Review

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# An Overview of Autonomic Regulation of Parotid Gland Activity: Influence of Orchiectomy

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**Key Words**
Amylase · $Na^+$-$K^+$-ATPase · Orchiectomy · Parotid gland · Rat

**Abstract**
The parotid gland participates in the digestive process by providing fluid, electrolytes and enzymes that facilitate the onset of digestion. Neurotransmitters, hormones and biologically active peptides regulate its activity. The autonomic system is the main regulatory mechanism of the gland. Sympathetic stimulation induces amylase release through $\beta_1$-receptor activation and few fluid secretion by $\alpha_1$-receptor activation. The parasympathetic system controls basal activity of the gland acting on $M_1$ and $M_3$ muscarinic acetylcholine receptors and induces the secretion of fluid saliva rich in electrolytes through the modulation of ion channels and the $Na^+$-$K^+$-ATPase activity. In addition, its activation induces amylase release. The mechanisms involved in amylase secretion by isoproterenol and carbachol, as well as the mechanism of the cholinergic regulation of $Na^+$-$K^+$-ATPase activity and the changes observed after orchiectomy, are the scope of this review.


**Abbrevations used in this paper**

| | |
|---|---|
| 4-DAMP | 4-diphenylacetoxy-N-methylpiperidine methiodide |
| CaM | calmodulin |
| EGF | epidermal growth factor |
| FGF | fibroblast growth factor |
| IP | inositol phosphate |
| Ki | equilibrium inhibition constant |
| mAChR | muscarinic acetylcholine receptor |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | phospholipase C |

## Parotid Gland

As an exocrine gland of the digestive tract the parotid gland supplies a variety of proteins, fluids and electrolytes that play key roles in maintaining the environment of the oral cavity, and in facilitating the onset of the digestive process. The components of saliva are mainly produced by acinar cells and are conveyed to the oral cavity by a cell-lined duct system, where the fluid and electrolyte components are subjected to secondary modifications [Castle and Castle, 1998].

Structurally, the parotid gland comprises four major epithelial compartments: acinar cells and intercalated, striated and excretory ducts [Denny et al., 1997]. The acinar cells secrete amylase, an important digestive enzyme, and produce saliva that is isotonic with plasma.

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The release of salivary components by acinar cells is regulated by neuronal stimuli. Acinar cells are richly innervated by both sympathetic and parasympathetic nerve fibers [Emmelin, 1987]. Sympathetic nerve excitation elicits protein-rich saliva, whereas parasympathetic stimulation induces fluid secretion with low protein concentration. These receptor systems seem to act in collaboration with other transmitters as histamine [Hashioka, 1995], nitric oxide [Rosignoli and Pérez-Leirós, 2002], substance P [Iwabuchi and Kimura, 1998], prostaglandins [Abdel-Hakim, 1994] or cannabinoids [Busch et al., 2004b].

Parotid gland function can be altered by changes in dietary consistency, radiation therapy, medications and viral, genetic or autoimmune diseases [Herrera et al., 1988]. Besides, salivary glands are tissues sensitive to hormones [Kurihara et al., 1996].

### Orchiectomy

Like the ovaries, the testicles have two functions: gametogenesis and hormone production. Gametogenesis is controlled in part by the follicle-stimulating hormone secreted by the anterior pituitary gland and by testosterone, which is synthesized locally. Leydig cells, upon the influence of luteinizing hormone, produce testosterone, the main androgen secreted by the testicle. Thus, when testicles are removed, the animal is deprived of the effects of testosterone.

In gonadal-intact animals, testosterone and its active metabolite, 5α-dihydrotestosterone, bind to intracellular steroid receptor (androgen receptor) and modulate target organ functions. Castration induces a decrease in serum testosterone levels being almost immeasurable 21 days after orchiectomy. In contrast, in testosterone-substituted orchiectomized rats (1 mg/100 g body weight), after 7 days of treatment, a significant increase in serum testosterone levels is noted [Busch et al., 2004a].

### Salivary Glands and Sex Steroids

The existence of an interaction between sex hormones and salivary glands of rodents has long been documented, beginning with the pioneering works of Lacassagne [1940] and Bruillard and Delsuc [1941]. In 1940, Lacassagne stressed the difference between the histologic structure of the male and female mouse submandibular glands. It was indicated that the female submandibular gland assumes the appearance of the male submandibular gland when the animal receives testosterone and, that the male submandibular gland resembles the female gland in structure when the male animal is treated with folliculin. Lacassagne suggested that these variations observed in the structure of the glands might correspond to differences in the quality of saliva excreted by the two sexes. Bruillard and Delsuc, in 1941, reported that in the parotid and retrolingual glands of the rat there are differences in the size of the cells and the diameter of the acini after gonadectomy and injection of testosterone, observations that were coincident with those described by Lacassagne in the submandibular gland. Shafer and Muhler, in 1953, described the histologic changes observed in the granular tubules of the submandibular gland after gonadectomy.

After these pioneering works, the existence of sexual dimorphism in the rat and mouse submandibular gland that disappeared after castration was confirmed. In the rat submandibular gland, the granular tubules of male rats are larger than those of females. Castration seems to abolish these differences by inducing involution of tubular tissue in males. Whereas treatment with testosterone seems to reconstitute tubular tissue in castrated males, a similar dose of estradiol benzoate elicits a reduction in tubular size in ovariectomized females [Rins de David et al., 1991]. In the mouse, male and female submandibular glands can also be distinguished. The male type has a well-developed duct system, while the acini are moderately developed. In contrast, the female type of this gland bears large acini with a poorly developed duct system. Estrogens given exogenously to males cause a shift in the structure of the gland towards the female type. The reverse is observed when testosterone is given to females [Sawada and Noumura, 1991]. In submandibular glands of the adult hamster, a marked sexual dimorphism was revealed in 20.5- and 24-kDa proteins. These proteins are present in intact and castrated males but not in females. In females, ovariectomy induces the 20.5- and 24-kDa proteins to male levels. Estrogen administration to gonadectomized hamsters of either sex or to intact males obliterated these proteins [De, 1996].

The androgen-dependent expression of fibroblast (FGF) and epidermal growth factor (EGF) in submandibular glands of mice has been described. It was demonstrated that the expression of FGF-1 in the submandibular gland was androgen dependent by the striking gender differences in FGF-1 activity. In females, the gland contained one tenth or less of the FGF-1 activity of male glands. Castration reduced, while administration of tes-

tosterone to females increased FGF-1 concentrations in the gland [Okamoto et al., 1996]. EGF is a potent regulator of cell growth and differentiated function in many tissues and its levels are regulated by several hormones in a tissue-specific manner. In the mouse submandibular gland, the levels of immunoreactive EGF are highest in the male, where it is stored as a small peptide before being released. Testosterone increases EGF levels. Treating female mice with testosterone raises the level of EGF in the submandibular gland, which approaches those in the males after approximately 1–2 weeks. Conversely, castration of adult male mice causes EGF mRNA levels to begin to fall within 24 h and to reach 2% of the initial value at 2 weeks [Sheflin et al., 1996]. After ovariectomy, submandibular gland EGF first decreased to one third of preovariectomy levels, but after 10 days it started to increase and reached 3.5 times the preovariectomy level by day 80. Simultaneously, EGF mRNA increased. Testosterone treatment strongly augmented the levels of both EGF mRNA and EGF [Tuomela et al., 1990].

Ovariectomy also alters the secreted proteins in saliva. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of whole saliva of 50-day-ovariectomized rats showed the loss of a substantial number of proteins, including amylase and the acidic proline-rich proteins [Purushotham et al., 1993].

In contrast to the submandibular gland, the sexual dimorphism of the parotid gland is still questionable, although in one morphometric study on the human parotid gland it has been pointed out that the diameter of the striated duct in men is twice that in females [Posinovec, 1967]. Jezek et al. [1996] demonstrated that orchiectomy affected parotid gland morphology, an effect that can be prevented or alleviated by exogenously given testosterone. In orchiectomized animals 30–60 days after castration, a significant reduction in the volume of the acini and the duct system as well as a significant increase in the connective tissue was noted. The structure of the rat parotid acini is also changed by castration indicating a reduction in acinar-cell activity. Exogenously given testosterone can prevent or alleviate the mentioned effects of orchiectomy on the gland. Others reports describe a fatty degeneration of the parotid gland induced by ovariectomy [Shiraishi et al., 2000].

Salivary gland function is also regulated in part by thyroid and adrenocorticosteroid hormones [Kurihara et al., 1999]. In addition, the submandibular gland of female rats suffers changes in the expression and the activation of $\alpha_1$-adrenoceptors during different stages of the estrous cycle [Anesini and Borda, 2003]. The rat parotid gland is particularly affected by circadian variation in feeding activity/secretion and storage of amylase and other secretory products [Johnson, 1987].

We studied first the ability of the rat parotid gland of both sexes to secret amylase in response to sympathetic and parasympathetic stimuli and the effect of carbachol upon the $Na^+$-$K^+$-ATPase activity. Our results showed no gender differences in basal or stimulated amylase release or $Na^+$-$K^+$-ATPase activity in the rat parotid gland. Thus, the focus of our study was to evaluate the effect of orchiectomy and testosterone replacement on these two functional responses.

In order to take control of all the variables that may influence parotid gland activity, male albino rats of the Wistar strain of our own colony, weighing 250–300 g were used throughout. The animals were kept at 22 ± 2°C in a 14-hour light and 10-hour darkness photoperiod. The experiments were done always at the same hour, 10 a.m., and the night before Fed Purina chow but not water was withdrawn.

### Orchiectomy and Amylase Release

Salivary glands secrete numerous proteins that participate in salivary immunity, enamel stability and bactericidal activities or protect digestive enzymes from dietary tannins. The parotid gland provides 50% or more of the stimulated enzyme-rich saliva. Amylase is one of the enzymes that participate in the protective, digestive and antibacterial functions of the saliva and is the main enzyme secreted by the parotid gland. It is synthesized on the rough endoplasmic reticulum, secreted via the Golgi apparatus and stored in intracellular vesicles until release. The amylase granules discharge their content to the lumen by exocytosis.

Amylase activity is determined by activity assays using starch as standard substrate [Bernfeld, 1995]. Amylase activity is expressed in terms of milligrams of maltose ($C_{12}H_{22}O_{11} \cdot H_2O$) liberated in 3 min at 20°C by 1 ml of the enzyme solution. The amount of amylase contained in the gland is expressed as amylase activity/mg wet weight, and the level of amylase released into the medium is expressed as percent liberated from the total contained in the gland. Although the gold standard for saliva analysis is to collect it in vivo, via cannulation of the main duct, as described by Ulmansky et al. [1971], we used the method in vitro for amylase assays because this method let us to study the effect of a different drug in each gland.

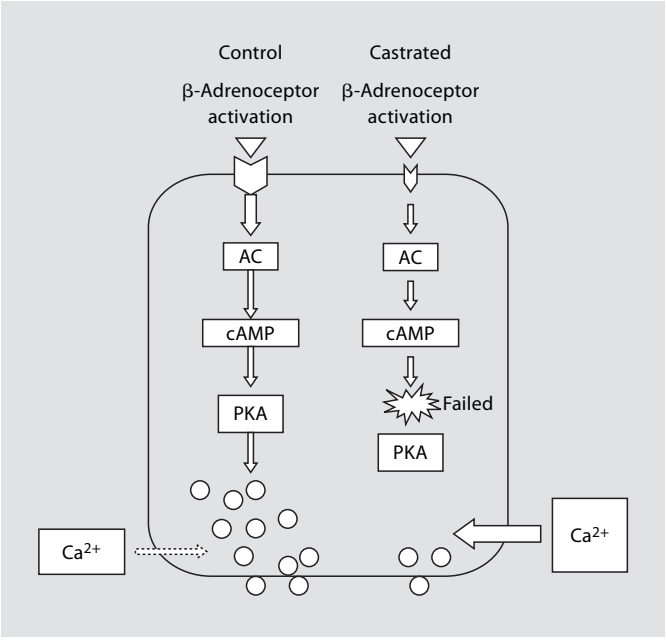


**Fig. 1.** Scheme illustrating the hypothetical role of cAMP and calcium-mediated effects in the regulation of amylase release in the parotid gland from control and castrated rats. AC = Adenylate cyclase. Data from Busch and Borda [2002].

**Table 1.** Amylase contained in the gland and resting amylase release by the parotid gland from control and 21-day-castrated male rats

| Parameter | Control | Castrated |
|---|---|---|
| Amylase contained in the gland, activity/mg wet weight | 22 ± 2 | 27 ± 2 |
| Resting amylase release, % of total | 4.5 ± 0.36 | 4.7 ± 0.5 |

Amylase contained in the gland is expressed as amylase activity/mg wet weight, where activity is expressed in terms of milligrams of maltose liberated in 3 min at 20°C by 1 ml of the enzyme solution (data from Busch and Borda [2002].

To judge from the data obtained by us, amylase synthesis and resting amylase release are not under testosterone control (table 1).

*β-Adrenoceptor Activation*

Amylase secretion from parotid acinar cells results from stimulus-regulated fusion (docking, priming and fusion-exocytosis) of the apical membrane and secretory granules that contain amylase. The secretory response to β-adrenergic agonists begins with the docking step that may be regulated by cAMP presumably through the phosphorylation of substrates by a cAMP-dependent protein kinase [Fujita-Yoshigaki, 1998; Fujita-Yoshigaki et al., 1999].

We observed [Busch and Borda, 2002] that isoproterenol induces an increase in cAMP and amylase release through the activation of $\beta_1$-adrenergic receptors. These effects of isoproterenol are inhibited by the inhibition of the enzyme adenylate cyclase. Although it is known that calcium participates in the fusion-exocytosis step, we find that amylase release induced by isoproterenol is not inhibited by the blockage of the calcium channels, probably because the contribution of calcium in this case is masked by the effect of cAMP-protein kinase A derivatives [Busch and Borda, 2002].

Castration alters the β-adrenergic pathway that leads to amylase secretion. The product, amylase, is decreased, the mechanism involved is altered, the production of second messenger (cAMP) is decreased and the number of β-adrenoceptors is decreased.

Our results show [Busch and Borda, 2002] that castration alters isoproterenol-induced amylase secretion in the parotid gland. The number of β-adrenoceptor binding sites, using the specific radioligand l-[4,6-propyl-$^3$H]dihydroalprenolol, is 28% minus than in controls and isoproterenol-induced cAMP accumulation is 25% decreased. The correlation observed indicates that the decrease in cAMP accumulation is a consequence of the decrease in the l-[4,6-propyl-$^3$H]dihydroalprenolol binding sites. Both effects are testosterone dependent.

However, isoproterenol-induced amylase release is 45% decreased after castration and it is not cAMP dependent. Besides, the accumulation of cAMP through the addition of dibutyryl cAMP does not increase amylase release in parotid glands from castrated animals. The secretory effect of isoproterenol, after castration, is abolished by the blockage of the calcium channels. The altered response to isoproterenol of parotid glands from castrated rats is testosterone dependent [Busch and Borda, 2002].

We propose that in parotid glands from castrated rats phosphorylation of a substrate involved in the amylase release evoked by cAMP-dependent protein kinase is altered. Under this condition isoproterenol induces amylase secretion by a mechanism that involves calcium influx, triggered by $\beta_1$-adrenoceptor activation (fig. 1). The increase in calcium would induce the fusion-exocytosis step only in vesicles that are primed near to the membrane. This hypothesis is supported by the fact that pa-

rotid glands from castrated rats do not respond to isoproterenol in a calcium-free medium [Busch and Borda, 2002].

*Muscarinic Acetylcholine Receptor Activation*

Calcium-mobilizing agonists stimulate phospholipase C (PLC) to catalyze the breakdown of phosphatidylglycerol, where inositol 1,4,5-triphosphate releases cellular calcium by interacting with specific receptor sites on the endoplasmic reticulum and diacylglycerol activates protein kinase C (PKC) [Horn et al., 1988]. Amylase secretion is mediated by the elevation in intracellular calcium ions [Fujita-Yoshigaki et al., 1999].

We observed that in the parotid gland carbachol triggers amylase release by the activation of the muscarinic acetylcholine receptor (mAChR) which, in turn, induces activation of PLC. The positive correlation observed between inositol phosphate (IP) accumulation and amylase release indicates that amylase release is the result of IP turnover (fig. 2). The effect of carbachol decreases when PLC or calcium calmodulin (CaM) are inhibited [Busch and Borda, 2003].

The selective antagonists of $M_1$ and $M_3$ muscarinic receptor subtypes, pirenzepine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), respectively, inhibit carbachol-induced amylase release and IP production. This fact supports the view that these receptor subtypes are important mediators of carbachol-induced biological effects in the parotid gland. The muscarinic receptor subtype $M_3$ has been described as the muscarinic receptor predominant in parotid glands from the rat [Dai et al., 1991] and mouse [Watson et al., 1996]. The second muscarinic receptor subtype described in salivary glands is the $M_1$ [Dai et al., 1991; Watson et al., 1996; Yamamoto et al., 1996; Pérez-Leirós et al., 2000].

The 4-DAMP potency in inhibiting carbachol-induced IP production and amylase release, expressed as $pA_2$, is 10 times greater than that of pirenzepine. This result is in agreement with the respective equilibrium inhibition constant (Ki) of the antagonists obtained by the competition-binding assays (fig. 3).

Castration modifies the muscarinic activation pathway that results in amylase secretion. The product, amylase, the second messenger, IP, and the number of muscarinic receptors are decreased while the mechanism involved does not change. On the other hand, castration alters $M_1$ muscarinic receptor subtype characteristics.

Our results show [Busch and Borda, 2003] that carbachol-induced amylase release and IP production are decreased after castration. Similar to control animals, a positive correlation between IP production and amylase release is observed (by carbachol), pointing out that the mechanism involved in amylase secretion was unchanged (fig. 2).

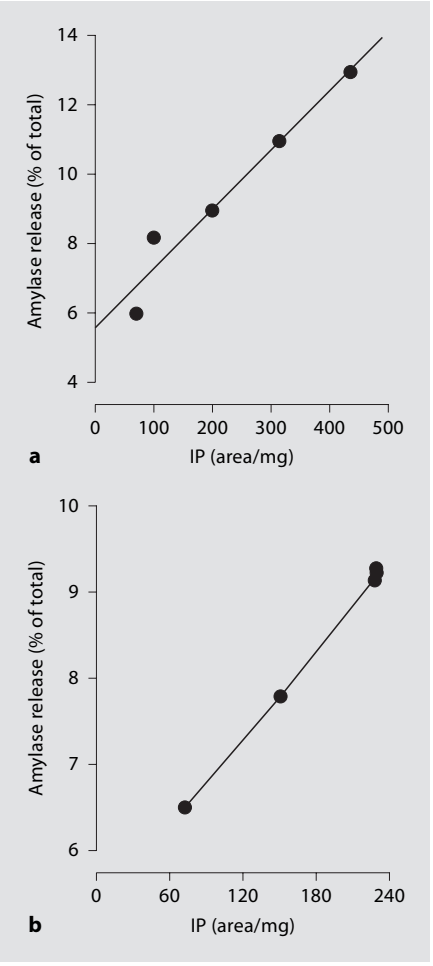


**Fig. 2.** Correlation between IP accumulation and amylase release in parotid slices from control (**a**) and castrated male rats (**b**). Amylase release was plotted as a function of the IP accumulation. Correlation values: 0.9747 and 0.9992 for control and castrated animals, respectively (data from Busch and Borda [2003]).

Binding studies using [$^3$H]quinuclidinylbenzilate show that mAChR expression was diminished after castration without any alteration in the equilibrium dissociation constant. This fact supports the view that the effect of castration on carbachol-induced IP production and amylase release is a consequence of a decrease in mAChR sites [Busch and Borda, 2003].

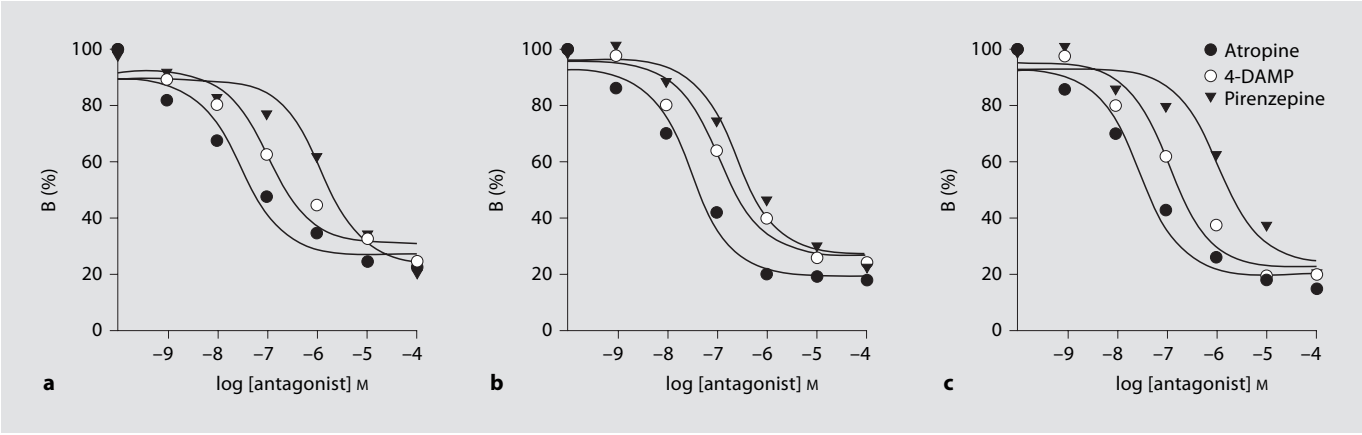


**Fig. 3.** Competition binding assays of muscarinic antagonists with [$^3$H]quinuclidinylbenzilate in parotid membranes from control (**a**), castrated (**b**) and castrated testosterone-treated (**c**) rats. % B = % [$^3$H]quinuclidinylbenzilate bound (from Busch and Borda [2003]).

We propose that carbachol by coupling mAChR induces IP accumulation which in turn leads to amylase release. Thus, in the parotid gland from castrated rats the decrement in the number of binding sites results in a decreased response to carbachol in inducing IP production, resulting in a decrease in the secretory response of the parotid gland to carbachol in evoking amylase release. Testosterone replacement restores binding parameters, IP production and amylase release in castrated rats to control levels [Busch and Borda, 2003].

The same muscarinic receptors subtypes, $M_1$ and $M_3$, are involved in amylase release in the parotid gland from castrated rats. However, here, 4-DAMP potency is equal to that of pirenzepine. In addition, competition-binding assays show that pirenzepine Ki decreases after castration (fig. 3). These changes could be due to an increase in the relation of $M_1/M_3$ expression, but since 4-DAMP $pA_2$ for both physiological responses and 4-DAMP Ki are not changed by castration, we propose that the differences observed with pirenzepine are due to changes in $M_1$ muscarinic receptor conformation that result in an increase in pirenzepine affinity. This effect of castration is testosterone dependent [Busch and Borda, 2003].

### Orchiectomy and $Na^+$-$K^+$-ATPase Activity

Epithelial cells regulate the flow of water by creating concentration gradients of osmotically active material. In salivary glands, which produce hypotonic saliva, the volume and the composition of this fluid vary with the type of stimulus used. Stimulation of cholinergic and α-adrenergic receptors results in the secretion of moderately large volumes of saliva with characteristic electrolyte composition, while stimulation of β-adrenoceptors causes the secretion of smaller volumes of saliva with different electrolyte composition [Martinez and Cassity, 1983]. Generally, fluid secretion is regulated by the combined action of four membrane transport systems, i.e. $Na^+$-$K^+$-ATPase, $Na^+$-$K^+$-$2Cl^-$ cotransporter, $Ca^{2+}$-activated $K^+$ channels in basolateral membranes and an apical conductive pathway for $Cl^-$, presumably involving $Ca^{2+}$-activated $Cl^-$ channels [Turner, 1993; Kurihara et al., 2000].

$Na^+$-$K^+$-ATPase is a ubiquitous membrane-bound enzyme that uses the energy released from ATP hydrolysis to maintain osmotic balance and to produce an electrochemical gradient of $Na^+$ and $K^+$ across the plasma membrane [Skou, 1988]. The enzyme not only maintains the membrane potential of excitable neural and muscle cells but also is involved in re-absorption of $Na^+$ in the kidney [Jorgensen, 1982] and in salivary glands [Kurihara et al., 1990].

$Na^+$-$K^+$-ATPase is found in the parotid gland along the apical and basolateral plasma membranes of acinar, striated and excretory duct cells. The apical $Na^+$-$K^+$-ATPase is proposed to participate in series with basolateral sodium and chloride entry pathways in driving secretory electrolyte fluxes [Speight and Chisholm, 1984; Conteas et al., 1986; Simson and Chao, 1994]. Furthermore, it is suggested that in acinar cells $Na^+$ is not only transported paracellularly but is also actively transported intracellularly into the luminal space by the $Na^+$-$K^+$-ATPase lo-

cated on luminal plasma membranes. Water is passively transported to the luminal space to form a plasma-like isotonic primary saliva, while in the duct cells the same ion is selectively re-absorbed intracellularly by the enzyme, which is found in abundance along the many infoldings of the basal plasma membranes, thus producing hypotonic saliva [Iwano et al., 1987].

*[$^3$H]Ouabain Binding Sites*

$Na^+$-$K^+$-ATPase consists of two subunits, a catalytic α-subunit and a glycosylated β-subunit [Jorgensen, 1982]. Both the α- and β-subunit occur as several isoforms, which are variably expressed in a cell-specific manner [Nomura et al., 1990; Horisberger and Rossier, 1992]. The α-subunit is involved in $Na^+$ extrusion, $K^+$ influx, and ATPase activity [Ewart and Klip, 1995]. The β-subunit seems to be an important regulatory component of the sodium pump [Ewart and Klip, 1995; Factor et al., 1998]. Inhibition of $Na^+$-$K^+$-ATPase with ouabain greatly reduces solute and water transport [Basset et al., 1987].

The hydrolytic activity of $Na^+$-$K^+$-ATPase is determined by the release of phosphorus from ATP and expressed as micromoles of phosphorus per milligram of protein per hour (μmol Pi/ mg protein h).

In the mammalian cortical collecting ducts, an acute increase in the intracellular concentration of $Na^+$ rapidly stimulates the $V_{max}$ of $Na^+$-$K^+$-ATPase activity and increases the number of active pump units, taken as the specific [$^3$H]ouabain binding [Barlet-Bas et al., 1990; Blot-Chabaud et al., 1990]. This fact strongly suggests that an inactive pool of $Na^+$-$K^+$-ATPase units can be rapidly activated under particular circumstances. The increased number of active $Na^+$-$K^+$-ATPase units may result from the translocation of pumps from an intracellular compartment to the plasma membrane and/or the activation of latent pump units already located at the plasma membrane. For instance, in mammalian collecting ducts, cAMP rapidly mobilizes an intracellular pool of $Na^+$-$K^+$-ATPase resident in the trans-Golgi network toward the plasma membrane [Gonin et al., 2001].

In the parotid gland, two ouabain binding sites with high and low affinity were present (fig. 4) [Busch et al., 2004a]. Castration induces an increase in the affinity of both high- and low-affinity binding sites and a decrease in the number of low-affinity binding sites for [$^3$H]ouabain (fig. 4). These effects are reversed by testosterone treatment. In spite of the changes observed in [$^3$H]ouabain binding parameters in membranes from castrated rats, the basal $Na^+$-$K^+$-ATPase activity is not modified by castration (table 2).

**Table 2.** Basal $Na^+$-$K^+$-ATPase activity in parotid glands from control and castrated rats

| Group | $Na^+$-$K^+$-ATPase activity μmol Pi/mg protein h |
|---|---|
| Control | 2.5 ± 0.30 |
| Castrated | 2.6 ± 0.24 |

Means ± SEM of six experiments (data from Busch et al. [2004a]).

The $Na^+$-$K^+$-ATPase α-subunit of the rat submandibular gland is regulated by steroid hormones [Kurihara et al., 1996]. We propose that the unchanged basal activity of $Na^+$-$K^+$-ATPase observed in the castrated group is due to the fact that only low-affinity binding sites are downregulated in castrated rats, in addition with an increase in affinity [Busch et al., 2004a]. This increase in [$^3$H]ouabain affinity may be related to castration-induced changes in the catalytic subunit [Kurihara et al., 1996].

*Muscarinic-Acetylcholine Receptor Activation*

The ion gradients that are necessary for a wide range of physiological processes are maintained by $Na^+$-$K^+$-ATPase, and regulation of the $Na^+$-$K^+$-ATPase plays a role in the hormonal and homeostatic responses of many cells. Regulation occurs at several levels: biosynthesis and degradation; reversible recruitment to and internalization from the plasma membrane; alteration of affinity for $Na^+$; and either stimulation or inhibition of activity [Sweadner and Feschenko, 2001].

Phosphorylation of the α-subunit by protein kinases has been proposed to mediate the regulation of the enzyme. The phosphorylation sites for protein kinase A (PKA; cAMP-activated) [Feschenko and Sweadner, 1994; Fisone et al., 1994] and PKC [Beguin et al., 1994; Feschenko and Sweadner, 1995; Fisone et al., 1995] have been identified. Rat $Na^+$-$K^+$-ATPase is phosphorylated in vitro by PKC predominantly at Ser-18 and by PKA at Ser-938 [Beguin et al, 1994]. The molecular mechanism of regulation is an open question, however, because seemingly inconsistent functional effects have been reported with different experimental systems, including either activation or inhibition ostensibly through the same kinase [Feschenko et al., 2000; Kreydiyyeh, 2000].

Another regulatory mechanism of $Na^+$-$K^+$-ATPase is the dephosphorylation followed by activation of the CaM-dependent protein phosphatase, calcineurin [Bertorello

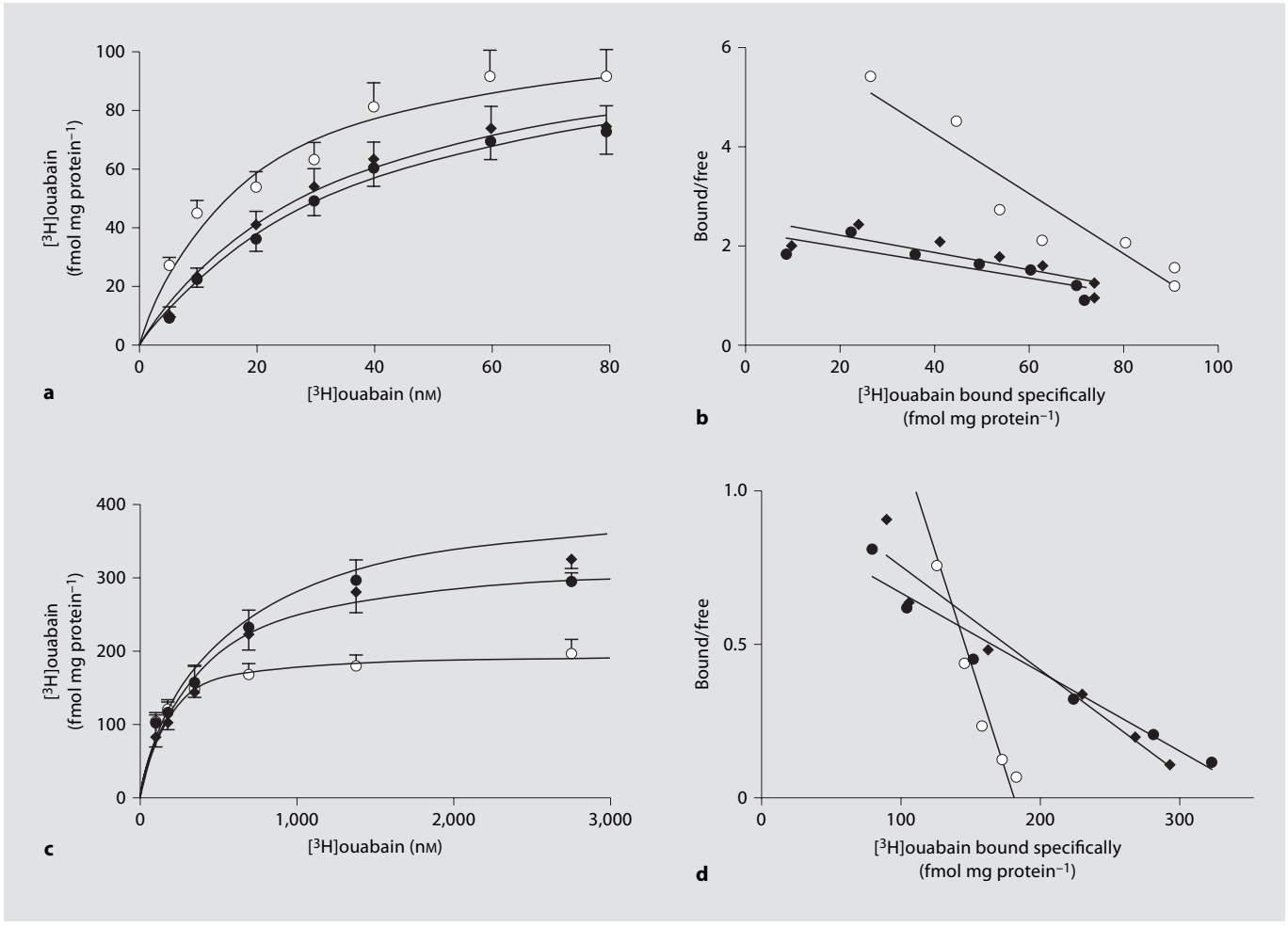


**Fig. 4.** Saturation curves (**a**, **c**) and Scatchard plots (**b**, **d**) of parotid gland membranes from control (●), castrated (○) and castrated testosterone-treated (◆) rats incubated with different concentrations of [$^3$H]ouabain. High- (**a**, **b**) and low-affinity (**c**, **d**) binding parameters are shown (from Busch et al. [2004a]).

et al., 1991]. In the parotid gland, carbachol inhibited $Na^+$-$K^+$-ATPase activity [Busch et al., 2004a]. The inhibitory effect of carbachol is abolished when PLC and CaM activities are blocked. However, when PKC is inhibited $Na^+$-$K^+$ pump activity increases, an effect that is prevented by inhibition of CaM. These results suggest that PKC is involved in the inhibitory action of carbachol.

We propose that, in the parotid gland, carbachol induces stimulation and inhibition of $Na^+$-$K^+$-ATPase activity, through the increase in intracellular $Ca^{2+}$ ions and activation of PKC, respectively. Thus, when PKC activity is blocked, the stimulatory effect mediated by $Ca^{2+}$ is unmasked [Busch et al., 2004a]. The stimulatory effect of the enzyme by calcium is confirmed through the increase in $Na^+$-$K^+$-ATPase activity observed with the calcium ionophore [Busch et al., 2004a]. On the other hand, there is evidence for the participation of CaM in the α-adrenergic stimulation of $Na^+$-$K^+$-ATPase activity in rat renal tubule cells [Aperia et al., 1992] and in the calcium stimulatory action in rat brain homogenates [Powis, 1985].

PKC-α was described as the principal calcium-dependent isoform associated with cholinergic stimulation in parotid cells [Terzian and Rubin, 1993]. Trifluoperazine, by inhibiting CaM, could modulate the availability of free cytosolic calcium and influenced the activation of PKC [Nishizuka, 1984]. Results obtained with renal proximal tubule cells from rats suggest that the $Na^+$-$K^+$-ATPase is an effector protein for PKC, and dopamine inhibition of enzyme activity is mediated by this kinase [Bertorello and Aperia, 1988].

Castration induces alterations in the muscarinic post-receptor pathway by inhibiting $Na^+$-$K^+$-ATPase activity, resulting in an opposite effect of carbachol.

Our results show [Busch et al., 2004a] that, in contrast with the inhibitory effect observed in the parotid gland from control rats, carbachol increases $Na^+$-$K^+$-ATPase activity in castrated rats. This activation is mimicked by a calcium ionophore and is inhibited in the presence of inhibitors of PLC and CaM. Based on the known effect of calcium on $Na^+$-$K^+$-ATPase activity, it is likely that in parotid glands from castrated rats $Ca^{2+}$ constitutes a positive signal for the $Na^+$-$K^+$ pump as in controls. In contrast, PKC activity is apparently not involved in the pathway triggered by carbachol in castrated rats, because its inhibition by staurosporine does not modify the effect of carbachol. Conversely, the direct activation of PKC with the phorbol 12-myristate 13-acetate decreases $Na^+$-$K^+$-ATPase basal activity in castrated rats. This result indicates that, even though PKC preserves the ability to inhibit the pump in castrated rats, carbachol fails to activate the kinase [Busch et al., 2004a].

Other evidence supporting the view that castration induces an alteration in the carbachol pathway comes from the effect of testosterone replacement. In this group, low concentration of carbachol induces activation of the pump, while at higher concentration inhibition is observed. This result suggests that a higher concentration of carbachol is needed to achieve activation of PKC in castrated testosterone-treated rats [Busch et al., 2004a]. Bearing in mind that castration induces a decrease in mAChR sites accompanied with a decrease in IP production [Busch and Borda, 2003] it is possible that these events are related to the failure of carbachol to induce PKC activation.

We propose that carbachol decreases $Na^+$-$K^+$-ATPase activity through the activation of PLC and the increase in intracellular $Ca^{2+}$ concentrations, which in turn activate PKC. This kinase is finally responsible for the inhibition of enzyme activity. In castrated rats, carbachol fails to activate PKC, and a stimulant effect of calcium is then observed (fig. 5).

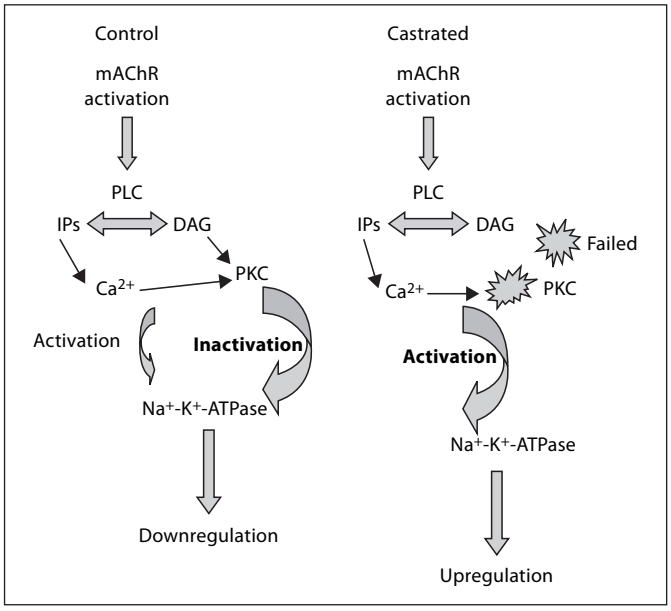


**Fig. 5.** Scheme illustrating the hypothetical role of $Ca^{2+}$ and PKC-mediated effects with opposing physiological actions in the regulation of $Na^+$-$K^+$-ATPase activity. DAG = Diacylglycerol; $Ca^{2+}$ = intracellular calcium (from Busch et al. [2004a]).

**Table 3.** Basal $K^+$ release in parotid glands from control and castrated rats

| Group | $K^+$ release, % of total |
|---|---|
| Control | 5.2 ± 0.45 |
| Castrated | 4.8 ± 0.50 |

Means ± SEM of four experiments (data from Busch et al. [2004a]).

*$K^+$ Release*

Slices of salivary glands incubated in physiological salt solutions release $K^+$ in vitro when exposed to appropriate secretagogues [Schneyer and Schneyer, 1964]. The net efflux of $K^+$ from salivary glands appears to be the result of two simultaneous and opposing mechanisms, namely, passive $K^+$ efflux induced by secretagogues and active $K^+$ re-uptake by the $Na^+$-$K^+$ pump [Martinez and Quissel, 1976]. $K^+$ efflux may be measured in an Instrumentation Laboratories flame photometer and expressed as percentage of the $K^+$ content in the gland [Martinez and Quissel, 1976; Busch et al., 2004a].

As observed in table 3, basal $K^+$ efflux from the parotid gland does not change after castration. Ouabain increases the basal net $K^+$ efflux indicating that an ouabain-sensitive $Na^+$-$K^+$-ATPase is responsible for the $K^+$ re-uptake during secretion [Busch et al., 2004a].

We observed that carbachol induces an increase in $K^+$ efflux. The magnitude of the net $K^+$ efflux, after carbachol stimulation, is affected by two opposing mechanisms, a loss via a calcium-activated $K^+$ channel [Lee and Turner, 1993] and a gain through $Na^+$-$K^+$-ATPase activ-

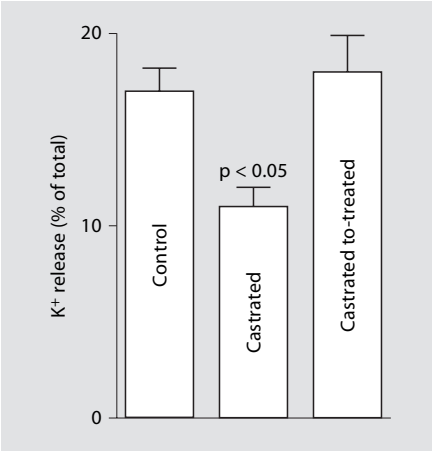


**Fig. 6.** Net $K^+$ efflux by parotid glands from control, castrated and castrated testosterone (To)-treated rats (data from Busch et al. [2004a].

ity [Martinez and Quissel, 1976]. Given that the increase in intracellular calcium concentration elicited by carbachol activates potassium and chloride channels [Petersen, 1992], and has the opposite effect on net $K^+$ efflux by $Na^+$-$K^+$-ATPase [Martinez and Camden, 1983], we propose that cholinergic agonists abolish the effect of the enzyme through activation of PKC.

Castration alters carbachol-induced $K^+$ efflux in the parotid gland. In castrated rats, the activation of the $Na^+$-$K^+$ATPase induced by carbachol resulted in an increase in $K^+$ uptake and a decrease in $K^+$ efflux. This effect is testosterone dependent (fig. 6). In addition, when carbachol-stimulated $K^+$ efflux is evaluated in the presence of ouabain, synergism is observed in control and castrated testosterone-treated rats and antagonism in castrated animals [Busch et al., 2004a].

## Orchiectomy and Proteins

The influence of castration on β-adrenergic and muscarinic-cholinergic receptor expression in the parotid gland was not due to a nonselective alteration in protein synthesis because the output of total proteins, as well as the concentration of total proteins in the parotid gland, did not differ significantly in both groups (controls and castrated animals) [Busch et al., 2006]. Thus, in spite of the anabolic effect of testosterone in various cells [Wilson and Griffin, 1980], receptor downregulation can be considered a selective action.

## Conclusion

This overview has concentrated on the limited contribution which pharmacological analysis can make to elucidate a physiological problem and has been heavily biased toward our own experiences. It is well known that salivary glands are target organs for androgens. Here, we accumulate data supporting alterations in the autonomic system in the parotid gland after orchiectomy. We describe a selective downregulation of β-adrenergic and muscarinic cholinergic receptor expression, which results in a decrease in the production of the second messenger and leads in a diminution of the effect. Protein kinases activated by cAMP, $Ca^{2+}$ or diacylglycerol pathways seem to be influenced by orchiectomy, and this fact results in a decrease or an opposite response of the parotid gland to β-adrenoceptors or muscarinic receptor agonists. Orchiectomy modifies the expression and affinity of the enzyme $Na^+$-$K^+$-ATPase and the regulatory mechanism induced by carbachol. Basal parotid gland activity is not changed after castration, indicating that the hormonal regulation is limited to autonomic stimulation.

## Clinical Implications

The effect of castration on the parotid gland could be related with xerostomia associated with aging. Although studies have determined that salivary gland function is well preserved in the healthy geriatric population and that xerostomia is a condition of systemic or extrinsic origin [Astor et al., 1999], it is known that women approaching the climacterium suffer changes in the mouth due to the decline in circulating estrogen levels and improved salivary flow with hormone replacement therapy [Laine and Leimola-Virtanen, 1996]. Then, in men, an alteration in the glands as a result of the decrease in testosterone levels cannot be disregarded.

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