympathetic chorda tympani nerve was stimulated. Consequently, the protein output in the latter experiments was somewhat larger than that in the rat glands. Stimulation by different transmitter does not seem to affect the protein output qualitatively but only quantitatively (Ekstrom et al., 1996).

Thus, in the rat salivary glands, methacholine evoked a profuse secretion poor in protein. The protein concentration therein was, however, twice as high in the parotid saliva than in the submandibular. In the rat parotid and submandibular glands, the inhibitory pattern was the same except that 4-DAMP, at the doses used, had a greater inhibitory effect on the protein output, which makes muscarinic M1 receptors unlikely to be directly connected to regulatory effects on protein secretion (Figure 16). In the ovine submandibular gland, the

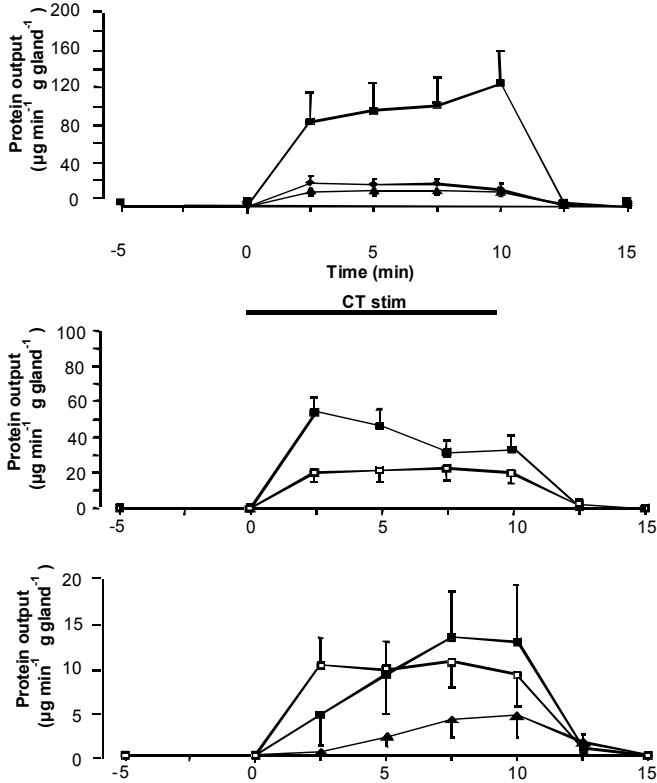


Figure 17. Upper panel: Changes in submandibular protein output in response to chorda tympani stimulation at 2 Hz continuously (▲), 20 Hz in bursts (1:10 s; ●) and 8 Hz continuously (■) for 10 min in 13 anaesthetized sheep.

Middle panel: Changes in submandibular protein output in response to chorda tympani stimulation at 2 Hz continuously for 10 min in the absence (□) and in the presence of methoctramine (100 µg kg⁻¹ I.V.; ■) in five anaesthetized sheep.

Lower panel: Changes in submandibular protein output in response to chorda tympani stimulation at 2 Hz continuously for 10 min in the absence of antagonists (□) and in the presence of pirenzepine (40 µg kg⁻¹ I.V.; ■) before and after administration of p-F-HHSiD (4 µg kg⁻¹ I.V.; ▲) in five anaesthetized sheep.

Vertical bars: S.E. M. value. Horizontal bar: duration of stimulation (CT stim).

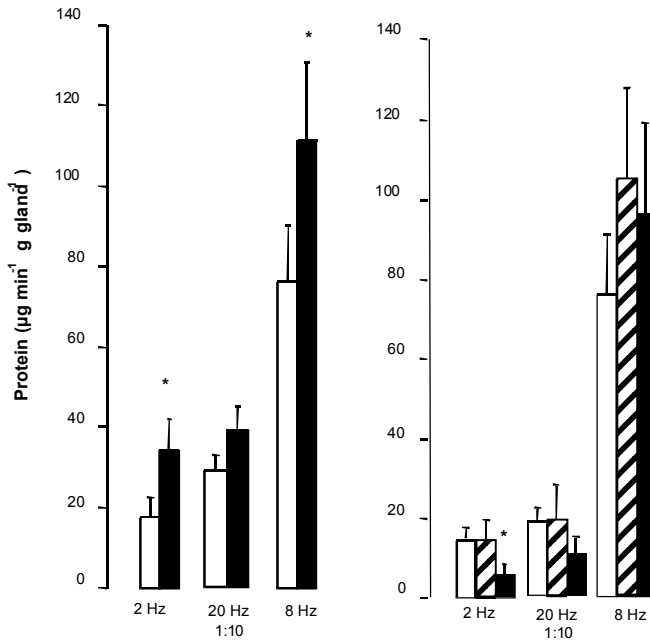


Figure 18. Changes in mean protein output over the 10 min stimulation period. The column doublets (left panel) and triplets (right panel) in each panel show from left to right the mean responses to chorda tympani stimulation at 2 Hz continuously, 20 Hz in bursts and 8 Hz continuously. The left panel show the mean responses in the absence (□) and presence (■) of methoctramine ($100 \mu\text{g kg}^{-1}$ I.V.) in five anaesthetized sheep and the right panel show the mean responses in the absence (□) and presence of pirenzepine ($40 \mu\text{g kg}^{-1}$ I.V.) before (▨) and after administration p-F-HHSiD ($4 \mu\text{g kg}^{-1}$ I.V.) in five other anaesthetized sheep. Vertical bars: S.E. M. * $P < 0.05$, ** $P < 0.01$.

protein concentration was approximately 30 % higher than in the rat, but this is, as discussed above, likely to have depended on the agonist versus nerve stimulation (Figure 17). Also, the neuronal release of peptidergic transmitters is enhanced by increased neuronal signalling intensity (Lundberg *et al.*, 1981a; Edwards *et al.*, 2003). As a consequence, electrical nerve stimulation delivered in bursts gives rise to a larger VIP release than the same number of impulses delivered at a continuous pattern. And in contrast to the fluid nerve stimulation

response in the ovine submandibular gland, burst stimulation at 20 Hz given every tenth second caused a larger output of protein than the continuous stimulation at 2 Hz, and further, 8 Hz stimulation caused a greater concentration in the saliva than 2 Hz. But as mentioned previously, this is no guarantee for absence of peptidergic influence on nerve response at 2 Hz. Namely, neuropeptides may be released at very low frequencies (<1 Hz; Ekstrom and Tobin, 1989) and could consequently potentiate a cholinergic response even at 2 Hz.

The intravenous administration of methoctramine combined with continuous stimulation at 2 and 8 Hz, caused significant increases of the saliva protein output by 90 and 45%, respectively. The increase in the protein output at 2 Hz occurred primarily during the initial few minutes. Likewise, methoctramine caused a significant increase during the first minutes (2-4) of stimulation at 20 Hz in bursts, but not of the output when assessed for the whole period. Whereas pirenzepine on its own did not affect the protein output at any frequency or pattern, the addition of p-F-HHSiD significantly reduced the output at 2 Hz. This decrease occurred in spite of four-fold increase in the protein concentration, much because of the markedly diminished fluid response (Figure 18). The initial increases in the protein output observed in the sheep both after the administration of pirenzepine and methoctramine may not necessarily be coupled to postsynaptic events. In view of the affinity profiles (see above) of the two antagonists, muscarinic M4 receptors seem plausible for mediating the protein output enhancing effect. In many organs, muscarinic M4 receptors have been described to modulate the transmitter release (Alberts, 1995; Kilbinger *et al.*, 1995; D'Agostino *et al.*, 1997). Tentatively, muscarinic M4 receptors may be the inhibitor receptor on parasympathetic nerve terminals in salivary glands also. Inhibitory muscarinic receptors have previously been described in the rat parotid gland and suggested to affect the release of acetylcholine as well as NANC transmitter(s) (Tobin, 1995, 1998). However, no characterization discrepancies regarding M2 and M4 receptors were made.

The current findings may support non-M1 receptor mediation by excitatory muscarinic receptors (M3/M5) in protein release, but more strongly indicate the occurrence of prejunctional muscarinic M4 receptors. Blockade of such inhibitory receptors would result in increased responses to electrical stimulation of the parasympathetic

responses. In view of the poor effect on the fluid response and on the pronounced effect on the protein output, as well as the intermediate effect on the vascular response, and further, in connection with effects even at high frequencies, the release of NANC transmitters, and VIP in particular, seems to be mainly affected by this prejunctional modulation.

III. Neuronal release of transmitter (paper IV)

Modulator roles of prejunctional muscarinic receptors on acetylcholine release from cholinergic nerve terminals have been demonstrated in peripheral organs of several species including man (Kilbinger *et al.*, 1991; Tobin, 1995; Tobin & Sjogren, 1995; Somogyi & de Groat, 1999; D'Agostino *et al.*, 2000; Larsen *et al.*, 2004). The modulating effects include facilitation as well as inhibition. While the facilitation preferentially seems to occur over short periods of intense neuronal activity, the inhibition seems to be the basic function occurring more or less constantly (Tobin, 1998, 2002). Pharmacological studies characterizing the muscarinic prejunctional inhibitory receptors suggest these to be of either the M2 or the M4 subtype (Kilbinger *et al.*, 1991; Alberts, 1995; D'Agostino *et al.*, 1997; Tobin, 1998; D'Agostino *et al.*, 2000; Tobin, 2002). In salivary glands, the modulator prejunctional muscarinic receptor subtypes have been characterized out of indirect functional observations. Direct inhibitory and facilitator effects by muscarinic receptors have been observed on the release of several NANC transmitters from parasympathetic nerves in salivary glands (Tobin *et al.*, 1991; Tobin *et al.*, 1994). However, in salivary glands the effect has been most thoroughly studied on the parasympathetic release of VIP (Lundberg *et al.*, 1981a; Tobin *et al.*, 1991; Edwards *et al.*, 2003). The inhibitory prejunctional effect seems to be unspecific for the release of transmitters co-localised in the nerve, as indicated by the muscarinic modulator effects on typical responses to acetylcholine and VIP (Tobin, 1998). Since VIP is co-stored with acetylcholine (Lundberg, 1981), the release of VIP is therefore likely to reflect a general effect on the neuronal transmitters being released, even though the release of each may vary depending on the intensity of neuronal activity. By considering VIP as a marker of the effect on transmitter release, the detection of VIP in the glandular blood effluent demands relatively high nerve stimulation frequencies, since the peptidergic transmitters are released in low amounts at low stimulation frequency. But even though the parasympathetic nerve was challenged with high frequency of stimulation, the amount of VIP being released is small considering the detection limit of the ELISA tests available. Consequently, a big animal such as the sheep allowing for withdrawal of blood samples of certain volumes to be analysed is a requirement. Any short-lived facilitation was not even in this big animal possible to

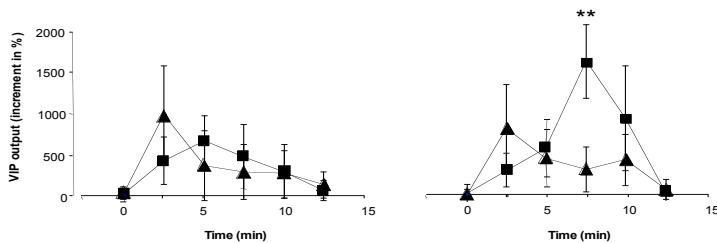


Figure 19. Comparison of the changes in VIP output in response to chorda tympani stimulation at 8 Hz continuously for 10 min (from point of time 0 to 10) in the absence (▲) and in the presence (■) of methoctramine (left column of panels; 100 µg/kg iv) in 5 anesthetized sheep and in the absence (▲) and in the presence (■) of pirenzepine (right column of panels; 40 µg/kg iv) in 6 anesthetized sheep. Values are means±S.E.M.

study in spite of employing a high frequency of stimulation (8 Hz).

Electrical stimulation of the parasympathetic chorda tympani nerve at 8 Hz induced significant increase outflow of VIP in the submandibular venous effluent plasma. When the chorda tympani nerve was challenged by electrical stimulation in the absence of antagonist, the VIP output rose by nine to eleven times. After the intravenous administration of methoctramine, the total mean VIP output over the 10 min stimulation was not significantly increased. In the presence of pirenzepine, no changes in the output occurred during the early phase of stimulation, whereas it was conspicuously increased in the later phase of the 10 min stimulation period (Figure 19).

In the current experiments, pirenzepine inhibited the flow of saliva. Even though the existence of a VIP-evoked as well as an atropine-resistant parasympathetic fluid response, muscarinic receptor stimulation is the principal stimulus for fluid secretion in the actual gland (Edwards et al., 2003). In view of the small VIPergic response, the pirenzepine inhibition of the flow of saliva is likely to be an effect on glandular muscarinic receptors and any blockade of postjunctional muscarinic receptors would override prejunctional effects increasing the release of VIP. However, all other parameters, *i.e.* protein output, vasodilatation and VIP release, were increased after pirenzepine administration, indicating a blockade of prejunctional inhibitory

receptors. In view of the general opinion that pirenzepine preferentially blockades muscarinic M1 receptors, it may seem rather unexpected that pirenzepine showed a greater potency than the “M2/M4”-selective antagonist methoctramine. However, the selectivity window of muscarinic antagonists is very narrow. Even though pirenzepine shows about 100 times greater affinity for M1 than for M2 receptors, it shows only about five times greater for the M1 over the M4 receptor (Caulfield & Birdsall, 1998). Furthermore, the affinity of the currently used antagonists, pirenzepine and methoctramine, is close to identical on M4 receptors. Nevertheless, while pirenzepine significantly increased the protein output, the vasodilatation and the release of VIP, methoctramine showed tendencies towards the same pattern. The reason to the poor effect of methoctramine could possibly be found in the pharmacology of the antagonist, which could include effects on nicotinic as well as on adrenergic receptors (Howell *et al.*, 1994; Usherwood, 2000; Melchiorre *et al.*, 2003) resulting in more varying responses. Notably, this was also indicated in the present thesis studies, in which methoctramine inhibited phenylephrine induced blood vessel contractions (paper II).

A striking phenomenon within the current results is that the release of VIP and the VIP archetypical responses, *i.e.* protein secretion and vasodilatation, went in parallel. This favours the idea that it is the release of VIP that is affected by the muscarinic antagonists and not direct anticholinergic effects on glandular secretory and vasodilator responses. The fact that the blockade caused increases in these responses also favours a blockade of inhibitory receptors. No muscarinic M2 receptors were detected in the immunohistochemical examination of the ovine submandibular gland, and pirenzepine showed at least the same inhibitory potency as methoctramine. Thus, it seems reasonable to conclude that in this gland, inhibitory muscarinic receptors of the M4 subtype are localized prejunctionally and that these modulate the release of transmitters.

However, the values of the VIP output should be interpreted with some caution. In the resting condition, the plasma VIP content of the venous and arterial samples was occasionally very close to the detection limit. However, the results could be considered as reliable, since clear progresses were found during the stimulation period, and further, since the typical VIP responses coincided with the release of VIP. Also, the

length of the experiment could have influenced the released amount of VIP into the venous blood since one sheep was submitted to a series of stimulation. It is possible that more VIP could be released from the nerve at the beginning of the experiment than after several periods of stimulation (it did not only include the 8 Hz stimulations, but also the 2 Hz and 20 Hz bursts stimulations). In contrast to the classical transmitters, VIP is synthesized in the nerve cell body and transported by the axonal flow to the nerve terminal (Dahlstrom et al., 1992). If the stimulation released more than is transported by the axonal flow, there will be a shortage of the transmitter in peripheral end of the neuron (Tobin, 1998). Thus, the experimental protocol may have hampered the detection possibilities of muscarinic prejunctional inhibition. The release may have been underestimated at the end of the protocol, which makes the conclusion from the data of the existence of inhibitory muscarinic receptors on nerve terminals in the ovine submandibular even more definite.

General discussion

In physiological responses, the integration of stimuli may be performed at different levels. So may the sensory input be, such as in salivary glands the result of mechanical or chemical stimulation at short or long duration and at varying intensity. In the central nervous system, the input may then be further modulated by nervous impulses from other areas, which consequently may result in varying efferent signals affecting salivary gland function. These efferent signals may not only employ different and interacting signals in the two autonomic nervous systems. They may also, by complex systems within each division, be further modulated. So is the release of transmitter affected by prejunctional receptors and the amount of co-stored transmitters modulated by the intensity of neuronal activity. The responses are thus not the result of a single type of transmitter or receptor, but the effects of the whole concert of interacting players either enhancing or inhibiting each other. Furthermore, the actual response from a salivary gland is also dependent on the integration of different types of functions within the gland. Needless to say, the blood flow through the gland must present fluid and molecules meeting the demands of the living cells necessary for its survival under normal and pathological conditions. However, the primary function of glandular cells, i.e., moistening, lubrication and digestion, put further requirements into focus (Pedersen et al., 2002). Namely, the quantity of fluid and essentials for synthesis of saliva content must be adapted to the circumstantial requirements.

An experiment on isolated tissues only gives indications for how a specific mechanism may affect functions. Even though studies within whole body preparations, such as within the anaesthetized animal, give better clues for the roles of the mechanisms, the number of interactions and variations are obviously numerous. Therefore, the importance of one particular receptor system for the homeostasis of the organism is hard to quantify out of experimental models. However, by describing the effects of muscarinic receptor subtypes on glandular functions in different species and by different techniques, a pattern of regulatory effects was searched for. Common findings in the different species would also imply general occurrence of the mechanisms in mammals, including humans.

More or less, all muscarinic receptors were detected in the salivary glands in the three species examined. In accordance to the orthodoxy idea, muscarinic M3 receptor subtype was doubtlessly the receptor evoking the most significant functional effects both regarding vascular and secretory responses. However, the postjunctional receptor subtype besides the M3 subtype, contributing considerably seems to be the muscarinic M1 receptor, which influenced both types of function. Also, its frequent occurrence supports this assumption, but the occurrence pattern indicated different roles. While muscarinic M3 receptors showed a general appearance, the muscarinic M1 receptors preferentially occurred in seromucous/mucous glands. These types of glands have been suggested to be particularly important during resting conditions, such as for meeting the demands of lubricating the oral cavity (Pedersen *et al.*, 2002; Brosky, 2007). It is also noteworthy that the blockade of muscarinic M1 receptors in the ovine submandibular gland was most efficient on the fluid response at less-intense stimulation. It should be kept in mind that muscarinic M5 receptors have been indicated to exert influence on the salivary secretions in the rat submandibular gland (Tobin *et al.*, 2002), but that the effect was small and did not favour the idea of any significant role for secretion. Furthermore, in the vasculature, the muscarinic receptor responses comprised relaxation, being at least partly NO dependent, as well as contractile components. In the arteries, a M1 receptor component, in addition to the M3 receptor relaxatory effects, may have contributed to the relaxation at less intense stimulation, while a non-M1 contractile component appeared at intense stimulation. According to the expression findings, this non-M1 receptor contractile effect is most likely to have been evoked by M3 receptors. In veins, contractions occurred, preferentially at less intense stimulation, which, however, were markedly reduced at intense stimulation, possibly indicating the inclusion of a relaxation component at high cholinergic activity. This venous contractile effect may either be induced by inhibition of NO-synthesis (possibly by M4 receptors) or more likely, by muscarinic receptor-induced contraction, presumably by M1 receptors. The expression pattern showing endothelial as well as more adventitial occurrence of muscarinic receptors supports the idea of cholinergic intrinsic and extrinsic systems in the regulation of the vasculature (Kummer & Haberberger, 1999). Tentatively, acetylcholine autoregulatory effects by release from endothelial and blood cells (Kawashima *et al.*, 1990; Kawashima & Fujii, 2000) may be of

significance for the basal regulation of blood flow. These effects may be mediated by endothelial muscarinic M1 and M3 receptors on the arterial part of the vasculature, whereas endothelial muscarinic M4 receptors may participate on the venous part. The intrinsic system may also include blood cell cholinergic systems, such as lymphocytes (Kawashima & Fujii, 2000) comprising acetylcholine synthesis as well as muscarinic receptors including the M5 subtype (Kawashima & Fujii, 2004), which may further complicate the picture. The activation of the extrinsic system, which presumably would have been done by electrical stimulation of the parasympathetic innervation in the ovine submandibular gland, induced cholinergic vasodilatation. Inhibition of this response by administration of “subtype-specific” antagonists did not agree to any specific receptor subtype, particularly not to M1 mediation. This may of course be the result of a composite cholinergic response or to the involvement of yet another type of muscarinic receptor subtype, possibly of the M5 subtype. All in all, a general feature for the muscarinic receptors according to “normal” physiological responses may be that in the case of resting conditions, the M1 receptors play a significant role, whereas muscarinic M3 receptors seem to mediate parasympathetic cholinergic responses in the situation of more conspicuous glandular activation. This theory is further supported by the fact that while pirenzepine had a very minor effect on the blood flow in the sheep, which were subjected to nerve stimulation, it had a tendency to a more pronounced effect on the methacholine-induced blood in the rat flow *in vivo* and a substantially greater effect on the methacholine-induced arterial relaxation *in vitro*.

The role of the seemingly dual cholinergic responses of arterial and venous preparations, that is, constriction at low cholinergic concentrations and possibly dilatation at large of veins and the opposite of arteries, may also be interpreted in the context of composite responses. The responses at low concentrations may reflect the effects during autoregulatory cholinergic mechanisms. Thus, there is an arterial vasodilatation and a tendency towards a constriction of the venous drainage. Accessory glands may secrete even in the absence of autonomic impulses (Emmelin, 1981). The autoregulation causing increase in the arterial (by dilatation) as well as in the venous (by constriction) hydrostatic pressure, may serve to preserve filtration conditions by compensating for the fluid and protein secretion. In other glands, arteriovenous anastomoses and/or myoepithelial mechanisms

have suggested to exert such effects (Lung, 1998). However, as mentioned above (Fraser & Smaje, 1977; Ohtani *et al.*, 1983), not all glands are provided with anastomoses, and further, if there is no autonomic signalling, some other mechanism is likely to contribute to preserving filtration according to the re-evaluated Starling hypothesis (Levick, 2004). In this context it is worth noting the findings by Lung (1998) when injecting acetylcholine and VIP intraarterially. While the hilar venous pressure rose gradually over a one minute period in spite of an instantaneous increase in blood flow in response to VIP, acetylcholine induced an immediate increase of both. The observations favour the idea of muscarinic receptor involvement in the regulation. Since the contractile response of salivary gland veins was markedly sensitive towards pirenzepine, as the permeability of glandular filtration was also, muscarinic M1 receptors (and possibly M4) are likely to exert the venous autoregulatory effect. Furthermore, M1 receptors may also contribute to the relaxatory effect on the arterial side; their occurrence on the endothelium and the effects of pirenzepine on arterial relaxation indicates this. Nevertheless, these effects seem to occur only at low intense stimulations. At intense stimulation, the responses were reversed indicating counteracting effects; for instance such as counteracting pronounced arterial NANC dilatator effects.

The current findings regarding protein secretion could also be interpreted in support for the assumption that muscarinic M1 receptors principally mediate effects during resting conditions. Assuming M1 receptors to cause some kind of basal effects, any pronounced cholinergic stimulation would add secretory products to the basal content. Since blockade by the antagonists favoured the idea of muscarinic M3/M5 receptors possibly evoking protein release, it does not contradict the assumption. However, the observations made on the protein output more strongly indicate modulation on the release of neuronal transmitters. The parasympathetic neuronal co-storage of acetylcholine and VIP (Lundberg, 1981), and that VIP is much more potent regarding protein secretion (Lundberg *et al.*, 1980), means that any variation of the relative release of the two would have impact on the saliva protein concentration. Since blockade by pirenzepine, and possibly methoctramine also, increased the protein output, prejunctional muscarinic M4 receptors are plausible to modulate parasympathetic transmitter release, which was supported by the observations on the nerve-evoked release of VIP.

The observations made on human labial glands showed the same occurrence pattern for the muscarinic receptor subtypes as was found in the examinations of the rat and the sheep. However, in patients suffering from Sjögren's syndrome, there was a conspicuous increase in the expression of muscarinic M3, M4 and, in particular of M5 receptors. This is in accordance with an previous report of M3 up-regulation (Beroukas *et al.*, 2002), as well as the occasional glandular hyperfunction occurring during the disease progression (Dawson *et al.*, 2005). M5 receptor up-regulation has been associated with inflammation in other tissues (Wood *et al.*, 2000; Giglio *et al.*, 2005; Eglén, 2006), but to my knowledge it has not been reported in man before. Thus these findings may strengthen the idea of a role for M5 in the cross-talk between the nervous and the immune system, regulating pathological mechanisms.

Concluding comments

Most autonomic responses in salivary glands, if not all, represent the composite effects of activation of different types and subtypes of receptors. The glands in all species examined showed some principal similarities; muscarinic M3 receptors were of utter importance for the nerve-evoked cholinergic responses, while muscarinic M1 receptors mediated effects by low intensity cholinergic stimulation. Tentatively, this could mean that nerve activity of certain intensity evokes responses that are primarily mediated by muscarinic M3 receptors, while cholinergic function under resting conditions, or at least at less intense stimulation, is mediated by the M1 subtype. The autoregulation of blood flow may represent an example of the latter. The nerve-evoked parasympathetic responses are modulated by prejunctional muscarinic receptors of the M4 subtype. Furthermore, in pathological conditions, other subtypes and systems may be recruited. The muscarinic M5 receptor is one such candidate.

Expression of muscarinic receptor subtypes and their cellular location.

All subtypes occurred in the salivary glands, but the degree of expression varied. In the glandular vasculature, muscarinic M3 receptors are expressed on endothelial and smooth muscle cells in the arteries, while muscarinic M1 receptors seem to be the most prominent in the endothelium. In veins, muscarinic M1 receptor occurs in the venous smooth muscle layer also. In the glandular parenchymal tissue seromucous/mucous glands, the M1 receptor was commonly expressed

besides the muscarinic M3 receptors. In vicinity to glandular acini as well as in adventitial tissue of more or less all kinds of glands, muscarinic M1, M4 and M5 receptors were expressed, interpreted as nerve terminal, and possibly also, myoepithelial cell expression of muscarinic receptors.

Functional characterization of the muscarinic receptor subtypes according to vascular effects

Submandibular as well as carotid arteries relaxed in response to muscarinergic stimulation, partially via a nitric oxide-dependent mechanism. Submandibular and jugular veins contracted. In the carotids, M1 receptors may evoke a large part of the relaxation, at least that representing the intrinsic system. However, at high concentrations of muscarinic agonists and during blockade of M1 receptors, a contractile response may appear, probably via M3 receptor mediation. In the jugulars, a muscarinic M1 receptor contraction is suggested. In the submandibular vein, the contraction occurs at low concentrations of agonist. The overall effect on the glandular blood flow is vasodilatation and this effect is mainly evoked by muscarinic M3 receptors. However, muscarinic M1 or M5 or possibly both receptors may participate. Since blockade of M1 receptors seemed to have greater effect when the muscarinic receptors were stimulated by an intravenous muscarinic agonist, than by stimulation by nerve stimulation, muscarinic M1 receptors are suggested to be involved in the intrinsic system. Furthermore, the muscarinic M1 receptor affected vascular filtration.

Functional characterization of the muscarinic receptor subtypes according to secretory effects

Muscarinic M1 receptors account for a significant proportion of the cholinergic parasympathetic secretory response, and at low intensity of nerve activity, their contribution may be larger than that of muscarinic M3 receptors. If any particular muscarinic receptor subtype contributes more than another to the secretion of protein, this receptor may be an excitatory muscarinic receptor of the M3 or possibly the M5 subtype.

Functional characterization of the muscarinic receptor subtypes according to neuronal transmission

Inhibitory muscarinic receptors of the M4 subtype may occur prejunctionally on parasympathetic nerve terminals. In the sheep, these

receptors potentially inhibited the release of VIP, but are likely to have an impact on the release of acetylcholine as well.

Thus, it is shown in the thesis that:

- All subtypes of the muscarinic receptor occur in the salivary glands
- Muscarinic M1 receptors accompany the M3 receptor both in the vasculature and in the secretory parenchyma
- The muscarinic M1 receptor expression as well as its function is particularly obvious in seromucous/mucous glands
- Muscarinic M1 receptors have contractile effects in veins and affect the permeability of the glandular (submandibular) vasculature
- Muscarinic M4 receptors are expressed on parasympathetic nerve terminals and modulate the release of transmitter
- Muscarinic M5 receptors are increased in Sjögren's syndrome, and are suggested to have a role in pathophysiological regulatory mechanisms.

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