Saliva and the Control of Its Secretion

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Abstract

The various functions of saliva—among them digestive, protective and trophic ones—not just limited to the mouth, and the relative contribution of the different types of gland to the total volume secreted as well as to various secretory rhythms over time are discussed. Salivary reflexes, afferent and efferent pathways, as well as the action of classical and non-classical transmission mechanisms regulating the activity of the secretory elements and blood

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vessels are in focus. Sensory nerves of glandular origin and an involvement in gland inflammation are discussed. Although, the glandular activities are principally regulated by nerves, recent findings of an "acute" influence of gastro-intestinal hormones on saliva composition and metabolism, are paid attention to, suggesting, in addition to the cephalic nervous phase, both a regulatory gastric and intestinal phase. The influence of nerves and hormones in the long-term perspective as well as old age, diseases and consumption of pharmaceutical drugs on the glands and their secretion are discussed with focus on xerostomia and salivary gland hypofunction. Treatment options of dry mouth are presented as well as an explanation to the troublesome clozapine-induced sialorrhea. Final sections of this chapter describe the families of secretory salivary proteins and highlight the most recent results obtained in the study of the human salivary proteome. Particular emphasis is given to the post-translational modifications occurring to salivary proteins before and after secretion, to the polymorphisms observed in the different protein families and to the physiological variations, with a major concern to those detected in the pediatric age. Functions exerted by the different families of salivary proteins and the potential use of human saliva for prognostic and diagnostic purposes are finally discussed.

1 Functions of Saliva: An Overview

Saliva exerts digestive and protective functions and a number of other functions, depending on the species, usually grouped under the heading "additional functions." *Digestive functions* include the mechanical handling of food such as chewing, bolus formation, and swallowing. The chemical degradation of food is by amylase and lipase—these enzymes continue to exert their activities in the stomach, amylase exerting its activity until the acid penetrates the bolus. The group of digestive functions also includes the process of dissolving the tastants, and thus allowing them to interact with the taste buds. If pleasant, taste sets up a secretory reflex of gastric acid as part of the cephalic regulation of gastric secretion. To the *protective functions* belong the lubrication of the oral structures by mucins, the dilution of hot or cold food, and spicy food, the ability of the buffer (by bicarbonate, phosphates, and protein) to maintain salivary pH around 7.0 (note that in many laboratory animals, the pH is higher, 8.5–9.0), the remineralization of enamel by calcium, the antimicrobial defense action by immunoglobulin A, α-defensins, and β-defensins, and wound healing by growth-stimulating factors such as epidermal growth hormone, statherines, and histatines. Additionally, saliva is necessary for articulate speech, for excretion (as discussed below), and for social interactions (such as kissing). Moreover, saliva exerts trophic effects. It maintains the number of taste buds. Further, it has recently become apparent that the composition of saliva secreted during fetal life may be of importance for the development of oral structures (Jenkins 1978; Tenouvo 1998; Mese and Matsuo 2007; Inzitari et al. 2009**;** Castagnola et al. 2011a). It has already been mentioned that the salivary enzymes accompanying the bolus are still active in the stomach. There are further examples of the fact that the action of saliva is not restricted to the mouth. Swallowed saliva protects the esophageal wall from being damaged by regurgitating gastric acid as is the case with a lowered tone of the lower esophageal sphincter (Shafik et al. 2005). The defense mechanisms of saliva protect the upper as well as the lower respiratory tract from infectious agents (Fig. 1).

Although the exocrine function of the salivary glands is in focus, it is worth noting that salivary glands have, in addition, excretory and possibly endocrine functions. Circulating non-protein-bound fractions of hormones, such as of melatonin, cortisol, and sex steroids, passively move into the saliva, as do a number of pharmaceutical drugs (Gröschl 2009). Interestingly, melatonin, when in the oral cavity, exerts antioxidative, immunomodulatory, and anticancerogenic effects (Cutando et al. 2007). Iodide is actively taken up by the glands by the same transport system as in the thyroid gland, a situation that may be deleterious for the salivary glands if the iodide is radioactive and is used in the treatment of thyroid tumors (Mandel and Mandel 2003). Salivary substances may appear in the blood as indicated by amylase and epidermal growth factor, which suggests endocrine functions of the glands (Isenman et al. 1999).

In animals, saliva may be secreted to lower the body temperature by evaporative cooling (panting of dogs and spreading of saliva on the scrotum and the fur by rats), for grooming (rats and cats) and, by salivary pheromones, to mark territory or to attract mates (mice and pigs); particularly, sex steroids of the saliva serve as olfactory signals (Gregersen 1931; Hainsworth 1967; Gröschl 2009).

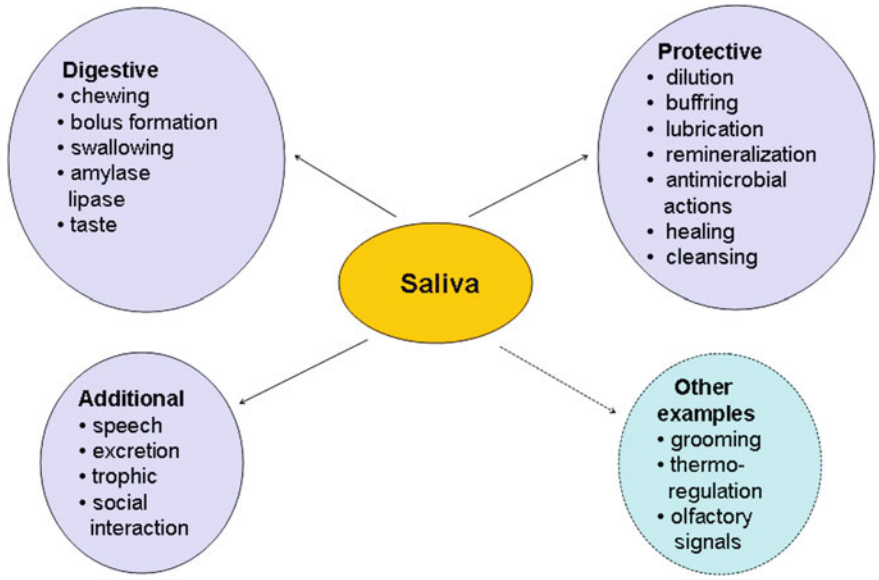

Fig. 1 Functions of saliva

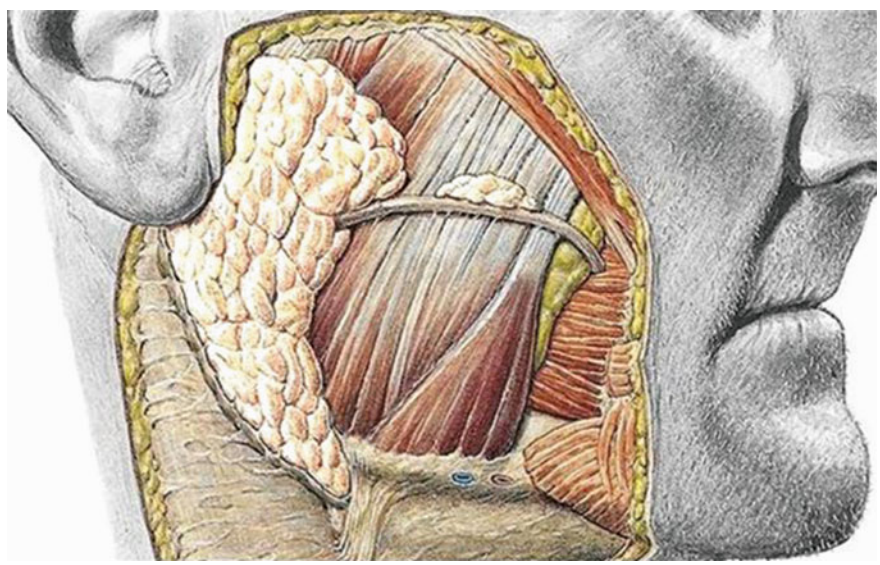

Fig. 2 Parotid gland and accessory gland. (With permission from Elsevier)

2 Major and Minor Salivary Glands and Mixed Saliva

Saliva is produced by three pairs of major glands, the parotids, the submandibulars, and the sublinguals, located outside the mouth, and hundreds of minor glands—each the size of a pinhead and located just below the oral epithelium (Figs. 2 and 3). As judged by magnetic resonance imaging, the volume of the parotid gland is about 2.5 times that of the submandibular gland and eight times that of the sublingual gland (Ono et al. 2006). Similar relationships are obtained when the comparisons are based on gland

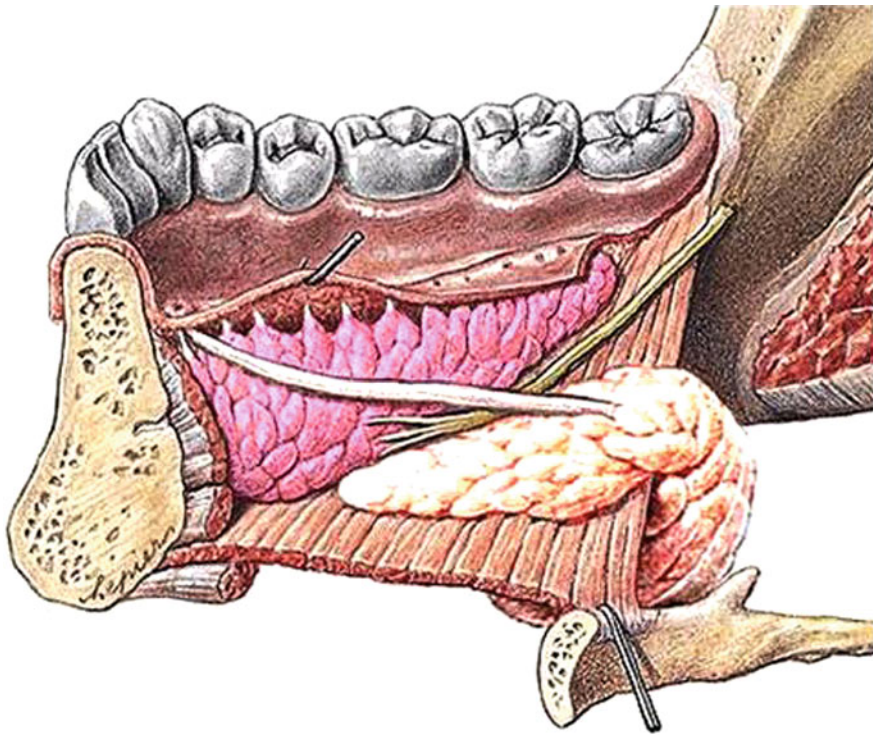

Fig. 3 Submandibular and sublingual glands. Note the many small ducts from the sublingual gland. (With permission from Elsevier)

weights, the parotid gland weighing 15–30 g (Gray 1988). The saliva from the parotid and submandibular glands reaches the oral cavity via long excretory ducts (7 and 5 cm, respectively), the parotid duct (also called Stensen's duct) opening at the level of the second upper molar, and the submandibular duct (Wharton's duct) opening on the sublingual papilla. In about 20% of the population, the parotid duct is surrounded by a small accessory gland. Sublingual saliva empties into the submandibular duct via the major sublingual duct (Bartholin's duct) or directly into the mouth via a number of small excretory ducts opening on the sublingual folder. Likewise, the saliva of minor glands, such as of the buccal, palatine (located just in the soft palate), labial, lingual, and molar glands, empties into the mouth directly via small, separate ducts just traversing the epithelium (Tandler and Riva 1986). Unless saliva is collected directly from the cannulated duct, the saliva in the mouth will be contaminated by the gingival crevicular fluid, blood cells, microbes, antimicrobes, cell and food debris, and nasopharyngeal secretion. Consequently, mixed saliva ("whole saliva") collected by spitting or drooling is not pure saliva, although the term "saliva" is usually used.

3 Spontaneous, Resting, and Stimulated Secretion

Some salivary glands have an inherent capability to secrete saliva (Emmelin 1967). The type of gland differs among different species. In humans, only the minor glands secrete saliva spontaneously. Although these glands are innervated and may increase their secretory rate in response to nervous activity, they secrete saliva at a low rate, without exogenous influence during the night. In daytime and at rest, a nervous reflex drive—set up by low-grade mechanical stimuli due to movements of the tongue and lips, and mucosal dryness—acts on the secretory cells, particularly engaging the submandibular gland (Fig. 4). In the clinic, the saliva secreted at rest is often called "unstimulated secretion," despite the involvement of nervous activity. With respect to stimulated secretion, the parotid contribution becomes more dominant: in response to strong stimuli, such as citric acid, the flow rate is about equal to that from the submandibular gland, whereas in response to chewing, the flow rate is twice as high as that from the submandibular gland. The total volume of saliva secreted amounts to 1–2 L per 24 h. The flow rate

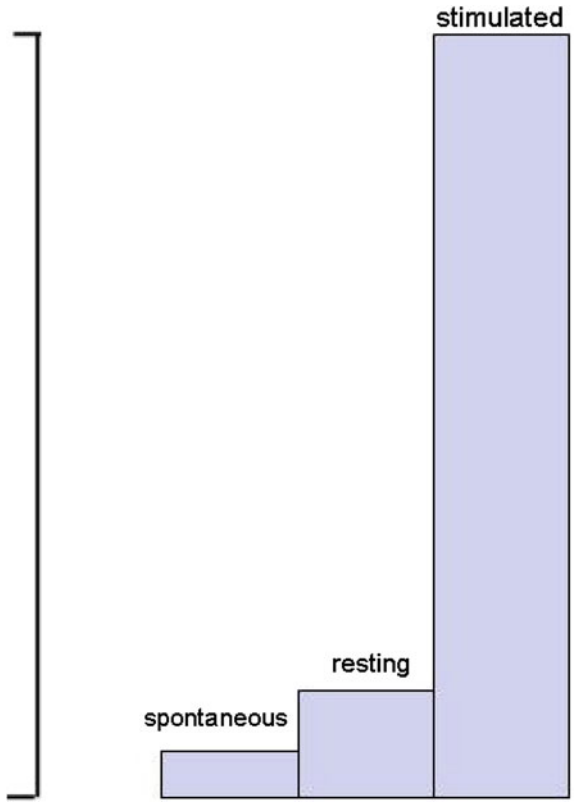

Fig. 4 Different rates of salivary flow

correlates with gland size, and is higher in males than in females (Heintze et al. 1983). The relative contributions of each type of gland to the total volume secreted are as follows: roughly 30% for the parotid glands, 60% for the submandibular glands, 5% for the sublingual glands, and 5% for the minor glands (Dawes and Wood 1973). Different types of glands produce different types of secretion. Depending on the reaction to the histochemical staining of the acinar cells for light-microscopy examination, the cells are classified as (basophilic) serous or (eosinophilic) mucous cells. The serous cells are filled with protein-storing granules and are associated with the secretion of water and enzymes, whereas the mucous cells are associated with the secretion of the viscous mucins stored in vacuoles. The parotid gland is characterized as a serous gland, the submandibular gland is characterized as a seromucous gland (10% mucous cells and 90% serous cells), and the sublingual gland and most of the minor glands are characterized as mucous glands. The deep posterior lingual glands (von Ebner's glands), found in circumvallate and foliate papillae close to most of the taste buds, are, however, of the serous type. Though, the contribution of the minor glands is small, they continuously, during day and night, provide the surface of the oral structures with a protective layer of mucin-rich saliva that prevents the feeling of mouth dryness from occurring. Together with the sublingual glands, they are responsible for 80% of the total mucin secretion per 24 h.

4 The Salivary Response Displays Circadian and Circannual Rhythms

On the whole, the flow rate of resting as well as of stimulated saliva is higher in the afternoon than in the morning (Ferguson and Botchway 1980; Dawes 1975), the peak occurring in the middle of the afternoon. Also the salivary protein concentration follows this diurnal pattern. In addition, the flow of the resting saliva is higher during winter than during summer, indicating a circannual rhythm (Elishoov et al. 2008). Just a small change in the ambient temperature (by 2 °C) in a warm climate is enough to inversely affect the flow rate (Kariyawasam and Dawes 2005).

5 The Diversity of the Salivary Response

Pavlov drew attention to the fact that the volume of saliva secreted and its composition vary in a seemingly purposeful way in response to the physical and chemical nature of the stimulus (see Babkin 1950). Not only does the secretion adapt "acutely" to the stimulus, but also long-term demands may induce changes in gland size and secretory capacity. The variety in the salivary response is attained by the involvement of different types of glands, different types of cells within a gland, different types of reflexes displaying variations in intensity, duration, and engagement of the two divisions of the autonomic innervation, different types of transmitter and varying transmitter ratios, different types of receptors, and various intracellular pathways either running in parallel or interacting synergistically (Fig. 5).

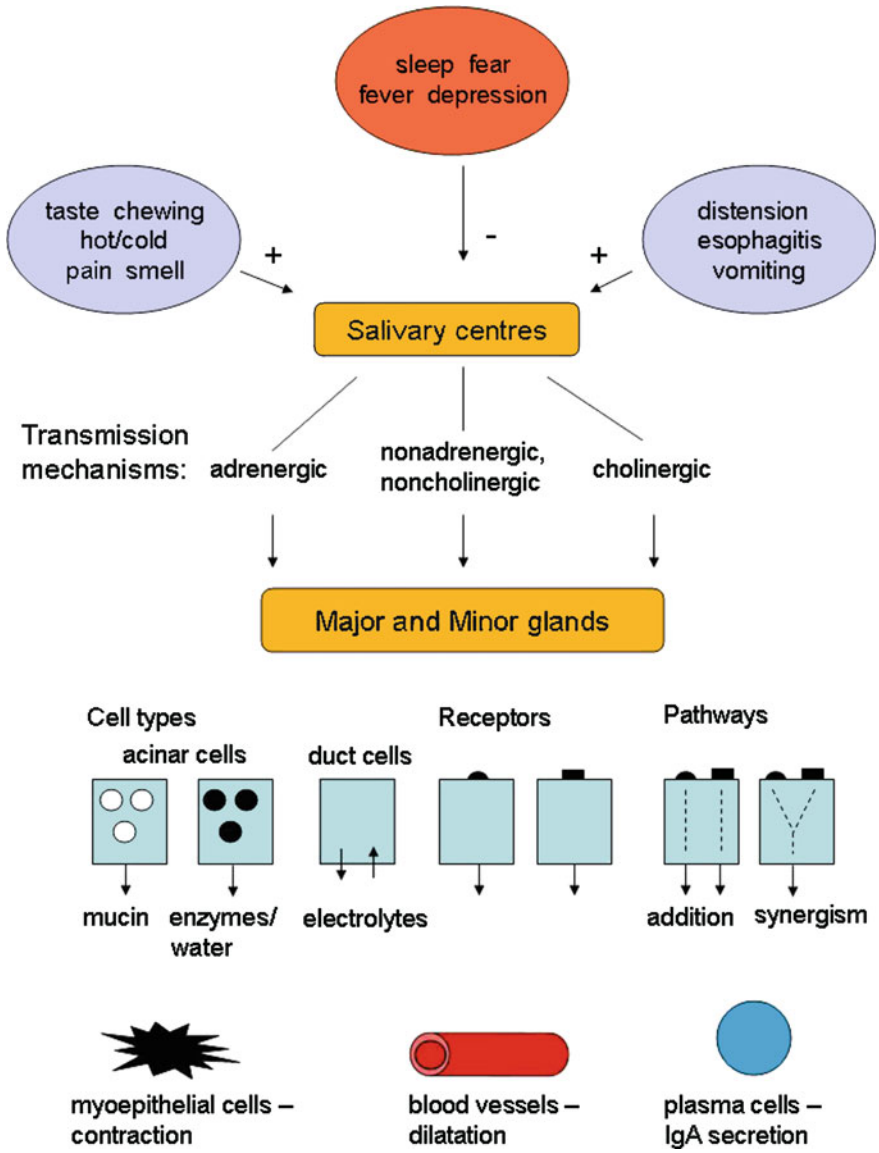

Fig. 5 Afferent and efferent nerves, and various elements of salivary glands

6 Afferent Stimuli for Secretion

Eating is a strong stimulus for the secretion of saliva (Hector and Linden 1999). A number of sensory receptors are activated in response to food intake: gustatory receptors, mechanoreceptors, nociceptors, and olfactory receptors (Fig. 5). All four modes of taste (sour, salt, sweet, and bitter) elicit secretion ("gustatory salivary reflex") but sour, followed by salt, is the most effective stimulus. Taste buds reside in the papillae of the tongue. The sensation of salt is particularly experienced at the tip of the tongue and that of bitter at the dorsum of the tongue, whereas the sensations of sweet and sour are experienced in between. Regions other than the tongue, in particular the soft palate, but also the epiglottis, the esophagus, the nasopharynx, and the buccal wall, also contain areas of taste buds. Chewing causes the teeth to move sideways, thereby stimulating mechanoreceptors of the periodontal ligaments ("masticatory salivary reflex"). In addition, gingival mucosal tissue mechanoreceptors are activated during chewing. Olfactory receptors are located at the cribriform plate,

i.e., at the roof of the nasal cavity, and they respond to volatile molecules of the nasal and the retronasal airflow (the latter arising from the oral cavity or the pharynx). Sniffing increases the airflow and thereby the access of stimuli to the receptor area. The epithelium containing the olfactory receptors has a rich blood supply. Interestingly, blood-borne odorants may pass through the vessel walls and stimulate these receptors. The submandibular glands, but not the parotid glands, are regulated by an "olfactory salivary reflex." Irritating odors, do, however, mobilize the parotid gland, in addition to the submandibular gland, in this case in response to the stimulation of epithelial trigeminal "irritant receptors." The nociceptors may also be activated in response to spicy food (e.g., chilli pepper). Thermal stimuli also influence the rate of secretion. Ice-cold drinks cause a greater volume of saliva to be produced than do hot drinks (Dawes et al. 2000). Dryness of the mucosa acts as yet another stimulus for secretion ("dry mouth reflex"; Cannon 1937). Salivary secretion as a consequence of pain is a well-known phenomenon, and both pain receptors and mechanoreceptors may cause secretion elicited by esophageal distension due to swallowing dysfunctions (Sarosiek et al. 1994). When applied unilaterally, the stimulus may evoke secretion from the glands of both sides. However, the secretory response is more pronounced on the stimulated side. Afferent signals arising from the anterior part of the tongue preferentially engage the submandibular gland, whereas signals arising from the lateral and posterior parts preferentially engage the parotid gland (Emmelin 1967). Patients suffering from chronic gastroesophageal reflux of acid may experience salivation in response to
acid directly hitting the muscle layers of a damaged esophageal wall ("esophageal salivary reflex"; Helm et al. 1987). This reflex is also elicited in healthy subjects (Shafik et al. 2005). Salivation is part of the vomiting reflex set up by a number of stimuli, including distension of the stomach and duodenum as well as of chemical stimuli acting locally or centrally. The phenomenon of conditioned reflexes has been tightly associated with salivary secretion since the pioneering work by Pavlov on dogs. In humans, however, it is difficult to establish conditioned salivary reflexes to sight, sound, or anticipation of food. The feeling of "mouth watering" at the sight of an appetizing meal is attributed to anticipatory tongue and lip movements as well as to an awareness of preexisting saliva in the mouth (Hector and Linden 1999).

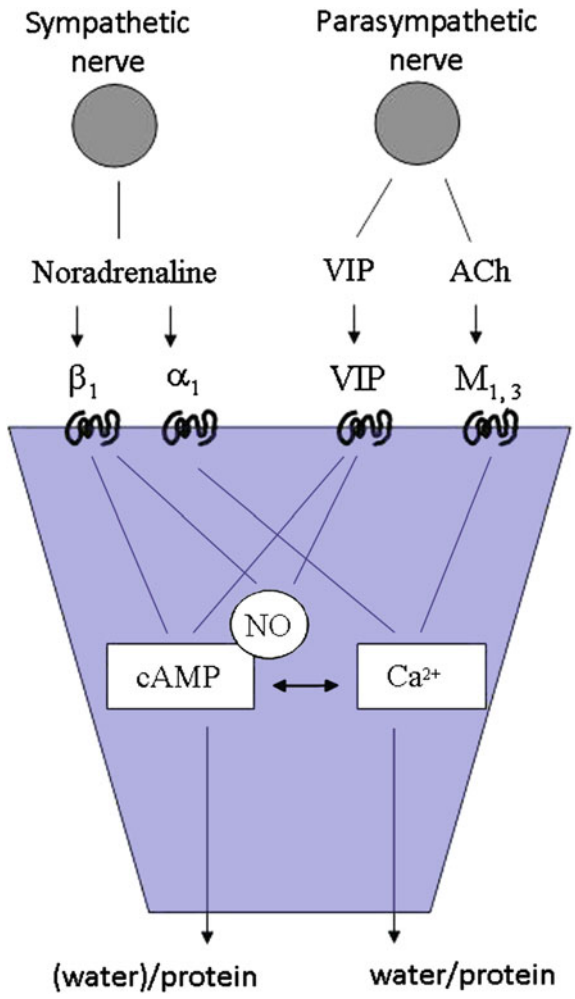

Fig. 6 Acinar cells: transmitters, receptors, and intracellular pathways

7 Efferent Stimuli for Secretion

Since the days of the ninetieth century pioneers of experimental medicine who were exploring the action of nerves, the secretion of saliva has been thought to be solely under nervous control (Garrett 1998). Recent studies, however, imply an "acute" role for hormones in the regulation of saliva composition (see below). The secretory elements (acinar, duct, and myoepithelial cells) of the gland are invariable richly supplied with parasympathetic nerves. The sympathetic innervation

differs in intensity between the glands, however. In humans, the secretory elements of the parotid glands are reported to be supplied with fewer sympathetic nerves than the submandibular glands, and the labial glands are thought to lack a sympathetic secretory innervation (Rossoni et al. 1979). The parasympathetic innervation is responsible for the secretion of large volumes of saliva, whereas, in the event of a sympathetic secretory innervation, the sympathetically nerve-evoked flow of saliva is usually sparse. Both the parasympathetic and the sympathetic innervations cause the secretion of proteins. Whereas gustatory reflexes activate both types of autonomic nerves, masticatory reflexes preferentially involve the activity of the parasympathetic innervation (Jensen Kjeilen et al. 1987). Since the accompanying flow of saliva is much greater in response to parasympathetic stimulation than to sympathetic stimulation, the salivary protein concentration is lower in parasympathetic saliva than in sympathetic saliva. In case of a double innervation of the secretory cells, parasympathetic and sympathetic nerves interact synergistically with respect to the response (Emmelin 1987). The secretion of saliva requires a large water supply from the circulation. Parasympathetic activity causes vasodilation, and the glandular blood flow may increase 20-fold.

8 Autonomic Transmitters and Receptors

Traditionally, acetylcholine is the parasympathetic postganglionic transmitter and noradrenaline the sympathetic postganglionic transmitter that act on the secretory elements of the glands (Fig. 6). Noradrenaline acts on α_1-adrenoceptors and β_1-adrenoceptors, whereas acetylcholine acts on muscarinic M1 and M3 receptors. The parasympathetic nerve of the salivary glands has been found to use other transmission mechanisms besides the cholinergic one, i.e., peptidergic (vasoactive intestinal peptide, calcitonin-gene-related peptide, substance P, neurokinin A, neuropeptide Y) and nitrergic (nitric oxide, NO) mechanisms (Ekström 1999a). The cotransmitters to acetylcholine may, on their own, evoke secretory effects and potentiate the acetylcholine-evoked responses (Ekström 1987). For instance, vasoactive intestinal peptide causes the secretion of proteins with no (or little) fluid. However, in concert with acetylcholine, both the protein and the fluid secretion are enhanced by vasoactive intestinal peptide. Although the parasympathetic innervation of the salivary glands contains the NO synthesizing enzyme NO synthase, NO of parasympathetic origin does not seem to take part in the regulation of the secretory activity. Instead, NO of intracellular origin is mobilized, and particularly upon sympathetic nerve activity (Ekström et al. 2007). With respect to the parasympathetic-evoked vasodilator response, both vasoactive intestinal peptide and NO, besides acetylcholine, are involved.

9 Secretory Units

The glands are divided into lobules, each lobule consisting of a number of secretory units composed of acini and ducts. The acini, the lumen of which is surrounded by the secretory cells, form a blind end, and the saliva produced passes through intercalated, intralobular, and excretory ducts before finally emptying into a main excretory duct; on its way through the duct system, the primary saliva is modified.

10 Fluid and Protein Secretion

Fluid and protein secretion is an active, energy-dependent process. The acinar cells are responsible for the secretion of fluid. They are also responsible for most of the protein secretion, whereas the duct cells contribute to a minor proportion of the total protein output. Large volumes of water are transported from the interstitium to the lumen by paracellular and transcellular passages in response to the osmotic force exercised by intraluminal NaCl. An intracellular rise in calcium concentration opens basolateral channels for potassium and apical channels for chloride. Potassium leaves the cell for the interstitium and chloride leaves the cell for the lumen. Next, the luminal increase in chloride concentration drags sodium, via paracellular transport, from the interstitium to the lumen and, as a result, water will move along the osmotic gradient produced by NaCl (Poulsen 1998; Melvin et al. 2005) (Fig. 7a and b).

The primary isotonic saliva formed in the acini undergoes changes during its passage through the duct system. The water permeability of the ducts is

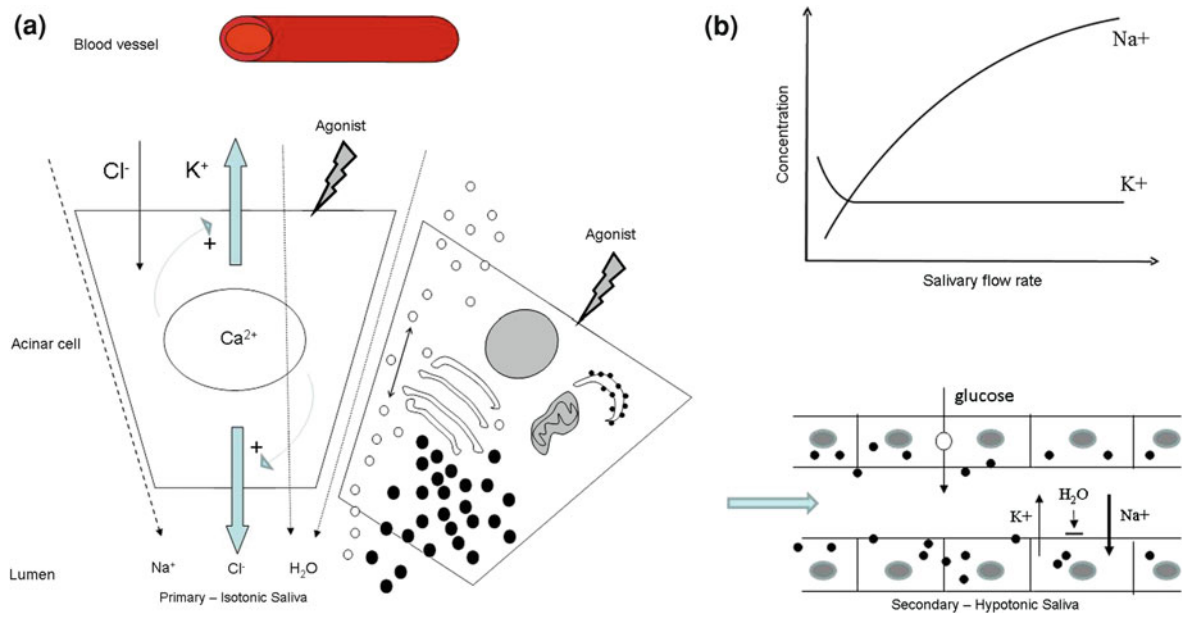

Fig. 7 **a** Acinar cells: water and protein secretion via vesicular and granular pathways—primary secretion. **b** Duct cells: modifications of saliva—secondary secretion

extremely small. Sodium and chloride are reabsorbed without accompanying water. A certain secretion of potassium and bicarbonate occurs at a lower rate than the rate of reabsorption of sodium and chloride. Consequently, the so-called secondary saliva that enters the mouth is hypotonic. The low salivary sodium concentration, one fifth of that of the primary saliva, makes it possible for the taste buds to detect salt at low concentrations.

The permeability of the duct system may increase under conditions that elevate the blood level of circulating catecholamines, released from the adrenal medulla, as illustrated by the appearance of glucose in the saliva in response to cold stress, mental stress, and physical exercise (Borg-Anderson et al. 1992; Teesalu and Roosalu 1993).

Immunoglobulins, in particular immunoglobulin A, are transported across the epithelial cells of acini and ducts. They are formed by plasma cells within the gland. After release to the interstitium, they form a complex with polymeric immunoglobulin receptor, which serves as transporter (Brandtzaeg 2009), a complex that splits in the saliva.

The secretion of proteins is of two types (Gorr et al. 2005). The constitutive (vesicular) secretion is a direct release of proteins as soon as they are synthesized by the Golgi vesicles. The constitutive secretion is responsible for a continuous secretion of several proteins without any ongoing external stimuli. The constitutive secretion is, however, also influenced

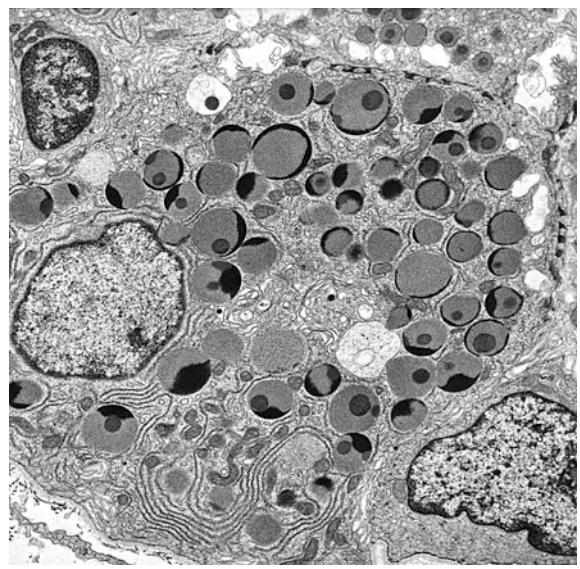

Fig. 8 Serous acinus of a human submandibular gland filled with secretory granules. Osmium maceration method. Magnification ×2,500. (Courtesy of Alessandro Riva, Cagliari University)

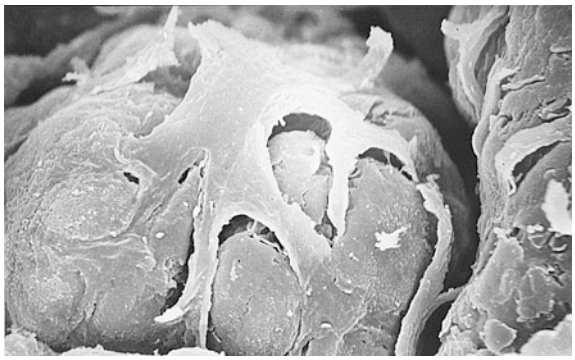

Fig. 9 Myoepithelial cells on the surface of, and embracing, a human parotid acinus. NaOH maceration method. Scanning electron microscope image, magnification ×2,000. (Courtesy of Alessandro Riva, Cagliari University)

by the nervous activity and, upon intense and prolonged stimulation, the importance of this pathway will increase concomitantly with the depletion of granules, as demonstrated experimentally (Garrett and Thulin 1975). Granular secretion is the regulated type of secretion. After synthesis, the proteins are stored in granules (Fig. 8). Upon stimulation, the granules empty their content of proteins into the lumen, i.e., the secretion occurs by exocytosis. The various routes for secretion may allow variations in the composition of the secretions (Ekström et al. 2009). Mobilization of the intracellular messenger adenosine 3′,5′-cyclic monophosphate (cAMP) by stimulation of β_1-adrenergic receptors and vasoactive intestinal peptide receptors is associated with protein secretion by exocytosis and a small volume response. Mobilization of the intracellular messenger Ca^{2+} by stimulation of muscarinic receptors (M1, M3) and α_1-adrenergic receptors is associated with fluid secretion—and particularly large volumes in response to muscarinic agonists—and protein secretion via vesicular secretion and, with intense stimulation, also via exocytosis (Ekström 2002). In acinar cells, agonists using cAMP may activate NO synthase of neuronal type but of nonneuronal origin to generate NO, which catalyzes the formation of guanosine 3′,5′-cyclic monophosphate (cGMP) (Sayardoust and Ekström 2003). The NO/cGMP pathway may contribute to the protein secretion partly by prolonging the action of cAMP (Imai et al. 1995), partly by catalyzing the generation of cyclic adenosine diphosphate ribose, which triggers the release of Ca^{2+} by its action on ryanodine-sensitive receptors of intracellular Ca^{2+} stores (Gallacher and Smith 1999) (Fig. 7).

The combined mobilization of Ca^{2+} and cAMP results in synergistic interactions with respect to both fluid and protein secretion (Ekström 1999a). Moreover, the two sets of autonomic innervations are also involved in protein synthesis. The nonadrenergic, noncholinergic mechanisms play a major role in parasympathetically nerve-induced protein synthesis (Ekström et al. 2000). The sympathetically nerve-induced protein synthesis is exerted via the two types of adrenergic receptors with a predominance for β-adrenergic receptors (Sayardoust and Ekström 2004). Importantly, the parasympathetic nonadrenergic, noncholinergic mechanisms have been shown to take part in the regulation of salivary gland activities under reflex activation due to taste and chewing (Ekström 1998, 2001; Ekström and Reinhold 2001).

11 Myoepithelial Cell Contraction

Myoepithelial cells display characteristics in common with both smooth muscle cells and epithelial cells. They embrace acini and ducts (Fig. 9). They receive a dual innervation, and both muscarinic receptors and α_1-adrenergic receptors cause the cells to contract; in some species, tachykinins also cause contraction (Garrett and Emmelin 1979). Myoepithelial cell contraction increases the ductal pressure, which may be of importance for the flow of high-viscosity mucin-rich saliva and for overcoming various obstacles to the flow. Moreover, the contraction of the myoepithelial cells may play a supportive role for the underlying parenchyma, particularly at a high rate of secretion.

12 Blood Flow

Salivary glands are supplied with a dense capillary network comparable with that of the heart (Edwards 1988; Smaje 1998). The capillaries are extremely permeable to water and solutes but not to macromolecules such as albumin. Parasympathetically induced vasodilatation may generate a 20-fold increase in gland blood flow, which ensures the secretory cells produce large volumes of saliva over a long period of time. The parasympathetic transmitter vasoactive intestinal peptide, besides acetylcholine,

plays a major role in the vasodilator response, which also involves the action of NO. Stimulation of the sympathetic innervation causes vasoconstriction by α_1-adrenergic receptors and neuropeptide Y receptors. However, the sympathetic innervation of the blood vessels of the gland is activated not in response to a meal but in response to a profound fall in systemic blood pressure in order to restore the blood pressure. The sympathetic vasoconstrictor nerve fibers originate from the vasomotor center and are separated from the sympathetic secretomotor nerve fibers taking part in alimentary reflexes (Emmelin and Engström 1960). Interestingly, the sympathetic nerve fibers innervating the blood vessels contain the potent constrictor transmitter neuropeptide Y, whereas the sympathetic secretomotor fibers lack this peptide (Ekström et al. 1996; Ekström 1999a, b).

13 Salivary Centers

The parasympathetic salivary center is located in the medulla oblongata and is divided into a superior and an inferior salivatory nucleus, and, in addition, an intermediate zone. The superior nucleus connects (the facial nerve) with the submandibular and the sublingual glands, whereas the inferior nucleus connects (the glossopharyngeal nerve) with the parotid gland (Emmelin 1967; Matsuo 1999). The intermediate zone makes connections with both the submandibular gland and the parotid gland. The sympathetic salivary center resides in the upper thoracic segments of the spinal cord. Higher centers of the brain exert both excitatory (glutamate) and inhibitory (γ- aminobutyric acid and glycine) influences on the salivary centers. The inhibitory influence is illustrated by the reduced flow of saliva associated with depression, fever, sleep, and emotional stress. Mouth dryness in response to stress is *not* a consequence of sympathetic activity: there are no inhibitory sympathetic fibers innervating the secretory cells (Garrett 1988).

14 Efferent Nerves

The parasympathetic preganglionic nerve fibers of the submandibular and sublingual glands leave the facial nerve and join, via the chorda tympani nerve, the lingual nerve to form the chorda-lingual nerve to reach the submandibular ganglion. The postganglionic nerve fibers of the submandibular ganglion innervate the submandibular and sublingual parenchyma (Rho and Deschler 2005). In humans, this ganglion is located outside the parenchyma of the two glands, which is in contrast to the intraglandular localization in many laboratory animals. The parasympathetic preganglionic nerve fibers of the parotid gland travel via the tympanic branch of the glossopharyngeal nerve (Jacobson's nerve), the tympanic plexus, and the lesser superficial petrosal nerve and, after relaying in the otic ganglion, the postganglionic nerve fibers are usually thought to reach the gland via the auriculotemporal nerve. With respect to the preganglionic innervation of the parotid gland, reflex studies suggest that not only fibers of the glossopharyngeal nerve but also fibers of the facial nerve (chorda tympani nerve) contribute, since cutting the chorda tympani nerve in the tympanic membrane reduces the response (Reicher and Poth 1933; Diamant and Wiberg 1965). The routes of the postganglionic cholinergic nerve fibers may differ as judged by extensive animal studies. Cholinergic nerve fibers may detach at an early stage from the auriculotemporal nerve, to reach the gland via the internal maxillary artery. Moreover, and in contrast to the general textbook view, the facial nerve passing through the parotid gland parenchyma, with its twigs, supplies the secretory cells with a cholinergic innervation that takes part in the reflex secretion (Ekström and Holmberg 1972; Khosravani et al. 2006; Khosravani and Ekström 2006). The facial nerve is therefore a potential contributor to the development of Frey syndrome (Dunbar et al. 2002). Frey syndrome is characterized by sweating, redness, flushing, and warming over the parotid region when eating. It develops over a period of months following parotid gland surgery, neck dissection, blunt trauma to the cheek, and chronic infection of the parotid area. It is considered to be due to aberrant regeneration of postganglionic parasympathetic cholinergic nerve fibers of the auriculotemporal nerve that innervate sweat glands and skin vessels following loss of the sympathetic postganglionic cholinergic innervation but may, in the light of a secretory role for the facial nerve, also involve regenerating parasympathetic postganglionic cholinergic nerve fibers of the facial nerve. Since botulinus toxin, preventing transmitter exocytosis, is more effective than the muscarinic

receptor antagonist atropine in the treatment of the syndrome, a cotransmitter or cotransmitters to acetycholine is/are likely to contribute to the symptoms; vasoactive intestinal peptide is such a cotransmitter (Drummond 2002).

The routes of the parasympathetic nerves of the minor glands (Tandler and Riva 1986) are via the buccal branch of the mandibular nerve with respect to the molar, buccal, and labial glands (postganglionic nerves originate from the otic ganglion), via the lingual nerve with respect to the lingual glands (Remak's ganglia, intralingually located), and via the palatine nerve with respect to palatine glands (sphenoplatine ganglion).

The sympathetic preganglionic nerve fibers ascend in the paravertebral sympathetic trunk to synapse with their postganglionic nerve fibers in the superior cervical ganglion, which then reach the glands via the arteries. However, their actual anatomical pathways are not completely defined, e.g., the parotid gland may be reached both via the external carotid artery and via intracranial routes (Garrett 1988).

15 Sensory Nerves of Glandular Origin

Pain in the salivary gland region is a well-known phenomenon in response to gland swelling upon inflammation or sialolithiasis. Although the pain is usually attributed to an increase in the intercapsular tension and activation of afferent nerves of the glandular fascia (Shapiro 1973; Leipzig and Obert 1979), sensory nerves occur in the glands and are therefore likely to be involved in the response. Nerve fibers showing colocalization of substance P and calcitonin-gene-related peptide are of sensory origin, and in the glands, these fibers are present in close connection with ducts and blood vessels (Ekström et al. 1988). The facial nerve and the great auricular nerve are pathways for nerves of this type of the parotid gland, originating from the trigeminal ganglion and dorsal root ganglia, respectively (Khosravani et al. 2006, 2008); in addition, the great auricular nerve innervates the parotid fascia (Zohar et al. 2002). The lingual nerve is thought to supply the submandibular and sublingual glands with sensory fibers of trigeminal origin. The periductal sensory nerves may serve protective functions. They may release defense substances from the duct cells (such as β-defensins) and by causing the myoepithelal cells to contract, noxious substances may be expelled and ductal distension may be overcome. Both substance P and calcitonin-gene-related peptide evoke protein extravasation and periglandular edema. Therefore, the perivascular sensory nerve fibers may be involved in gland swelling and gland inflammation. A role for sensory nerves in chronic inflammation has been pointed out, for instance, in asthma. In analogy, there might be a role for these nerves in chronic salivary gland inflammation. The levels of both substance P and calcitonin-gene-related peptide increase following extirpation of the superior cervical ganglion (Ekström and Ekman 2005), a phenomenon that may be associated with the clinical condition of parotid postsympathectomy pain upon eating (Schon 1985).

16 Hormones

Animal experiments demonstrate a long-term influence of sex steroids, growth hormone, and thyroid hormones on salivary gland metabolism, morphology, and secretory capacity (Johnson 1988). In humans, the development of postmenopausal hyposalivation illustrates the consequence of the loss of the continuous influence of estrogen and progesterone (Meurman et al. 2009). The opposite, i.e., excessive salivation, has been reported during pregnancy (Jenkins 1978). Apart from the effect of circulating catecholamines from the adrenal medulla in response to sympathetic activity, little attention has been paid to a short-term hormonal influence on the glands and their secretion. Aldosterone-induced ductal uptake of sodium (without water), lowering the sodium concentration of the saliva, is a well-known phenomenon in the parotid gland of the sheep, but in humans the effect of aldosterone is small (Blair-West et al. 1967). Recent animal investigations on the effect of some gastrointestinal hormones—gastrin, cholecystokinin, and melatonin, the latter found in large amounts in the intestines—do, however, imply that the secretory activity of salivary glands, like other exocrine glands of the digestive tract, are under the control of both nerves and hormones, and that the secretion from the salivary glands can be divided into three separate phases depending on the location from where the stimulus for secretion arises during a meal (Cevik Aras and Ekström 2006, 2008; Ekström and Cevik

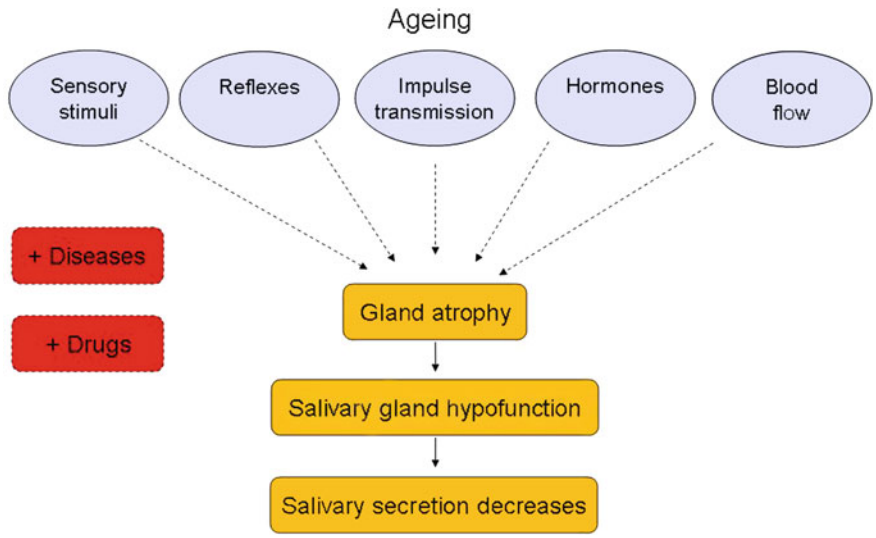

Fig. 10 Physiological changes at old age contributing to hyposalivation

Aras 2008; Cevik Aras et al. 2011). Thus, in addition to the well-known cephalic phase (nerves), a gastric phase (gastrin) and an intestinal phase (cholecystokinin and melatonin) may regulate salivary gland secretion. The hormones cause the secretion of proteins and stimulate the synthesis of secretory proteins but have little effect on the volume response. Ongoing studies show that human glands, like animal glands, are supplied with receptors for the three hormones and further, in vitro, release proteins from pieces of human gland tissues upon administration of the hormones (Riva et al. 2010).

Gastrointestinal hormones such as cholecystokinin, gastrin, and melatonin exert anti-inflammatory actions on salivary glands (Cevik Aras and Ekström 2010).

17 Trophic Effects of Nerves: Gland Sensitivity to Chemical Stimuli and Gland Size

When the amount of a drug required to elicit a certain submaximal biological response diminishes, the tissue is referred to as being supersensitive (Emmelin 1965; Ekström 1999b). Salivary glands, in particular, have been used as model organs to explore the phenomenon of supersensitivity. Depriving the glands of their receptor stimulation by trauma, surgery, or the pharmacological action of drugs results in the gradual development of denervation supersensitivity. The sensitization is most pronounced in response to the loss of influence of the postganglionic parasympathetic nerve. Restoration of a functional innervation normalizes the sensitivity. Experimentally, variations in the gland sensitivity can be brought about in animals supplied with functionally intact reflex arcs by varying the intensity of the reflex stimulation, the gland subjected to disuse (liquid diet) being more sensitive to stimuli than the gland subjected to overuse (chewing-demanding pelleted diet)—thus illustrating that the state of "normal sensitivity" is indeed a relative phenomenon (Ekström and Templeton 1977). Supersensitivity is attributed to intracellular events rather than to a change in the number of receptors on the cell membrane. The phenomenon is usually regarded as nonspecific but it seems, in fact, possible to demonstrate agonist-specific patterns associated with the degree of disuse of the various intracellular pathways (Ekström 1999b).

As might be expected, under physiological conditions the gland size is of primary importance for the volume response of the gland. Preclinical studies show that when the chewing-demanding diet is changed to a liquid diet in rats, the parotid gland loses about 50% of its dry weight, the amount of saliva secreted as a response to submaximal muscarinic stimulus is reduced by 40%, and the maximally evoked muscarinic volume response is reduced by 25% (Ekström and Templeton 1977). Parasympathetic postganglionic denervation causes a profound decrease in gland weight (by 30–40%). However, loss of the action of acetylcholine on the gland is probably

not the cause: prolonged treatment with the muscarinic antagonist atropine results in no decrease in weight. Instead parasympathetic nonadrenergic, noncholinergic transmission mechanisms maintain the gland weight, and induce mitotic activity in the glands (Ekström et al. 2007). The nature of the transmitter or transmitters involved is unknown.

As previously pointed out, salivary glands are supplied with β_1-adrenergic receptors (Ekström 1969); however, the sympathetic system seems to play a minor role in the regulation of gland size under physiological conditions. Although the β-adrenergic agonist isoprenaline is known to cause gland swelling after prolonged treatment of asthma and isoprenaline in preclinical studies is known to increase gland weights severalfold (Barka 1965), sympathetic denervation only slightly, if at all, reduces gland weight. In agreement, treatment with the β_1-adrenergic receptor antagonist metoprolol causes only a small decrease in gland weight (Ekström and Malmberg 1984). It should be noted that the severalfold gain in weight caused by isoprenaline does not correspond to a similar increase in secretory capacity (Ohlin 1966).

18 Ageing

The secretory capacity is usually thought to decline with age; however, functional data do not support such an assumption (Vissink et al. 1996; Nagler 2004; Österberg et al. 1992). No doubt, the proportion of fat and fibrovascular tissue gradually increases with time and consequently, the proportion of functional parenchyma decreases. However, despite these morphological changes, the secretory volumes of unstimulated and stimulated saliva are only slightly affected, if at all. With respect to the composition of saliva, the individuality of the glands comes to light since the parotid saliva composition is considered unchanged whereas the mucin secretion of the mucous/seromucous glands as well as the immunoglobulin A secretion of the labial glands is thought to decrease (Fig. 10).

A number of events associated with ageing will make salivary gland functions particularly vulnerable, and in concert, these events may eventually have implications for the production of the saliva. For instance, the intensity of the reflex activity diminishes owing to reduction in the number of olfactory and taste receptors as well as loss of teeth; the neuroglandular junction widens, diminishing the concentration of transmitters acting on the receptors; the blood levels of the sex steroids decrease; and the blood perfusion of the glands is reduced. To this list of changes, diseases and pharmaceutical drugs are added. In 70-year-olds, 64% of women and 55% of men were found to be receiving medication in a recent Swedish study; the average number of drugs was 4.0 for women and 3.3 for men (Johanson 2011).

19 Xerostomia, Salivary Gland Hypofunction, and Dry Mouth

Usually, the salivary secretion is estimated after an overnight fast or 2 h after a meal (Birkhed and Heintze 1989; Navazesh and Kumar 2008). To collect whole unstimulated/resting saliva, the subject, sitting in a chair, is instructed to swallow and then to lean the body forward, allowing the saliva to drip passively through a funnel into an (ice-chilled) graduated (or preweighed) cylinder for 15 min. The stimulated whole saliva is usually collected over 5 min: by chewing paraffin wax, usually at a fixed frequency (e.g., 40 or 70 strokes per min); by citric acid applied either on the dorsum of the tongue for 30 s or as a solution (2.5%) held in the mouth for 1 min; or by sucking a lemon-flavored candy. The saliva pouring into the mouth is spat into a cylinder, preferentially at fixed intervals. The secretion is expressed per milliliters per minute or per milligrams per minute (the density of saliva is assumed to be 1.0 g/ml).

In humans, salivary ducts are not usually cannulated to measure the flow of saliva from individual glands. However, by applying the Lashley–Crittenden "cup" over the orifice of the parotid duct, one can record the flow of parotid saliva. Devices of various types have been constructed for the collection of submandibular/sublingual secretion—but here, saliva from the two types of gland is mixed. By the so-called Periotron method, saliva from the minor glands can be estimated (Eliasson and Carlén 2010). A filter paper is placed over a small area of the oral epithelium, and the fluid collected on the filter paper is measured using the change in conductance to indicate fluid.

An unstimulated flow rate of whole saliva less than 0.1 ml/min and a stimulated flow rate of whole saliva less than 0.7 ml/min are considered to indicate *salivary gland hypofunction* (Ericsson and Hardwick 1978). *Xerostomia* is the subjective sensation of dryness of the oral mucosa. Importantly, xerostomia and salivary gland hypofunction may or may not be related phenomena—only about 55% of those complaining of xerostomia show, by objective measurement, a decrease in saliva volume (Field et al. 1997; Longman et al. 1995). The term "dry mouth" refers to the oral sensation of dryness with or without the demonstration of salivary gland hypofunction.

The thickness of the fluid layer covering the oral mucosa varies markedly, being 70 μm at the posterior dorsum of the tongue and 10 μm at the hard palate (DiSabato-Mordaski and Kleinberg 1996; Wolff and Kleinberg 1998). The volume of saliva in the mouth is dependent not only on the secretion of saliva but also on evaporation, absorption of fluid through the oral mucosa, and swallowing. Mouth breathing and speaking are the main causes of the fluid loss by evaporation; the hard palate with its thin fluid layer is directly exposed to the flow of inspired air (Thelin et al. 2008). An excess of saliva in the mouth elicits a swallowing reflex. Usually, the volume of saliva that enters the mouth at rest exceeds the volume lost by evaporation and swallowing. Despite wide differences in the rate of unstimulated secretion, a decrease by about 50% of this secretion in an individual will give rise to the sensation of oral dryness (Dawes 1987; Wolff and Kleinberg 1999). In this case, the thickness of the saliva film of the anterior dorsum of the tongue and the hard palate is less than 10 μm. It is also from these locations that the subject experiences the most pronounced symptoms of xerostomia (Wolff and Kleinberg 1999). A decrease in the labial secretion by only 20% is correlated to the feeling of oral dryness (Eliasson et al. 1996).

20 Causes of Dry Mouth

The prevalence of dry mouth is 15–40%. The condition is more common among women and increases with age (Österberg et al. 1984; Nederfors et al. 1997). Dry mouth dramatically impairs the quality of life (Ship et al. 2002; Wärnberg et al. 2005), and is both a physical and a social handicap. It is associated with difficulties in chewing, swallowing, and speaking. The lips are cracked and dry. Taste acuity weakens and oral mucosal infections, dental caries and halitosis develop. Among known causes of dry mouth are chronic gland inflammation as Sjögren syndrome, diabetes, depression, head and neck radiotherapy, radioiodide therapy, HIV/AIDS, orofacial trauma, surgery, and use of medications (Grisius and Fox 1988). Drugs presently in use may interfere with the reflexly elicited secretion at the level of the central nervous system and/or at the level of the neuroglandular junction. In this connection, it should be remembered that the salivary glands are effectors of the autonomic nervous system and that they are supplied with the same set of receptor types as other effector organs of this system. Consequently, when a dysfunction of an effector within this system is treated by interfering with the transmission mechanisms, e.g., overactive urinary bladder (by muscarinic receptor antagonists) or hypertension (see below), the functions of the salivary glands are invariably influenced. Drugs with antimuscarinic actions cause a marked reduction in the volume of saliva produced. Although the volume is not always changed to any great extent, the composition of the saliva may have undergone changes resulting in the subjective feeling of oral dryness. The use of drugs belonging to the cardiovascular category or the psychotropic category is particularly correlated with a decreased rate of secretion as a side effect. Antihypertensive drugs may block α_1- adrenergic receptors and β_1-adrenergic receptors, and stimulate prejunctional (neuronal) α_2-adrenergic receptors (which inhibits the transmitter release). Although diuretics in in vitro experiments influence various electrolyte exchange processes in the glands and dry mouth is a common complaint in response to the treatment with diuretics, the salivary flow rate in humans is only slightly affected, if at all (Atkinson et al. 1989; Nederfors et al. 1989). Oral mucosal tissue dehydration has been suggested as cause of the dry mouth feeling. Antiarythmics block β_1-adrenergic receptors and exert anticholinergic effects. Apart from the central action of antidepressants, this group of drugs blocks peripherally the muscarinic receptors. Antipsychotics have not only antimuscarinic actions but also have anti-α_1-adrenergic actions. Importantly, when one set of receptor type is blocked, not only is

the response mediated by this particular receptor abolished, but the synergistic interaction provided by the receptor is also abolished.

Several hundred drugs are said to be xerogenic, and dry mouth is the third most common side effect of drug treatment. It is important to realize that reference guides to drugs causing dry mouth are usually put together on the basis of the sensation of oral dryness rather than on the basis of the actual measurement of the saliva output. There is a correlation between the total intake of the number of drugs and dry mouth (with or without hyposalivation). The use of four drugs or more increases the probability that the phenomenon of dry mouth will occur. If the number of drugs is increased, the chance of consuming a drug producing dry mouth by itself or by its interaction with other drugs is likely to increase.

21 Treatment of Dry Mouth

The options to treat dry mouth are, unfortunately, limited and focused on maintaining the salivary reflexes by flavored gums or lozenges, or by the use of salivary substitutes such as artificial saliva, oral rinses, and oral gels. These treatments are of short duration. In addition, scrutiny of the medication list may make it possible to achieve a reduction in the number of drugs taken by the patients or in the dose of individual drugs and, in addition, replacement with drugs with less xerogenic effects may be effected. A number of drugs for systemic use have been introduced, such as parasympathomimetics, cholinesterase inhibitors, the bile stimulating agent anethole trithione, the mycolytic agents bromhexine and guafensin, the immune-enhancing substance interferon-α, the cytoprotective amifostine, and the antimalarial drug hydroxychloroquine. In many cases, the clinical effects are questionable, and moreover some of these drugs are associated with serious side effects. The parasympathomimetics pilocarpine (Salagen®) and cevimeline (Evoxac®) stimulate the flow of saliva but may also cause nausea, sweating, gastrointestinal discomfort, respiratory distress, urges to empty the bladder, and hypotension. Recent clinical trials using topical application of the cholinesterase physostigmine on the oral mucosa have demonstrated local treatment of dry mouth as an alternative approach to systemic treatment (Khosravani et al. 2009). After diffusion of the drug through the mucosal barrier, the underlying mucin-producing minor glands are stimulated to secrete saliva, while at the same time the systemic effects are minimized. The patient suffering from dry mouth should maintain meticulous oral hygiene, including the use of a fluoride-rich gel, frequent visits to the dental hygienist, and in addition, avoiding food and beverages that are sweet, acidic, or carbonated.

22 Sialorrhea

Neuromuscular dysfunctions associated with cerebral palsy, Parkinson disease, amyotrophic lateral sclerosis, and stroke are examples of conditions that cause drooling. Under these conditions, saliva pools in the mouth owing to lack of swallowing rather than to an increased rate of secretion of saliva (Young et al. 2011). An increase in the rate of secretion may occur in the treatment of Alzheimer disease and myasthenia gravis owing to the medication with reversible cholinesterases (Freudenreich 2005; Ecobichon 1995). Sialorrhea is reported as a side effect of clozapine in about one third of patients under treatment for schizophrenia (Praharaj et al. 2010). Clozapine is an atypical antipsychotic drug used when traditional antipsychotics fail to treat schizophrenia. During the night, patients are troubled with choking sensations and the aspiration of saliva. The situation may be so bothersome that the drug regimen is discontinued. The phenomenon has been largely unexplained, and some authors have referred it to a weakened swallowing reflex. A number of various categories of drugs have been suggested for the treatment of clozapine-induced hypersalivation, usually with limited success and with side effects of their own (Sockalingam et al. 2007). Recent preclinical studies have shown that both clozapine and its main metabolite *N*-desmethylclozapine exert mixed actions on the salivation (Ekström et al. 2010a, b; Godoy et al. 2011). Upon reflex secretion, the two drugs decrease the flow of saliva by antagonistic actions on muscarinic M3 receptors and α_1-adrenergic receptors. During sleep and at rest, an agonistic action by the drugs on muscarinic M1 receptors maintains a low-grade, continuous flow of saliva.

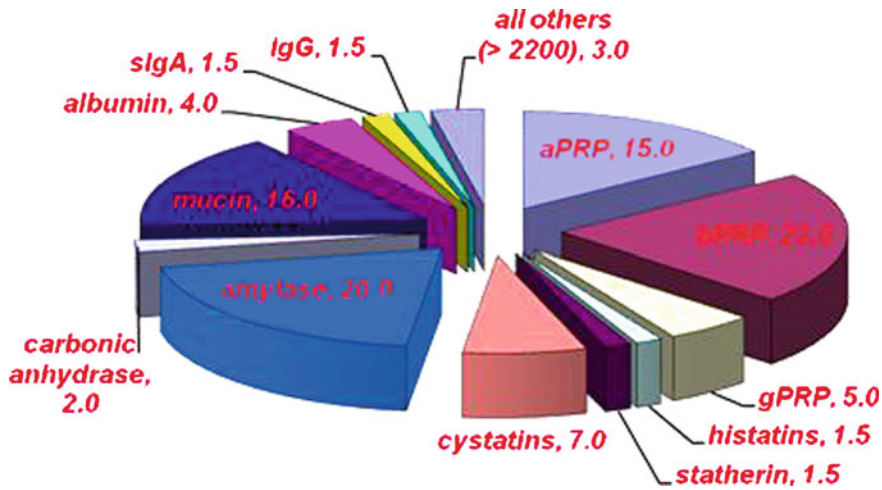

Fig. 11 Approximate percentages (w/w) of the different protein families present in human adult whole saliva, assuming a comparable contribution of parotid and submandibular/sublingual glands. (Modified from Messana et al. 2008b)

23 Protein Components of Human Saliva and Posttranslational Modifications

The recent availability of mass spectrometry (MS)-based techniques applicable to the study of complex protein mixtures has stimulated the effort to obtain a qualitative and quantitative comprehensive understanding of the protein composition of saliva.

Indeed, MS techniques are capable of identifying and quantifying thousands of protein components in complex samples. The mass spectrometer makes it possible to obtain precise mass values through the measure of the mass-to-charge ratio (m/z) of the ions generated from peptides and proteins at the source. Selected ions may also be submitted to a fragmentation process (this technique is called MS/MS), and the determination of the m/z ratio of the fragments allows the peptide structure to be investigated. Thus, the power of MS rests in the possibility to obtain information on not only the exact mass of a given peptide/protein, but also on its sequence. Two main strategies may be used to investigate protein mixtures: the top-down and the bottom-up approaches. In the bottom-up approach, the nonfractionated sample is submitted to digestion, typically by trypsin, and the resulting digestion mixture is fractionated and analyzed by MS. Thus, presence and quantification of the proteins in the sample are inferred from the ensemble of identified digestion peptides, supposing that any peptide identified derives from a unique protein. Even though this approach is of high throughput, the digestion step introduces a limitation, since relevant naturally occurring cleavages may obviously not be disclosed. The top-down approach overcomes this problem, since peptide and protein separation followed by MS analysis is performed with undigested samples. However, top-down platforms often cannot cover the entire proteome, because some proteins can escape from the analysis (e.g., proteins insoluble in acidic milieu). High-performance liquid chromatography (HPLC) is more suitable than gel electrophoresis as a separation step technique for the analysis of the salivary proteome, since it is mainly represented by peptides and small/medium-sized proteins. Moreover, with respect to gel electrophoresis, HPLC offers the advantage that MS analysis can be performed online, i.e., peptides and proteins are submitted directly to the ion source of the MS apparatus.

24 The Salivary Proteome

Most of the about 2,400 different proteins of whole saliva characterized in recent years by proteomic studies are not of glandular origin but probably originate from exfoliating epithelial cells and oral microflora. Proteins of gland secretion origin should not be more than 200–300 in number, and they represent more than 85% by weight of the salivary proteome (Fig. 11). They belong to the following major families: α-amylases, carbonic anhydrase, histatins, mucins, proline-rich proteins (PRPs), further divided in acidic, basic, and basic glycosylated PRPs, statherin, P–B peptide, and salivary-type (S-type) cystatins.

Table 1 Families of major salivary proteins: function, origin, genes, name of mature proteins, and main posttranslational modifications (*PTMs*)

Family	Function	Origin	Gene	Mature proteins	Other PTMs
α-Amylases	Antibacterial, digestion, tissue coating	Pr Sm/Sl	*AMY1A*	α-Amylase 1	Disulfide bond, N-glycosylation, phosphorylation, proteolytic cleavages
Acidic PRPs	Lubrication, mineralization, tissue coating	Pr Sm/Sl	*PRH1*, *PRH2*	Db-s, Pa, PIF-s, Pa 2-mer, Db-f, PIF-f, PRP-1, PRP-2, PRP-3, PRP-4, P–C peptide	Disulfide bond, further proteolytic cleavages, phosphorylation, protein network
Basic PRPs	Binding of tannins, tissue coating	Pr	*PRB1*, *PRB2*, *PRB3*, *PRB4*	II-1, II-2, CD-IIg, IB-1, IB-6, IB-7, IB-8a (Con1−/+), P–D, P–E, P–F, P–J, P–H, PRP Gl 1–8, protein N1, salivary PRP Po	Disulfide bond (Gl 8), further proteolytic cleavages, N- and O-glycosylation, phosphorylation, protein network
Glycosylated PRPs	Antiviral, lubrication				
Carbonic anydrase VI	Buffering, taste	Pr Sm	*CA6*	Carbonic anhydrase 6	Disulfide bond, glycosylation
Cystatins	Antibacterial, antiviral, mineralization, tissue coating	Pr Sm/Sl	*CST1*, *CST2*, *CST3*, *CST4*, *CST5*	Cystatin SN, cystatin SA, cystatin C, cystatin S, cystatin D	Disulfide bond, O-glycosylation, phosphorylation, sulfoxide, truncated forms
Histatins	Antifungal, antibacterial, mineralization, wound-healing	Pr Sm/Sl	*HTN1*, *HTN3*	Histatin 1, histatin 2, histatin 3, histatin 5, histatin 6	Further proteolytic cleavages, phosphorylation, sulfation
Lactoferrin	Antibacterial, antifungal, antiviral, innate immune response	All salivary glands	*LTF*	Lactoferrin	Disulfide bond, glycosylation, phosphorylation
Lysozyme	Antibacterial	Pr Sm	*LYZ*	Lysozyme C	Disulfide bond
Mucins	Antibacterial, antiviral, digestion, lubrication, tissue coating	All salivary glands	*MUC5B*, *MUC19*, *MUC7*	Mucin-5B, mucin-19, mucin-7	Disulfide bond, N- and O-glycosylation, phosphorylation
Peptide P–B	Not defined	Pr Sm/Sl	*SMR3B* (*PROL3*)	Proline-rich peptide P–B	Proteolytic cleavages
Statherins	Inhibits crystal formation, lubrication, mineralization, tissue coating	Pr Sm/Sl	*STATH*	Statherin, statherin SV2	Phosphorylation, proteolytic cleavages, protein network

Modified from Castagnola et al. (2011b)
PRP proline-rich protein, *Pr* parotid, *Sm* submandibular, *Sl* sublingual, *GCF* gingival crevicular fluid

The function, origin, and encoding genes of the major salivary proteins are reported in Table 1, together with the name of mature proteins and the main posttranslational modifications occurring before, during, and after secretion.

Histatins are a family of small peptides, the name referring to the high number of histidine residues in their structure. All the members of this family arise from histatin 1 and histatin 3, which share very similar sequences and are encoded by two genes

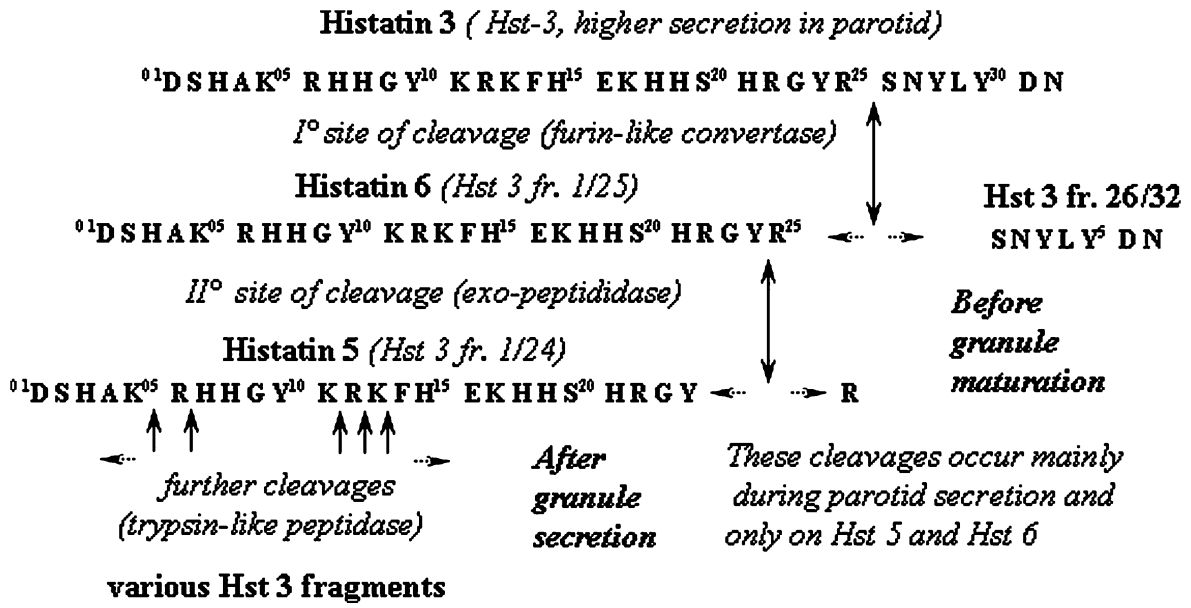

Fig. 12 The sequential proteolytic cleavage of histatin 3, which generates histatin 6 and histatin 5, before granule maturation, and a multitude of other fragments after granule secretion. (From data reported in Castagnola et al. 2004; Messana et al. 2008a)

(*HTN1* and *HTN3*) located on chromosome band 4q13 (Sabatini and Azen 1989). Statherin is an unusual tyrosine-rich 43-residue phosphorylated peptide involved in oral cavity calcium ion homeostasis and tooth mineralization (Schwartz et al. 1992). Its gene (*STATH*) is localized on chromosome band 4q13.3 (Sabatini et al. 1987), near the histatin genes. Usually P–B peptide is included in the basic PRP family. However, it is the product of *PROL3* gene localized on chromosome band 4q13.3, very close to the statherin gene, and several characteristics of P–B peptide suggest a functional relationship with statherin (Inzitari et al. 2006). Cystatin S, SN, and SA are salivary cystatins; they are inhibitors of cysteine proteinases and this property suggests its role in the protection of the oral cavity from pathogens and in the control of lysosomal cathepsins (Bobek and Levine 1992). Cystatin S1 and cystatin S2 correspond to monophosphorylated and diphosphorylated cystatin S, respectively. The loci expressing all the S-type cystatins (*CST1–CST5*) are clustered on chromosome band 20p11.21 together with the loci of cystatins C and D. Whereas cystatin SA seems to be specifically expressed in the oral cavity, cystatin S and SN have also been detected in other bodily fluids and organs, such as tears, urine, and seminal fluid (Dickinson 2002; Ryan et al. 2010). Human salivary acidic PRPs consist of five principal isoforms codified by two distinct loci called *PRH1* and *PRH2* localized on chromosome band12p13.2. They show acidic character in the first 30 amino acid residues of the N-terminal region; the remaining part is basic and, similarly to basic PRPs, shows repeated sequences rich in proline and glutamine. Basic and glycosylated (basic) PRPs are the most complex group of salivary peptides, encoded by four different genes named *PRB1–PRB4* clustered on chromosome band 12p13.2. Numerous homologous and unequal crossing-overs are present within the tandem repeats of the third exon, producing frequent length polymorphisms.

Salivary amylases consist of two families of isoenzymes, called A and B, each family comprising three isoforms whose differences are connected to different posttranslational modifications (Scannapieco et al. 1993).

Salivary mucins are divided in two distinct classes: the large gel-forming mucins (MG1) and the small soluble mucins (MG2). MG1 represents a heterogeneous family of 20×10^6–40×10^6 Da glycoproteins expressed by *MUC5B*, *MUC4*, and *MUC19* genes (Offner and Troxler 2000; Thomsson et al. 2002). MG2, a much smaller mucin of 130–180 kDa, is the product of the *MUC7* gene mapped to chromosome bands 4q13–4q21 (Bobek et al. 1996). Mucins are

composed of approximately 15–20% protein and up to 80% carbohydrate, present largely in the form of serine and threonine O-linked glycans (Strous and Dekker 1992; Gendler and Spicer 1995). The polypeptide backbone can be divided into three regions. The central region contains tandemly repeated sequences of eight 169 amino acids. This domain serves as the attachment site for the *O*-glycans, and each mucin has a unique, specific tandem-repeat sequence. Many mucins with monomeric molecular masses greater than 2×10^6 Da form multimers more than ten times bigger than that size.

25 Polymorphism of the Salivary Proteome

The human salivary proteome shows high interindividual variability. The different isoforms of salivary proteins may be genetic in origin (different alleles codifying acidic PRPs, basic PRPs, mucins, cystatins; differential splicing), but may also derive from several posttranslational modifications which occur during the trafficking of the proteins through the secretory pathway and after secretion.

One of the better known examples of polymorphism and modifications occurring before, during, and after secretion concerns acidic PRPs. The two loci which encode acidic PRPs have different alleles. The *PRH2* locus is biallelic, and the expression products are PRP-1 and PRP-2. There are three alleles of the *PRH1* locus and they express Pif-s (parotid isoelectric-focusing variant, slow), Db-s (double band, slow), and Pa (parotid acidic protein) proteins (Inzitari et al. 2005). All the isoforms are N-terminally modified (pyroglutamic moiety) and are subjected to phosphorylation before granule storage. The major derivatives are diphosphorylated, but low levels of monophosphorylated and triphosphorylated forms are also detected in saliva. Another important modification is the cleavage. Before granule storage PRP-1, PRP-2, Pif-s, and Db-s are in part cleaved at the Arg-106 residue by a specific enzyme of the convertase family. Cleavage generates four truncated derivatives, called PRP-3, PRP-4, Pif-f, and Db-f, and a common C-terminal peptide of 44 amino acids, called P–C peptide (Messana et al. 2008a). The Pa isoform is not cleaved, since the Arg-106 → Cys substitution eliminates the consensus sequence recognized by the proteinase. However, the cysteine residue generates a disulfide bridge and only the Pa dimeric form may be detected in whole saliva.

Also histatins, statherin, P–B peptide, and principally basic PRPs undergo proteolytic cleavage before granule storage and during secretion, but the entire forms of basic PRPs, differently from the other salivary proteins, are not detected in saliva (Messana et al. 2008a). Following proteolytic cleavage, many salivary peptides are also subjected to the removal of C-terminal residues by the action of specific carboxypeptidases, and this modification is considered an event common to all the secretory processes (Steiner 1998). An important example concerns the formation of histatin 5 from histatin 6. The two histatins derive from the parent peptide of 32 amino acid residues called histatin 3. Histatin 3, from the presence of the RGYR↓ convertase consensus sequence recognized by an unknown, but specific, proteinase acting before granule storage, generates histatin 6 (histatin 3 fr 1/25). Subsequently, an unknown carboxypeptidase removes the C-terminal arginine residue, generating histatin 5 (histatin 3 fr 1/24). Sequentially, histatins 5 and 6 are subjected to further proteolytic cleavages after granule secretion as shown in Fig. 12 (Castagnola et al. 2004; Messana et al. 2008a).

Before granule storage salivary proteins are also subjected to phosphorylation, glycosylation, and sulfation. MG1, MG2, glycosylated PRPs, and amylase are salivary glycosylated proteins (Ramachandran et al. 2006). The glycomoiety may be N- and/or O-linked and the sugars show the same architectures demonstrated for other glycoproteins (Guile et al. 1998). In the same way, the tyrosylprotein sulfotransferase involved in the polysulfation of histatin 1 seems to be the same enzyme acting in other tissues (Cabras et al. 2007).

The salivary proteome changes dynamically also after secretion under the action of endogenous and exogenous enzymes, the latter derived from microorganisms resident in the oral cavity. For instance, it has been demonstrated that a glutamine endoproteinase localized in dental plaque—likely of microbial origin—generates in the oral cavity a lot of small fragments (from seven to 20 amino acid residues) from different basic PRPs (Helmerhorst et al. 2008). Another important modification occurring in the oral cavity is the formation of cross-linked derivatives of salivary proteins, generating a protective

Table 2 Different contributions to salivary peptides and proteins

Peptide or family	Parotid glands	Sm/Sl glands	Plasma exudate	GCF
Acidic PRP (all the isoforms)	●●●●	●●●		
Basic PRP	●●●●			
Basic glycosylated PRP	●●●			
Histatin 3	●●●●	●●●		
Histatin 1	●●●	●●●		
Statherin	●●●●	●●●●		●
P–B peptide	●●	●●●●		●
"S-type" cystatins	●	●●●●		
Amylase	●●●●	●		
MG1		●●●●		
MG2		●●●		
Albumin (HSA)			●●	●●
Thymosins β_4 and β_{10}			?	●●
α-Defensins 1–4			●	●●

Modified from Messana et al. (2008b)
GCF gingival crevicular fluid, *HSA* human serum albumin, *Four circles* high contribution, *three circles* medium contribution, *two circles* low contribution, *one circle* very low contribution, *question mark* unknown

proteinaceous network on tooth surfaces (enamel pellicle) and oral mucosa. This protein film is important for the integrity of tooth enamel, because it acts as a boundary lubricant on the enamel surface (Douglas et al. 1991). Moreover, interactions between pellicle proteins and bacterial surfaces are responsible for specificity of the bacterial colonization during the earliest stage of plaque formation (Gibbons and Hay 1988). This protein network could also interact with the oral epithelial cell plasma membrane and its associate cytoskeleton and might contribute to the mucosal epithelial flexibility and turnover. Histatins, statherin, and acidic PRPs are among the proteins involved. It has been indeed demonstrated that acidic PRPs, statherin, and the major histatins are substrates of oral transglutaminase 2 and they participate in cross-linking reactions (Yao et al. 1999).

26 Physiological Variability

The composition of oral fluid varies depending on various factors. It has already been reported that the contribution of the different salivary glands to whole saliva in resting and stimulated conditions is different, and parotid saliva is the prevalent contributor to stimulated saliva. It has been also demonstrated that the protein composition of mixed submandibular/sublingual saliva is different from that of parotid saliva (Table 2). For instance, the levels of acidic PRPs, histatin 1, and α-amylases are higher in parotid saliva than in submandibular/sublingual saliva. Conversely, S-type cystatins are more concentrated in submandibular/sublingual saliva. Furthermore, the secretion of some peptides is gland-specific: basic PRPs are secreted only by the parotid glands. Finally, among the other proteins detected in whole saliva, α-defensins 1–4 and β-thymosins 4 and 10 originate mainly from gingival crevicular fluid (Pisano et al. 2005).

As a consequence, the salivary output is characterized by variations not only of the flow rate but also of the protein concentration and composition.

Age is another important factor affecting protein saliva composition. A recent study performed on human preterm newborns demonstrated the profound difference in the protein composition of their saliva with respect to that of adults (Castagnola et al. 2011a). Indeed, in saliva from preterm human newborns, more than 40 protein masses usually undetected in adult saliva were revealed. Among them, stefin A and stefin B (three isoforms), S100A7 (two isoforms), S100A8, S100A9 (eight isoforms), S100A11, S100A12, small PRP-3 (two isoforms), lysozyme C, thymosins β_4 and

β_{10}, antileukoproteinase, histone H1c, and α- and β-globins were identified. The salivary concentration of these proteins decreased as a function of postconceptional age, reaching the values observed in full-term newborns at about 270 days of postconceptional age, and the values observed in adult whole saliva later in development. Interestingly, the shape of decrease for many proteins was different, suggesting that the variations were connected to coordinate and hierarchical actions of these proteins. Many of the identified proteins are candidates as tumor markers in the adult. This observation led to the suggestion that during fetal development, the interplay between these proteins contributes to the molecular events that regulate cell growth and death. A preliminary study showed that salivary glands are responsible for the high levels of oral thymosin β_4 detected in preterm newborn saliva, whereas in adult saliva this peptide is primarily derived from crevicular fluid (Inzitari et al. 2009; Nemolato et al. 2009). These studies suggest that salivary glands switch their secretion to adult salivary proteins only after the normal term of delivery.

Whereas basic PRPs in whole saliva do not reach their mature concentrations until the age of adolescence (Cabras et al. 2009), other proteins show mature levels as early as an age of 3 years or show variable concentrations as a function of age, i.e., acidic PRPs, histatin 5, histatin 6, histatin 1, and cystatin S. For instance, acidic PRPs show a minimum of concentration around 6–9 years of age, probably in connection with events occurring in the mouth during the replacement of the deciduous dentition. A process called "exfoliation" might cause a decrease in the concentration of specific salivary protein and peptides, owing to their recruitment to dental and gingival surfaces. The higher concentration of histatin 1 around 3–5-years of age is of particular interest, since it may be associated with its recently demonstrated wound-closing properties (Oudhoff et al. 2008).

27 Function of Salivary Proteins

No doubt exists about the fundamental role of saliva and its protein content in the protection of oral mucosa and teeth. It is enough to consider the devastating macroscopic effects detectable in the oral cavity of patients affected by severe Sjögren syndrome. The mucosal epithelium is subjected to wounds and infections. The dental arc is compromised by recurrent periodontitis and caries. It is, however, very difficult to establish, at the molecular level, not only the specific role played by each salivary protein in oral protection but also the interactions between the different salivary proteins in their protection of the mouth and, since saliva is swallowed, of the entire digestive tract. Some roles seem evident, such as the lubricating and protecting role of mucins, and the buffering properties of carbonic anhydrase, as reported in Table 1. The high concentration of salivary amylase is traditionally associated with starch predigestion. However, owing to the low enzymatic activity of the enzyme, some researchers are convinced that oral amylase plays a presently not specified role in the protection of the mouth.

The information obtained by recent proteomic studies are a clue and a stimulus for the understanding of the roles of the different families of salivary proteins in the oral cavity. For instance, it is challenging to decipher the significant qualitative and quantitative differences in gland secretions, which suggest specific molecular requirements for different oral districts. Other suggestions could emerge from the variations observed in protein composition during the pediatric age which could offer valuable information on possible functions. Except for their tannin-binding properties (Lu and Bennick 1998), the function of basic PRPs is still almost completely obscure. Recent studies demonstrated that an unidentified component of the basic PRP family displays antiviral activity against HIV (Robinovitch et al. 2001), and a peptide fragment of ten amino acid residues considerably inhibits *Propionibacterium acnes* growth (Huang et al. 2008), revealing interesting antiviral properties for peptide fragments related to basic PRPs.

Acidic PRPs are responsible for the modulation of the salivary calcium ion concentration and are involved in the formation of acquired enamel pellicle and oral mucosal pellicle, networks originating from cross-linking of the proteins caused by the action of transglutaminase 2. However, no information is available on the functional differences exerted by the entire and truncated isoforms or on the possible role of P–C peptide, the C-terminal peptide deriving from the cleavage of all the isoforms of acidic PRPs.

Most salivary peptides and proteins are directly or indirectly involved in innate immunity and in the modulation of the oral microflora (Gorr 2009). In this

respect, the antifungal activity shown by histatin 3 and its fragments on *Candida albicans* species is particularly interesting. Recently, it was demonstrated that histatin 3 binds to heat shock cognate protein 70 (HSC70) during the G1/S transition in human gingival fibroblasts (Imamura et al. 2009); it prevents ATP-dependent dissociation of the HSC70–p27 complex, and it induces DNA synthesis. These findings suggest that histatin 3 may also be involved in oral cell proliferation.

Recently, it was shown that histatin 1 displays wound-healing activity (Oudhoff et al. 2008). Interestingly, histatin 1 induced cell spreading and migration in a full-skin human wound model; however, the peptide did not stimulate cell proliferation. N- to C-cyclization potentiated peptide activity 1,000-fold, indicating that a specific peptide conformation was responsible for the effect (Oudhoff et al. 2009a). The minimally active domain was found to be fragment 20–32 of the parent histatin peptide. The wound-healing effect was strongly inhibited by mucin-5B, probably by blocking reepithelialization. Interestingly, histatin 1 stimulated wound closure of primary cells of both oral and nonoral origin (Oudhoff et al. 2009b), which suggests a therapeutic application of histatin 1 derived peptides in the treatment of skin wounds.

Statherin is a singular salivary phosphopeptide of 43 amino acid residues involved in the inhibition of calcium phosphate precipitation and in the formation of acquired enamel pellicle (Schüpbach et al. 2001). However, statherin may have other relevant oral functions implicated in the formation of the oral epithelial protein pellicle, and it probably has a functional connection with the P–B peptide, whose function is still completely obscure (Messana et al. 2008b).

28 Pathological Modifications

Saliva is a very attractive bodily fluid for the diagnosis of diseases for several reasons: (1) collection of saliva is usually economical, "safe," "easy," and can be performed without the help of health care workers, allowing home-based sampling; (2) collection of saliva is considered an acceptable and noninvasive process by patients because it does not provoke any pain (and so saliva can be easily collected for patients in the pediatric age range) (Tabak 2001). Nowadays, saliva is used effectively for the detection of specific antibodies (i.e., HIV, hepatitis C), hormones, and pharmaceuticals (i.e., drugs of abuse). However, the widespread use of saliva for diagnostics is complicated by the above-reported dynamism and polymorphism that characterizes the salivary proteome. The present and future analytical ability of proteomic techniques to contemporaneously quantify the great variety of possible translational salivary states will inevitably lead to defining "individual salivary profiles." The challenges are to establish the differences between particular polymorphisms or posttranslational modifications connected with diseases and further, to determine whether these differences inhibit or promote the development of specific diseases.

The salivary proteome presents several unique proteins; thus saliva-based diagnostics may provide information complementary to that from blood- and urine-based diagnostics. Since about one quarter of the salivary proteome overlaps with the plasma proteome (Loo et al. 2010), it will be important to establish if disease-linked plasma modifications are reflected in the saliva secreted in order to rely upon noninvasive tests for disease screening, detection, and monitoring.

Indeed, several studies have shown that systemic diseases may affect the human salivary proteome. The salivary biomarkers characterized so far show satisfactory clinical sensitivity and specificity (i.e., good prediction of the patients with the disease and normal values in healthy subjects). An interesting example concerns the detection of low phosphorylation levels of three salivary peptides (statherin, histatin 1, acidic PRPs) in a subset (about 60%) of patients with autism spectrum disorder (Castagnola et al. 2008). A set of salivary proteins has been shown to display a different concentration in children affected by type 1 diabetes compared with healthy subjects (Cabras et al. 2010), i.e., a significant increase of amounts of the short form of S100A9, α-defensins 1–3, and various fragments deriving from P–C peptide paralleled by a decrease of the amounts of P–C peptide, statherin, P–B peptide, and histatins 3, 5, and 6.

Many studies have addressed the early detection of different oral tumors such as oral squamous cell carcinoma (Jou et al. 2010; Shintani et al. 2010; Hu et al. 2008) and head and neck squamous cell carcinoma (Dowling et al. 2008; Ohshiro et al. 2007; de Jong

et al. 2010; Chen et al. 2002). Because the research groups used different proteomic platforms, it is not surprising that the results may differ. Jou et al. (2010), using 2D electrophoresis followed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS, found the level of salivary transferrin to be increased in patients with oral squamous cell carcinoma. Shintani et al. (2010), using surface-enhanced laser desorption/ionization TOF analyses, showed an increase in the level of a truncated form of cystatin SN. Hu et al. (2008), using both liquid chromatography–MS/MS and 2D electrophoresis, found increased amounts of protectin, catalase, profilin, and S100A9 for oral squamous cell carcinoma. The salivary proteome of patients affected by primary Sjögren syndrome has also been extensively investigated (Giusti et al. 2007; Ryu et al. 2006; Peluso et al. 2007; Fleissig et al. 2009). The principal platform utilized was based on 2D electrophoresis followed either by MALDI-TOF MS or by electrospray ionization MS/MS analyses of the tryptic protein digests. Some controversial results were, however, obtained. For instance, whereas Giusti et al. (2007) found the level of salivary α-amylase decreased, Fleissig et al. (2009) found it increased, suggesting that the search of new biomarkers has to be performed in a large number of patients and validation of most of the results reported is necessary.

Even though it is a demanding task, for widespread introduction of saliva-based diagnostics it is mandatory to define proper reference proteomes and further, to standardize analytical procedures. As for blood and urine samples, the time and site of specimen collection, as well as the definition of specific treatments for sample stabilization, need to be established. Thus, high-throughput proteomic approaches, applied under standardized conditions, will result in the introduction of simple, sensitive, and specific analytical procedures to demonstrate salivary biomarkers in clinical practice.

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