Calcium signalling in tissue: diversity and domain-specific integration of individual cell response in salivary glands

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Summary

Organ function requires coordinated multicellular activities, which may require proper control of cell signalling dynamics at the supracellular level. By using high-speed confocal microscopy, we studied how calcium signalling is organised in the dissociated rat parotid gland.

Salivary gland function is accomplished primarily by the compartmentalized epithelial domains, acini and ducts, the former involved in the production of primary saliva and the latter involved in its modification. Upon muscarinic stimulation with carbachol, both domains showed an increase in intracellular free calcium concentration ([Ca2+]i) with distinctive spatiotemporal kinetics, as indicated by the fluo-3 fluorescence. Acini responded initially, and the ducts followed with a time lag of more than 0.3 second. Cells comprising an acinus responded synchronously, whereas those in the ducts responded heterogeneously with respect to the latency period, magnitude of response and the requirement of extracellular calcium to raise [Ca2+]i. ATP also elicited a non-synchronous [Ca2+]i response in the duct domain, under a pattern different from that of carbachol. The synchronous oscillations seen in the acinar domain were made asynchronous by octanol, an agent known to inhibit gap-junction function. Accordingly, a gap junction component, connexin 32, was immunolocalised predominantly between the acinar cells. Moreover, expression of the type 2 inositol (1,4,5)-trisphosphate receptor [Ins(1,4,5)P3R] was homogeneous in the acinar domain but heterogeneous in the duct domain. Together, these data suggest that the calcium signalling system in salivary glands is constructed specifically according to the tissue architecture.

Movies available on-line

Key words: Calcium signalling, Salivary gland, Gap junction, Ins(1,4,5)P3 receptor, Epithelia

Introduction

An organ is a mass of diverse cells possessing various morphological and functional identities. The diversity of cells ensures that organs develop a highly elaborate multicellular system to adapt complex physiological demands. An important premise involved in this system is the harmonisation of each cell activity so as to achieve organ function adequately and efficiently. In most organs, the mechanisms underlying this harmonisation remain largely unknown.

Cellular activities in virtually all cell types are, despite tremendous diversities in their expression, regulated by common intracellular signalling systems, and calcium is one important signalling molecule involved in the regulation of diverse cell functions (Berridge et al., 2000). In response to adequate stimuli, [Ca2+]i increases, oscillates and decreases, leading to the activation, modulation and termination of cell function. It is likely that the integration of multicellular activity in the organ is achieved through the regulation of Ca2+ signalling. Although extensive studies have elucidated subcellular mechanisms responsible for Ca2+ signalling, little is known of its regulation at the supracellular level.

The salivary gland is an exocrine organ capable of secreting fluid and macromolecules under the autonomic nerve regulation (Garrett, 1987). It is composed of tubular epithelia that can be divided into two major domains. The distal end is the secretory unit, the acini, where the primary saliva is produced. At the proximal part are the ducts, which modify the primary saliva by absorbing or secreting certain ions, such as Na+, Cl– and HCO3– (Young et al., 1987). By using high-speed confocal microscopy, we previously studied Ca2+ signalling in the rat salivary ducts (Yamamoto-Hino et al., 1998) and acini (Takemura et al., 1999). Millisecond analyses demonstrated that [Ca2+]i responses in the ducts started in some ‘pioneer cells’ and then spread to the neighboring cells, showing asynchrony and heterogeneity (Yamamoto-Hino et al., 1998), whereas those in the acini occurred synchronously (Takemura et al., 1999). These data prompted us to study whether the Ca2+ signalling system in salivary glands is constructed specifically according to the tissue architecture.

In the present study, we analysed details of calcium signalling in the rat parotid gland. We dissociated the glands with collagenase to obtain morphologically and functionally intact preparation of acini and ducts (Segawa et al., 1985). These specimens were loaded with fluo-3AM and stimulated with calcium mobilising agents carbachol (CCh) and ATP in the presence or absence of extracellular Ca2+. Changes in [Ca2+]i of acini and ducts were analysed by high-speed confocal microscopy both at the regional level and at the single...
cell level. The possible role of intercellular communication through the gap junction was studied functionally by octanol pretreatment and morphologically by connexin 32 immunohistochemistry. The distribution of inositol (1,4,5)-trisphosphate receptor type 2 [Ins(1,4,5)P_3R2], a receptor molecule involved in the release of Ca^{2+} from intracellular stores, was also examined.

**Materials and Methods**

**Cell dissociation**

Male Wistar rats (6-10 weeks) were killed by ether anaesthesia and exsanguination. Parotid glands were cut into small pieces and digested with collagenase (CLS4, Worthington Biochemical Corporation, Lakewood, NJ) at 3 mg/ml for 20 minutes at 37°C in Dulbecco’s modified Eagle medium (DMEM) containing 25 mM Hepes with constant shaking and gentle pipetting. Following digestion, the dissociated glands were washed twice with DMEM and then incubated with 10 μM fluo-3AM (Molecular Probes, Eugene, OR) for 20 minutes at room temperature. After washing, the dissociated tissues were resuspended in Krebs-Ringer Hepes (KRH) medium (120 mM NaCl, 5.4 mM KCl, 1.0 mM CaCl_2, 0.8 mM MgCl_2, 11.1 mM glucose, 20 mM Hepes) containing 0.2% bovine serum albumin. For omission of extracellular Ca^{2+}, Ca^{2+}-free KRH was supplemented with 0.2 mM EGTA. The dissociated tissues were able to respond to CCh and ATP for up to 1 hour in KRH at room temperature.

**High-speed confocal microscopy**

The fluo-3-loaded dissociated tissues were placed on the coverslip coated with Cell Tak (Collaborative Biomedical Products, Bedford, MA) (Segawa, 1999). They were viewed with a inverted light microscope (Oz with InterVision software, Noran Instruments Inc., Middleton, WI). For stimulation of the dissociated tissues, 10 μM carbachol (Wako Pure Chemical Industries, Osaka, Japan) or 100 μM ATP (adenosine 5′-triphosphate: Sigma Chemical, St Louis, MO) were added directly onto the tissues. When necessary, pretreatment with octanol (Wako Pure Chemical Industries) was performed at 3 mM for 3 minutes before addition of CCh. The specimens were observed with the plan apo objective lenses ×20, ×40, ×60 with NA 0.75, 1.00 and 1.20, respectively. The plane of approximately 20-50 μm depth from the coverslip was observed. Confocal images were taken every 8-33 milliseconds. Excitation was performed with an Ar/Kr laser at 488 nm and the emission signals were collected through a 515 nm barrier filter. Pseudo-ratio imaging was performed by dividing raw fluorescence images by an image performed with an Ar/Kr laser at 488 nm and the emission signals were collected through a 515 nm barrier filter. Pseudo-ratio imaging was performed by dividing raw fluorescence images by an image immediately before addition of CCh or ATP, representing the resting distribution of fluo-3 fluorescence in the specimens. The pseudo-ratio images were used for the quantitative measurement.

**Immunohistochemistry**

Pieces of parotid glands were fixed with 10% formalin for 1 hour at 4°C, and cut in a cryostat at 20 μm. The sections were pretreated with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 4°C for 1 hour. They were then treated with mouse monoclonal antibodies against Ins(1,4,5)P_3R2 (IgG1, KM1083) (Sugiyama et al., 1994) and connexin 32 (IgG1, Zymed Laboratories, Inc., CA) at 4°C for overnight. Control specimens were incubated solely with BSA-PBS. After washing with PBS, the sections were treated with FITC-labelled anti-mouse IgG1a and RITC-labelled anti-mouse IgG1, mounted with anti-fader reagent Fluoro-Guard (Bio-Rad Lab., CA) and observed under the conventional confocal microscope (Bio-Rad MRC 1024, Nilsson Bio-Rad Lab., Tokyo, Japan). Serial confocal images were taken along the Z-axis at a distance of 1.5 μm and then reconstructed into the ‘through focus’ image by summatizing each image.

**Results**

**Morphology of the dissociated glands**

Digestion of rat parotid glands with collagenase yielded relatively large cell clumps. Scanning electron microscopy of these specimens (Fig. 1) showed that the ducts exhibit the elongated tubular structure, whereas acini reveal the round structure located at the end of the duct segment. A lot of acini were grouped together, so that the overall morphology of acini-duct complex revealed the ‘bunch of grape’-like appearance. Comparable images were obtained by using confocal microscopy with transmitted light imaging (Fig. 2B).

**Changes in [Ca^{2+}]_i in acini and ducts in response to CCh**

Following application of CCh to the tissues, both acinar and ducital segments exhibited a rise in fluo-3 fluorescence intensity, indicating a rise in [Ca^{2+}]_i. Fluo-3 loading was efficiently applied in the outer layers of cell clumps so that the response of acini was defined in those located in the periphery of the specimen. Fig. 2 shows the typical response. Acini were
always the first to respond (0.32-2.5 seconds after CCh; mean=0.76 seconds, n=28), and the ducts followed (0.87-3.1 seconds after CCh, mean=1.70 seconds, n=12) with a time lag of 0.38-2.0 seconds (mean=1.0 seconds, n=12). We did not observe any propagation of Ca²⁺ waves from acini to the ducts. Instead, within the ducts, the rise in [Ca²⁺]i started in some ‘pioneer cells’ and then spread to the neighboring cells (Fig. 2C), as described previously in the ducts of submandibular glands (Yamamoto-Hino et al., 1998).

[Ca²⁺]i response to CCh and ATP in the acini and ducts: analyses at the regional level

The response of acini and ducts to CCh and ATP was analysed by the consecutive application of each reagent in the presence or absence of extracellular Ca²⁺. Acini and ducts exhibited a distinct response pattern. A typical example is shown in Fig. 3 (n=14).

When CCh was applied to the specimens in the absence of extracellular Ca²⁺ (Fig. 3C), [Ca²⁺]i increased in the acinar area but not in the ductal area. However, addition of Ca²⁺ to the perfusion medium resulted in a rise in [Ca²⁺]i in the ductal area (Fig. 3D). After washing the specimen thoroughly with Ca²⁺-free KRH, and adding ATP in the absence of extracellular Ca²⁺, the acinar response was weak or negligible (Fig. 3E). By contrast, the ductal area exhibited a rise in [Ca²⁺]i, although the response was transient and [Ca²⁺]i soon declined. When Ca²⁺ was added to the perfusion medium under these conditions, the ductal area exhibited a rise in [Ca²⁺]i and showed a sustained plateau (Fig. 3F).
When the application protocol was reversed and ATP was applied prior to CCh, the acinar and ductal responses were similar to those described above (not shown). Thus the sequence of application of ATP and CCh did not seem to alter the pattern of acinar and ductal response.

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The magnitude of the response and the latency period differed from one cell to another.

Effect of octanol on the synchronised \([\text{Ca}^{2+}]_i\) response in the acini

10 \(\mu M\) CCh treatments often caused an oscillation in the \([\text{Ca}^{2+}]_i\) response in acinar cells (Fig. 5A,C). The oscillatory
response occurred synchronously among the cells within an acinus. Gap junctions have been shown to be involved in the synchronised [Ca2+]i response in several cell systems (Stauffer et al., 1993; Guerineau et al., 1998). We thus analysed the effects of octanol, a gap junction inhibitor, on the acinar cell response. Following pretreatment of the specimen with octanol for 3 minutes, the response became asynchronous (Fig. 5B,D).

Distribution of connexin 32 and Ins(1,4,5)P3R2 in the acini and ducts
The involvement of gap junctions in the observed [Ca2+]i response was further analysed by immunohistochemistry using connexin antibody. As shown in Fig. 6A, positive immunofluorescence was found predominantly in the acinar area, whereas the ductal area was mostly devoid of positive signals. An exceptional area was the distal end of the intercalated ducts, in which a positive reaction was sometimes detected. In an attempt to discover the intracellular molecule responsible for the supracellular regulation of Ca2+ signalling, we also observed the distribution of Ins(1,4,5)P3R2. Ins(1,4,5)P3R2 immunofluorescence was detected along the apico-lateral area of acinar cells (Fig. 6B). This area corresponds to the intercellular canaliculi, a specialised form of the lumen in the rat parotid acini (Takemura et al., 1999). In the ducts, strong immunofluorescence was observed along the apical area; many cells showed positive signals, but some cells were found to exhibit weak or negative fluorescence. Thus, in contrast to the acini, the distribution of Ins(1,4,5)P3R2 in the acini and ducts. Control specimens treated without primary antibodies did not reveal any specific staining.

Discussion
Exocrine organs function is primarily achieved by the compartmentalized epithelial domains, acini and ducts, which also exhibit characteristic morphological specialisation (Young and van Lennep, 1978; Pinkstaff, 1980). In the present study, we found that the pattern of Ca2+ signalling is also distinct between acini and ducts in the rat parotid gland in response to CCh and ATP stimulation. Acinar cells within an acinus responded synchronously and behaved as a functional syncitium, whereas ductal cells responded heterogeneously, showing diverse signalling kinetics. In addition, the molecular distribution of connexin 32 and Ins(1,4,5)P3R2 was distinct between the two domains. Collectively, these data indicate that the Ca2+ signalling system in the rat parotid gland is organised in parallel with the tissue architecture.

Many investigators have observed the elevation of [Ca2+]i in the acini and ducts of salivary glands in response to CCh and ATP stimulation (Valdez and Turner, 1991; Gromada et al., 1993; Dinudom et al., 1993; Hurley et al., 1993; Soltos et al., 1993; Hurley et al., 1994; Jorgensen et al., 1995; Xu et al., 1996; Lee et al., 1997a; Lee et al., 1997b; Tojyo et al., 1997a; Tojyo et al., 1997b; Bird et al., 1998). As discussed previously, the time course of [Ca2+]i elevation proceeds rapidly, making it difficult to clarify the spatiotemporal details of Ca2+ signalling dynamics in the tissue (Takemura et al., 1999). High-speed confocal microscopy of dissociated glands allowed analysis of the changes in [Ca2+]i of the order of a millisecond, with precise measurement of acinar and ductal responses from the supracellular to the subcellular level.

CCh stimulation caused a rise in [Ca2+]i in the acini before
We did not observe any Ca\(^{2+}\) waves from acini to ducts; thus direct propagation of Ca\(^{2+}\) signals from acini to ducts seems unlikely. 3D propagation of Ca\(^{2+}\) signals out of and into the plane of focus might be another explanation. Since CCh triggers fluid secretion from the acini, it is also possible that acinar fluid elicited the ductal response from the luminal side (Xu et al., 1996; Lee et al., 1997a; Lee et al., 1997b). However, it was noted that the presence of acini was not obligatory to initiate the ductal response (Fig. 3). This indicates that the ductal system has its own intrinsic control system for [Ca\(^{2+}\)]\(_i\) mobilisation. Based on the fact that the Ca\(^{2+}\) response started in certain cells from which the Ca\(^{2+}\) wave then propagated to neighboring cells (Fig. 2), it is likely that these cells act as ‘pioneer’ or ‘pacemaker’ cells to control the regional ductal response (Yamamoto-Hino et al., 1998).

The pattern of Ca\(^{2+}\) response to CCh and ATP in the presence or absence of extracellular Ca\(^{2+}\) was considerably different in acini and ducts (Fig. 3). The acinar domain responded well to CCh but not to ATP, and the responsiveness to CCh did not require the presence of extracellular Ca\(^{2+}\). This indicates that the acinar domain raises [Ca\(^{2+}\)]\(_i\) by the release of Ca\(^{2+}\) from the intracellular pool upon muscarinic receptor stimulation. Purinergic receptor stimulation seems to have little effect on both the intra- and extracellular mechanisms of Ca\(^{2+}\) mobilisation in the acinar domain. By contrast, the ductal domain responded both to CCh and ATP, but in the absence of extracellular Ca\(^{2+}\) the responsiveness to CCh was abolished. This suggests that the observed ductal response to CCh occurs mostly through the entry of Ca\(^{2+}\) from the extracellular fluid.

Alternatively, the release of Ca\(^{2+}\) from the intracellular store might require the presence of extracellular Ca\(^{2+}\) in the case of CCh stimulation. In the same duct, ATP triggered both the release of Ca\(^{2+}\) from the intracellular pool (Fig. 3E) and the Ca\(^{2+}\) entry from the extracellular fluid (Fig. 3F). However, it should be pointed out that the response of individual duct cells varied from one to the other (Fig. 4). This indicates that the intracellular signalling cascade downstream of receptor activation is diverse among the duct cells.

Extensive studies have elucidated that Ca\(^{2+}\) signalling involves a molecular cascade of reactions, including phosphoinositide turnover and the activation of receptors/channels present on the plasma membrane and the intracellular stores, leading to the elevation of [Ca\(^{2+}\)]\(_i\) either by entry of Ca\(^{2+}\) from the extracellular fluid or release of Ca\(^{2+}\) from the intracellular stores (Berridge et al., 2000; Meldolesi and Pozzan, 1998; Michikawa et al., 1996). Our findings suggest that such subcellular mechanisms are not equally activated in the cells of duct domain. Since salivary ducts are known to be composed of heterogeneous cell populations (Sato and Miyoshi, 1988; Sato and Miyoshi, 1998), it is possible that the heterogeneity of Ca\(^{2+}\) signalling reflects the heterogeneity of cell types. Whether this involves the heterogeneity of ER Ca\(^{2+}\) stores (Meldolesi and Pozzan, 1998) remains to be elucidated. Further studies are needed to clarify the biological significance of diverse Ca\(^{2+}\) signalling occurring in the duct domain.

In contrast to the ducts, acini exhibited a synchronous [Ca\(^{2+}\)]\(_i\) response (Fig. 5). Gap junctional communication has
Ca\textsuperscript{2+} signalling in the tissue

been implicated in synchronised intercellular Ca\textsuperscript{2+} signalling (Stauffer et al., 1993; Guerineau et al., 1998). In mammalian salivary glands, the presence of gap junctions between the acinar cells has been shown by electron microscopy (Dewery and Barr, 1964; Hand, 1972; Nagato and Tandler, 1986) and connexin immunohistochemistry (Hirono et al., 1995; Lee et al., 1998; Shimono et al., 2000). Although there are debates as to whether gap junctions are present between the duct cells (Dewery and Barr, 1964; Hirono et al., 1995; Lee et al., 1998), our findings indicate that the ductal domain is mostly devoid of gap junctions and, if present, they are located at the distal end of the intercalated ducts close to the acini (Fig. 6). The immunofluorescence spots observed in the intercalated duct area presumably correspond to the gap junctions between myoepithelial cells (Lee et al., 1998) or intercalated duct cells themselves (Dewery and Barr, 1964; Hirono et al., 1995). Whatever the explanations are, previous data and our studies indicate that the acinar domain is the principal site where gap junctions are present in salivary glands. The abolishment by octanol of the synchronised Ca\textsuperscript{2+} response in the acini (Fig. 5) strongly suggests that the acinar domain acts as the functional syncitium through the gap junction.

Immunohistochemical distribution of Ins(1,4,5)P\textsubscript{3}R2 was homogeneous in the acini but heterogeneous in the ducts (Fig. 6). The distribution of Ins(1,4,5)P\textsubscript{3}R2 in the intercellular canaliculi area of salivary acini has been observed by us (Yamamoto-Hino et al., 1998; Takemura et al., 1999) and by others (Lee et al., 1997a). Heterogeneous expression of Ins(1,4,5)P\textsubscript{3}Rs in salivary ducts has also been noted by our previous study (Yamamoto-Hino et al., 1998). Heterogeneous expression of Ins(1,4,5)P\textsubscript{3}Rs has also been observed in airway epithelia (Sugiyama et al., 1996), kidney collecting ducts (Monkawa et al., 1998) and gastrointestinal epithelia (Matovick et al., 1996), and thus is considered as the common feature in tubular epithelia. Since Ins(1,4,5)P\textsubscript{3}Rs play key roles in the release of Ca\textsuperscript{2+} from the intracellular stores (Berridge et al., 2000; Meldolesi and Pozzan, 1998; Michikawa et al., 1996), the particular tissue distribution of Ins(1,4,5)P\textsubscript{3}R2 is likely to determine the type of Ca\textsuperscript{2+} signalling: either acinar (synchronised) or ductal (non-synchronised).

Our observations indicate that acini and ducts create distinct local Ca\textsuperscript{2+} signalling communities. Since acini and ducts constitute the elementary units of exocrine organs, the organised Ca\textsuperscript{2+} signalling might have some impact on the mechanisms underlying the harmonised organ function. Coordination of Ca\textsuperscript{2+} signalling according to the tissue unit has been observed in the liver (Nathanson et al., 1995; Robb-
Gaspers and Thomas, 1995), and suggested to facilitate diverse cells to perform the integrative, organ-level response. Distinct Ca\textsuperscript{2+} signaling among different domains (i.e. the acini and ducts), may help to develop the versatility needed for the organ to perform its complex functions.

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References


