Introduction

The key to understanding the mechanism of salivary secretion is in identifying the individual components of the secretory process and in visualising how these components fit together. Difficulties in understanding arise because there are a lot of components and more than one way of assembling them.

Salivary secretion may be defined as “A unidirectional movement of fluid, electrolytes and macromolecules into saliva in response to appropriate stimulation”. This simple statement encapsulates most aspects of the secretory process and points towards what is probably the most important and topical aspect of secretory physiology. The critical words in the statement are stimulation, fluid, electrolytes, macromolecules and unidirectional.

‘Stimulation’ encompasses the neural mechanisms that integrate the response to salivary stimuli, such as taste, and the processes within each salivary acinar cell that communicate between the nervous system and the secretory machinery.

‘Fluid’, ‘electrolytes’ and ‘macromolecules’ describe defining components of saliva. The unique viscoelastic and antibacterial properties of saliva stem largely from its protein component. The electrolyte content adds acid buffering and remineralization capabilities and the fluid vehicle dilutes and clears the oral environment.

The only way to achieve a ‘Unidirectional’ movement of fluid, electrolytes and macromolecules across a cell is if one end of the cell behaves differently from the other. It has always been obvious that one end of a secretory acinar cell looks different from the other, what is much less obvious is that the polarity extends to every aspect of cell function, including the control of secretion.
Stimulation

Neural Control of Salivation

Figure 1 The first step in stimulus-secretion coupling is release of a neurotransmitter.

Afferent pathways:
taste; facial (VII) and glossopharyngeal (IX) nerves to solitary nucleus in the medulla. Also input from higher centres in response to smell etc.

Efferent pathways:
Parasympathetic; sublingual and submandibular from facial nerve via submandibular ganglion. Parotid from glossopharyngeal via otic ganglion.
Sympathetic post ganglionic from cervical ganglion of sympathetic chain.

The neural control of secretion is outlined in figure 1. The primary stimulus for salivation is taste [1] and afferent input is carried to the solitary nucleus in the medulla via the facial (VII) and glossopharyngeal (IX) nerves. Input from other senses, such as smell and sight are also integrated in the solitary nucleus. Parasympathetic efferent pathways for the sublingual and submandibular glands are from the facial nerve via the submandibular ganglion and for the parotid gland from the glossopharyngeal nerve via the otic ganglion. These pathways regulate fluid secretion by releasing acetylcholine (ACh) at the surface of the salivary gland acinar cells. Macromolecule secretion is regulated by noradrenalin (NorAd or norepinephrine, US) release from sympathetic nerves. Sympathetic post ganglionic pathways are from the cervical ganglion of the sympathetic chain. The division between parasympathetic and sympathetic control of different aspects of the secretory process is blurred slightly because parasympathetic nerves may also release peptides, such as substance P and Vasoactive Intestinal Polypeptide (VIP) and also, NorAd will also bind to Ca\(^{2+}\)-mobilising a adrenergic receptors [2].
Second messengers carry the secretory stimulus from the nerves into the secretory cells and provide a flexible coupling between the intracellular and extracellular environments with built in amplification. Amplification is one of the most significant aspects of 2nd messenger signalling because it transduces a very small extracellular stimulus into a large intracellular event [3].

As shown in figure 2, fluid secretion is activated by binding of ACh to muscarinic M3 receptors, macromolecule secretion by binding of NorAd to β adrenergic receptors. Both of these receptors belong to the seven membrane-spanning domain G-protein-linked receptor superfamily. Ligand binding to members of this family of receptors causes activation of an associated heterotrimeric G-protein by replacement of bound GDP with GTP. The activated a-subunit of the G-protein dissociates from the βγ subunits and in turn activates a target enzyme [4]. The target enzyme in fluid secretion is phospholipase C (PLC, activated by Gaq) and in protein secretion adenylate cyclase (activated by Gas). The G-protein a subunit is self inactivating because it has an intrinsic GTPase activity. Once GTP is hydrolysed to GTP the a subunit and the enzyme it has activated switch off again. Nevertheless, the relatively slow rate of GTP hydrolysis means that a single activated target enzyme can process many molecules of substrate before it inactivates.
Adenylate cyclase and cyclic-AMP

Figure 3 The third step in macromolecule stimulus-secretion coupling is production of cAMP

Adenylate cyclase, activated by Ga<sub>s</sub> converts ATP into cAMP.

The next and all subsequent steps in macromolecule secretion are regulated by cyclic-AMP (cAMP) which is the product of adenylate cyclase (figure 3). Cyclic AMP was the first 2<sup>nd</sup> messenger to be identified, in fact the term ‘2<sup>nd</sup> Messenger’ was coined to describe the actions of cAMP. All of the activities of cAMP are mediated through protein kinase A (pKA or cAMP-dependent protein kinase). At rest, pKA is a tetramer composed of 2 catalytic subunits and 2 regulatory subunits. When cAMP binds to pKA, the catalytic subunits separate from the regulatory subunits and become active [4]. Protein kinase A (as its name suggests) phosphorylates proteins, not the proteins that comprise the macromolecule component of saliva but rather the cellular proteins responsible for its synthesis and secretion. Phosphorylation is a very common mechanism of upregulating the activity of cellular proteins. A characteristic of cAMP dependent cellular processes is that upregulation depends not on increased activity of a single enzyme or process but rather on increased activity of many processes. Downregulation of cAMP dependent processes, including macromolecule secretion, is accomplished by a reduction in cAMP levels mediated by the enzyme cAMP phosphodiesterase [4]. Phosphodiesterase activity is itself subject to many regulatory factors, including G-protein coupled receptor activation.

Phospholipase C, Inositol 1,4,5 trisphosphate and Calcium

The stimulus for fluid secretion, initiated by binding of ACh to muscarinic M3 receptors and activation of G-a<sub>q</sub> and PLC, continues with the soluble product of PLC activity inositol 1,4,5 trisphosphate (IP<sub>3</sub>) [4], see figure 4. IP<sub>3</sub> acts by binding to IP<sub>3</sub> receptors on endosomes, such as the endoplasmic reticulum (ER), and releasing the Ca<sup>2+</sup> stored within. The Ca<sup>2+</sup> content of the ER is maintained at a much higher concentration (~1mM) than that of the cytoplasm (~100nM) by Ca<sup>2+</sup> ATPase activity so that activation of a Ca<sup>2+</sup> channel is sufficient to raise cytosolic Ca<sup>2+</sup> activity by
diffusion from the Ca\(^{2+}\) stores. IP\(_3\) receptors are Ca\(^{2+}\) channels, activated by IP\(_3\) binding [5].

**Figure 4** The third step in fluid and electrolyte stimulus-secretion coupling is an increase in intracellular Ca\(^{2+}\) activity.

IP\(_3\) receptors are also sensitive to cytosolic Ca\(^{2+}\) activity and stay open for longer when [Ca\(^{2+}\)]\(_i\) is raised. This property of the receptor can dramatically enhance the Ca\(^{2+}\) mobilising properties of IP\(_3\) by positive feedback or Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) [5]. The Ca\(^{2+}\) signal may be further amplified by Ca\(^{2+}\) release through ryanodine receptors, a second Ca\(^{2+}\) channel also present on the ER of acinar cells [6]. Ryanodine receptors are also Ca\(^{2+}\) sensitive and contribute to CICR. The sensitivity of ryanodine receptors to Ca\(^{2+}\) may be ‘set’ by the cytosolic concentration of cyclic ADP ribose, a product of βNAD produced by ribosyl cyclase regulated by cyclic GMP and possibly Nitric Oxide levels [6]. The Ca\(^{2+}\) signal is therefore actively propagated through the acinar cell by an explosive release of Ca\(^{2+}\) from stores, triggered by IP\(_3\) amplified by Ca\(^{2+}\) and carried by both IP\(_3\) and ryanodine receptors [7] (figure 5).
In addition to mobilising stored Ca\(^{2+}\), the secretory process can also utilise extracellular Ca\(^{2+}\). Ca\(^{2+}\) influx across the plasma membrane is stimulated by depletion of the intracellular Ca\(^{2+}\) stores by a mechanism that is still poorly understood, but which probably depends on conformational coupling between the IP\(_3\) receptors and plasma membrane Ca\(^{2+}\) influx channels [8]. Downregulation of the Ca\(^{2+}\) signal depends mainly on Ca\(^{2+}\) ATPase activity to pump the Ca\(^{2+}\) back into the stores or out of the cell.

**Macromolecules**

Proteins are synthesized inside secretory vesicles by ribosomes (R). Secretory vesicles mature and are stored until a secretory stimulus is received.
Macromolecules cannot cross the plasma membrane. At first sight, this might seem to be an insurmountable problem for a protein secreting cell but the secret to protein secretion is to synthesize proteins for export within endosomes (figure 6). Topologically at least, these proteins are never inside the cell and so do not have to cross the cell membrane to get out. Proteins are secreted when the containing vesicle fuses with the plasma membrane in the process of exocytosis.

Synthesis of secretory proteins begins with gene transcription and manufacture of messenger RNA to carry the sequence information from the nucleus to ribosomes in the cytoplasm. Secretory proteins start with a ‘signal sequence’ which targets the developing polypeptide to the ER where it is N-glycosylated and folded into the correct three-dimensional structure. Small membrane vesicles carry proteins from the ER through several layers of the golgi apparatus for additional processing and ‘packaging’ for export. Proteins move by default onwards from the ER; those destined to remain in the cell contain specific ‘retention sequences’ to segregate them from secretory proteins. Secretory proteins are concentrated within golgi condensing-vacuoles and stored in secretory vesicles. As these mature they are transported close to the apical membrane. In response to a secretory stimulus, secretory vesicles fuse with the plasma membrane and discharge their contents outside the cell [1, 9].

The secretory process may be divided into four stages. Synthesis, segregation & packaging, storage and release. Each of these stages is regulated by phosphorylation of target proteins by cAMP dependent pKA.

Therefore an increase in cAMP stimulates
- transcription of genes for salivary proteins (e.g. PRP’s).
- posttranslational modification (e.g. glycosylation)
- maturation and translocation of secretory vesicles to the apical membrane.
- exocytosis.

Thus, an increase in the level of cAMP within the cell will stimulate every step involved in protein secretion [2, 10] (figure 7).
The role of cAMP in regulation of exocytosis in salivary acinar cells is unusual if not unique. Exocytosis is much more commonly regulated by \( \text{Ca}^{2+} \), even in the functionally very similar pancreatic acinar cells [11]. Both second messengers have some regulatory function in all protein secreting cells, but only in salivary glands does cAMP have such a central role. The molecular components of exocytosis have been extensively studied in other cell types and the key players, soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) are also present in salivary gland acinar cells. It seems unlikely therefore that there is a fundamental difference in the mechanism of exocytosis between salivary gland cells and other cell types. Secretory vesicles have SNAREs (v-SNARES) which recognise plasma membrane SNAREs (t-SNARES) and the two form tight complexes that link the two membranes and mediate the three steps in regulated exocytosis; docking, priming and fusion [10]. In neuronal cells, for example, secretory vesicles are docked and primed and await a \( \text{Ca}^{2+} \) signal to trigger exocytosis. Perhaps in salivary gland acinar cells, secretory vesicles wait for a secretory stimulus at an earlier cAMP-dependent ‘brake’ point [10] (figure 8).
Exocytosis occurs in three stages; docking, priming and fusion. The fusion process itself is Ca\(^{2+}\) dependent. However, earlier stages of the process, e.g. ‘docking’ could be cAMP dependent. In salivary gland cells, this step is the rate limiting ‘brake’ point in exocytosis.

Not all secreted proteins originate in salivary gland cells. Saliva also contains plasma proteins, for example the immunoglobulin, IgA. IgA is no more able to cross the plasma membrane than any other protein and so crosses acinar cells in a membrane vesicle. Receptors for IgA on the basolateral membrane of the acinar cells bind IgA which is taken ‘within’ the cell by endocytosis. Following transcytosis of the vesicles containing IgA, the immunoglobulin is released into the saliva by exocytosis [12] (figure 9).

Polymeric IgA and IgM are transported across salivary gland cells by the polymeric immunoglobulin receptor (pIgR). The pIgR binds its ligand at the basolateral surface and is internalized into endosomes. Here it is sorted into vesicles that transcytose it to the apical surface. At the apical surface the pIgR is proteolytically cleaved, and the large extracellular fragment is released together with the ligand.
Fluid and electrolytes

Fluid secretion is inevitably a process with multiple steps because biological systems cannot actively transport fluid as such. The only way of moving fluid rapidly across a tissue is by osmosis. Therefore, as shown in figure 10, fluid secreting tissues, including salivary acinar cells, concentrate electrolytes by active transport and the concentration gradient forces water to move.

Acinar cells use active transport to increase concentrate Cl− concentration inside the cell so that activation of an apical membrane Cl− channel allows Cl− to leave down its electrochemical gradient into the lumen of the acinus. Na+ crosses the acinar cells to maintain electroneutrality and the movement of Na+ and Cl− create the osmotic gradient across the tissue and water follows. The pivotal step, the single step that determines whether or not a cell is secreting is activation of the apical membrane Cl− channel. This step is regulated by increased [Ca2+]. A cell wide increase in [Ca2+] will also activate the basolateral K+ channel which keeps the membrane potential at a high negative value and thus preserves the driving force for Cl− efflux.

Electrolyte led fluid transport movement is always isotonic. Once isotonicity is reached, there is no additional driving force for water movement. The ability of salivary glands to generate an hypotonic saliva lies with the striated ducts. Striated duct cells pump electrolytes from the primary saliva by

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Figure 10 Fluid secretion follows electrolyte secretion.

The Na+/K+ ATPase makes direct use of ATP to pump Na+ out of the cell and create an inwardly directed Na+ gradient. This energises the Na+/K/2Cl− cotransport system (1) which in turn concentrates Cl− above its electrochemical potential (2). Increased [Ca2+], opens the Ca2+-dependent K and Cl channels and Cl crosses the apical membrane into the lumen of the acinus (2). Na+ follows Cl− across the cell to maintain electroneutrality and the resultant osmotic gradient moves water (3).
active transport. At first sight, it might seem that will simply reverse the secretory process, but the striated ducts are impermeable to water, so there can be no osmotically driven water reabsorption. The basic outline of this secretory process was identified as the ‘two stage hypothesis’ by Thaysen et al in 1954. The fluid secretory process in the acinar cells has a much greater capacity than the electrolyte reabsorptive process in the ducts. This is why the composition of saliva changes with flow rate. At low, resting, flow rates, saliva moves slowly through the ducts and the striated ducts are able to substantially modify the composition of the saliva. At high, stimulated, flow rates, the saliva passes rapidly through the ducts with little alteration. The composition of saliva at high flow rates more closely resembles that of the primary saliva produced by the acinar cells.

Figure 11  Bicarbonate secretion

Carbon dioxide inside cells is converted to HCO₃⁻ and H⁺ by carbonic anhydrase. HCO₃⁻ is secreted across the apical membrane of the cell through an anion channel (2). H⁺ are actively extruded across the basolateral membrane by Na⁺/H⁺ exchange energised by the Na⁺ gradient which is created by the action of the Na⁺/K⁺ ATPase (1). If protons were not lost from the cell, carbonic anhydrase would be unable to generate HCO₃⁻.

The secretory process for bicarbonate is similar to that for Cl⁻ inasmuch as bicarbonate is concentrated within acinar cells and released following receipt of a secretory stimulus. Details of the process for bicarbonate are much less well understood than for Cl⁻. In most salivary glands, uptake is probably via a carbonic anhydrase mediated process that depends ultimately on Na⁺/H⁺ exchange and the Na⁺ gradient. Efflux is probably via a bicarbonate permeable channel (figure 11). The Ca²⁺-dependent Cl⁻ channel is bicarbonate permeable and bicarbonate efflux via this channel would be the most simple mechanism for bicarbonate secretion. Qualitatively at least, bicarbonate secretion will be as effective as Cl⁻ secretion as a mechanism for driving fluid movement [2]. Bicarbonate is one of the electrolytes reabsorbed by the striated ducts and bicarbonate concentration in resting saliva is consequently low. A failure to reabsorb bicarbonate at high flow
rates is the simplest explanation of the much higher bicarbonate concentration in stimulated saliva. Acinar cells secrete macromolecules and fluid and electrolytes. Striated duct cells reabsorb electrolytes. Intercalated ducts lie between the acini and the striated ducts and seem to function more like acinar cells that striated duct cells. They probably make little contribution to protein secretion but may have an important role in bicarbonate and fluid secretion.

**Water channels.**

There are two possible routes for water to take across the cell, either through the tight junctions between the cells (paracellular) or across both the apical and basolateral membranes (transcellular). There has been much discussion as to which is the dominant route and little evidence to distinguish absolutely between them [13]. The intrinsic water permeability of the plasma membrane is very low and both apical and basolateral membranes must therefore contain water channels to facilitate transcellular water transport. Water channels in salivary acinar cells are members of the aquaporin (AQP) family. Aquaporins are membrane proteins composed of 4 subunits, each of which has 6 membrane spanning domains that form a water permeable pore. Aquaporins come in two types, one of which transports only water and another which is also permeable to glycerol. Neither type conducts ions [14]. There are at least 10 mammalian aquaporin isoforms and AQP5 has been localised to the apical membrane of salivary gland acinar cells. AQP5 knockout mice show a 60% reduction in stimulated flow in airway mucosal glands which would suggest that at least this proportion of water flow is transcellular [15].

**Unidirectional**

![Figure 12 Histological polarity](image)

Acinar and striated ducts are very obviously polarised. Acinar cells (A) have a high density of secretory vesicles at the apical pole (1) and striated duct cells (B) have basal infoldings and a high density of mitochondria (2).
Under any normal circumstance the secretory process works only one way. The unidirectionality of secretion is achieved by the barrier function of the acinar and duct cells in separating blood from saliva and, at a cellular level, by polarisation of structure (figure 12) and function. Every cell type involved in salivary secretion is polarised in one way or another. Acinar and duct cells are connected together by tight junctions, which also form the division between the apical membrane which faces into the lumen of the gland and the basolateral membrane which faces the blood. The different properties of these two membranes are fundamental to the polarisation of cell function necessary for unidirectional secretion.

Striated ducts are so called because in longitudinal section, their basolateral side has a striped appearance. The stripes are caused by many infoldings of the basal membrane, crammed full of mitochondria (figure 12). A high density of mitochondria, close to the plasma membrane is usually indicative of primary active transport, in this case the Na⁺/K⁺ ATPase. The most obviously defining feature of acinar cells is the apical pole of the cell, densely packed with secretory vesicles. From a functional perspective, the apical pole of the acinar cells is where all the most critical events occur. Secretory vesicles are directed by the actin cytoskeleton towards the apical pole of the cell and exocytosis occurs almost exclusively at the apical pole.

The key event in fluid secretion, activation of the Ca²⁺-dependent anion channel also occurs at the apical pole. There is growing evidence to indicate that the controlling Ca²⁺ signal originates at the apical pole of the cell and under certain circumstances, may be restricted to this pole of the cell [16] (figure 13).

![Figure 13 Local Ca²⁺ signals](image)

*Sequential Ca²⁺ image maps taken over 20s of a single mouse submandibular acinar cell loaded with the Ca²⁺ sensitive dye fura-2 and stimulated with 20nM ACh. The Ca²⁺ signal manifests only at the apical pole, at the bottom of the image. Each Ca²⁺ response lasted < 500 ms*

Calcium signals are very ‘expensive’ in terms of the metabolic cost of holding [Ca²⁺] at nanomolar levels and, because sustained elevated Ca²⁺ levels are cytotoxic, potentially dangerous to the cell. Spatially restricted Ca²⁺ signals may be an elegant resolution to both of these problems. It has proved very challenging to elucidate the mechanisms underlying local Ca²⁺ signals, not least of all, how the cell stops the signal from propagating across the cell by CICR. A partial answer to this question may simply be that spatially restricted Ca²⁺ signals are very brief. The apical origin of the
Ca\(^{2+}\) signal is slightly odd, given that the ER, which is thought to be the primary source of stored Ca\(^{2+}\), is almost exclusively distributed through the basolateral region of the cell. Secretory vesicles, which have a very obvious apical location, have been proposed as a possible Ca\(^{2+}\) store [17].

Figure 14  Ca\(^{2+}\) tunnelling

An alternative, and more widely accepted, mechanism depends on the reticulate nature of the ER. In this model, Ca\(^{2+}\) is stored at the basolateral pole of the cell and ‘tunnels’ through the ER to the apical pole where it is released [18] (figure 14). This last mechanism also offers the intriguing possibility that the actin cytoskeleton that shapes the dynamic structure of the ER and regulates transport or secretory vesicles to the secretory pole of the cell, might also have a role in the control of fluid secretion [19].

**Pharmacological control of fluid and electrolyte secretion**

Every step of stimulus-secretion coupling is potentially vulnerable to dysfunction under pathological conditions. The challenge for secretory physiologists studying autoimmune xerostomic conditions, such as Sjögren’s syndrome, is to find the points at which the immune response could damage the secretory process [20, 21]. There is a growing body of evidence to suggest that severe glandular atrophy is the end-stage of Sjögren’s syndrome and that glandular hypofunction occurs much earlier in the pathology of the condition. Contrarywise, every step of stimulus-secretion coupling is
a potential point for therapeutic intervention. Stimulation of fluid secretion by activation of muscarinic receptors is one of the more obvious and accessible entry points. Pilocarpine, a naturally occurring alkaloid, is probably the best known therapeutic cholinomimetic agent and is distributed under the trade name ‘Salagen’. Cevimeline (evoxac) is another cholinomimetic agent used therapeutically in the US which may have a higher specificity for muscarinic M3 receptors than pilocarpine and potentially, therefore fewer side effects. The side effects of therapeutic application of salagan (15-30mg/day) or evoxac (90 mg/day), sweating etc. are usually tolerated in preference to dry mouth (see chapter 4). ACh itself is of little use therapeutically because it is so rapidly metabolised. Saliva production may be blocked by cholinergic receptor antagonists, such as atropine, which compete with ACh for muscarinic receptors and prevent the effects of parasympathetic stimulation on fluid and electrolyte secretion. The most common cause of dry mouth is as a side effect of xerogenic drugs used to treat other conditions.

Microfluorimetry, electrophysiology and molecular biology are proving to be a powerful combination with which to study secretory mechanisms. For example: fluorescent probes for subcellular components, such as the ER, mitochondria or the nucleus may be used to visualise these organelles in living cells and determine their role in signal transduction. Caged agonists or second messengers may be used to provide precise spatial mapping of intracellular responses and so further refine our understanding of cellular polarisation. Genes for key elements of the secretory machinery can be linked to fluorescent markers and expressed and visualised in isolated acinar cells by transient transfection. Gene knockouts can help pinpoint the function of specific proteins, such as AQP5 or ACh M3 receptors. There is now great scope and great potential in turning these powerful techniques towards understanding glandular pathologies.
References


