Visualizing form and function in organotypic slices of the adult mouse parotid gland


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The salivary gland is an established and valued model for studying exocrine gland development (2, 9, 29), protein synthesis and trafficking (5), Ca2+ signaling (3, 12, 30), and fluid secretion (6, 10, 20, 28) in health and disease (33). Previous studies on salivary gland function have primarily used whole animal or enzymatically dispersed acini or single cells. However, imaging cellular and subcellular processes such as Ca2+ signaling or exocytosis in vivo is difficult to resolve and intravital imaging methods require expensive, specialized instrumentation such as a multiphoton microscope. In contrast, conventional live-cell imaging methods in combination with enzymatically dispersed single acinar cells or small acinar clusters have been used extensively to study exocrine secretion and Ca2+ dynamics. However, isolated cells exhibit altered morphology and there is debate as to whether dissociated acinar cells retain normal function (4, 24). In addition, these highly polarized epithelial cells rapidly lose many of their morphological and functional characteristics in primary culture. Other groups have attempted to resolve this by modifying culture conditions or developing cell lines. Although some success has been achieved using immortalized parotid cell lines, no cell line to date recapitulates the native acinar cell.

Recently, a method for primary culture of enzymatically dispersed rat parotid acinar cells that retained the capacity for agonist-induced amylase secretion was reported (11). In these cells, the synthesis and exocytotic release of secretory granules was maintained for up to 48 h. Although a leap forward, dispersed cells rapidly lost polarity and the possibility that ductal cells differentiated into acinar cells could not be excluded. Moreover, these cultures lacked three-dimensional acinar structure and the complexities of salivary gland tissue microenvironment. Therefore, we took a different approach to address this problem. We developed a thin slice preparation of mouse parotid gland fragments and introduced short-term culture methods to advance our understanding of Ca2+ and secretory dynamics in the parotid. Freshly prepared slices retained lobular structure including acinar clusters, ducts, vasculature and autonomic nerve fibers. Because no enzymes were used in the preparation, the complement of cell surface receptors was largely unaffected. Moreover, the thin slice was amenable to optical measurements of Ca2+ dynamics and exocytotic activity using standard wide-field microscopy. Furthermore, short-term culture (24–48 h) of slices preserved subsets of acinar cell clusters that retained typical polarized morphology, subluminal F-actin distribution, Ca2+ signaling, and secretory dynamics largely indistinguishable from freshly prepared slices.

In this study we report a significant advance in developing a salivary gland model system that more closely resembles the intact gland and demonstrate the potential of the parotid slice preparation to study integrative exocrine gland function in health and disease.

Materials and Methods

Preparation of Mouse Parotid Gland Slices

Whole parotid glands were quickly dissected from 18- to 25-g male NIH Swiss Webster mice (Charles River) following euthanasia by CO2 asphyxiation and puncture of the heart. Excess fat was removed with fine forceps and the gland was cut with a razor blade into a few large pieces in slice saline solution containing (in mM) 140 NaCl, 5 KCl, 1 MgCl2, 10 Na-HEPES, 10 glucose, 0.8 thioctare, 0.4 ascorbic acid; pH 7. To stabilize and preserve architecture of tissue fragments for slicing, 2 ml of a 3% (wt/vol) low-temperature gelling point agarose solution (Sigma Chemical, type VIIA) in nominal Ca2+.

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containing slice saline was prepared and warmed to 90°C until the solution became clear. The agarose solution was then maintained at \( \sim 35^\circ C \), and the pieces were embedded. The tissue-agarose mixture was then gelled at 4–8°C and the block trimmed so that minimal agarose (just enough edge to manipulate the slice with fine forceps) remained around the tissue. The block was then glued to the tissue stand of a Vibratome 1000 Classic, and the chamber was filled with ice-cold nominal Ca\(^{2+}\) slice saline. Tissue blocks were cut into 50-\( \mu \)m-thick sections and slices were kept at 25°C in physiological saline until recording. Experiments were performed 1–4 h after slice preparation. The remaining slices were maintained in culture at 37°C and 95% O\(_2\)-5% CO\(_2\) for 24–48 h in RPMI 1640 medium containing 2-mercaptoethanol, sodium pyruvate, HEPS, and 10% fetal bovine serum. All experiments using animals were approved by and carried out in strict accordance with the policies of the University of Toledo Institutional Animal Care and Use Committee and the University Committee on Animal Resources at the University of Rochester and conform to the “Guide for the Care and Use of Laboratory Animals,” National Institutes of Health Publication No. 85-23 (National Research Council, National Academy Press, Washington, DC, 1996).

**Digital Fluorescence and Time-Differentiated Imaging**

Fluorescence or transmitted light images were obtained using a Polychrome IV monochromator-based high-speed digital imaging system (TILL Photonics, Gräfelfing, Germany) ported to a Nikon TE2000 microscope equipped with DIC optics through a fiber optic guide and epifluorescence condenser. For measurement of intracellular Ca\(^{2+}\) concentration, slices were alternately illuminated with 340 or 380 nm light focused onto the image plane with a DM400 dichroic mirror and Nikon SuperFluo \( \times 40 \) oil-immersion objective and fluorescence collected through a 525 \( \pm \) 25 nm band-pass filter (Chroma Technologies, Brattleboro, VT). For experiments in which alternating transmitted light and fluorescent images were collected, a high-speed Uniblitz V535 optical shutter (Vincent Associates, Rochester, NY) was placed in the tungsten lamp illumination path. Pairs of transmitted light and fluorescence images (30- and 50-ms exposure, respectively) were obtained at 2 Hz. Time-differentiated images were generated by subtraction of each transmitted light frame by its preceding frame essentially as previously described. Time-differentiated imaging has been previously validated as a method of visualizing individual zymogen granule fusions (4).

**Immunofluorescence**

Organotypic tissue slices were fixed overnight with 4% paraformaldehyde. Fixed tissue was then rinsed three times in PBS and incubated in tissue permeabilization buffer comprised of PBS containing Ca\(^{2+}\) and Mg\(^{2+}\), 0.3% Triton X-100, and 0.1% bovine serum albumin for 30 min. For immunofluorescent localizations, tissue slices were incubated in blocking buffer (0.2% Triton X-100, 5% goat serum) for 1–2 h to block nonspecific IgG binding sites. Following two washes in PBS, sections were incubated overnight with appropriate polyclonal or monoclonal antisera in PBS-Triton (PBS-Tx) containing 5% goat serum at 4°C. Sections were rinsed in PBS-Tx and incubated for 1 h with Alexa Fluor 488- or Alexa Fluor 555-conjugated anti-monomoclonal/rabbit IgG as needed at room temperature. Anti-tyrosine hydroxylase (TH), anti-vesicular acetylcholine transporter (VAChT) or TUJ, a neuron-specific B-tubulin antibody, were used as sympathetic, parasympathetic, or general nerve fiber markers, respectively. F-actin was visualized by staining with Alexa Fluor 546-conjugated phalloidin (Invitrogen) for 30 min at room temperature. Nuclei were visualized by 4’,6-diamidino-2-phenylindole (DAPI) stain. Following several washes, stained sections were mounted on glass slides with Vectashield mounting medium.

**Confocal and Two-Photon Microscopy**

Images were acquired via a Leica TCS SP5 broadband confocal microscope system (Leica, Mannheim, Germany) equipped with argon and diode-pumped solid-state continuous-wave lasers and Coherent Chameleon XR tunable pulsed infrared laser source and coupled to a DMI 6000CS inverted microscope. Optical sections were obtained by use of a \( \times 10 \) objective [0.4 numerical aperture (NA)], \( \times 10 \) immersion oil objective (1.25 NA), or \( \times 63.0 \) (1.40 NA) oil-immersion objective. Alexa-488 and Alexa-546 dyes were excited by using 488- or 561-nm laser lines, respectively. DAPI was excited using multiphoton laser source tuned to 780 nm. Acquisition of emitted light was optimized via a tunable SP detector. A series of optical sections (0.5 \( \mu \)m thickness) were collected, rendered as projected images, or processed with Leica LAS, Image J (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2004) or Photoshop software packages. Real-time imaging of exocytotic activity using fluid phase fluorescent dye and two-photon microscopy was performed similar to that previously described (22). Briefly, slices were placed in 0.5 mM sulforhodamine B (SRB). For SRB excitation, the picosecond pulsed-IR laser was tuned to 830 nm and focused at the focal plane with a \( \times 63 \) water immersion objective (1.2 NA). Confocal and two-photon microscope studies were performed using resources of the Advanced Microscopy and Imaging Center at the University of Toledo Health Science Campus.

**Electron Microscopy**

For transmission electron microscopy, parotid slices were fixed with 3% glutaraldehyde for 1 h and postfixed for 2 h with 1% osmium tetroxide followed by treatment with saturated uranyl acetate for 1 h. Dehydration was carried out through a graded series of chilled ethanol solutions, with a final wash with 100% acetone. Cells were embedded in Spurr’s resin (Electron Microscope Sciences, Fort Washington, PA) and sections were collected on copper 300-mesh support grids. Sections were stained with uranyl acetate and lead citrate and examined by use of a Philips CM 10 transmission electron microscope.

**Cell proliferation and apoptosis.** Parotid slices were fixed overnight in 4% paraformaldehyde and incubated for 1 h at room temperature with PBS supplemented with 3% BSA, 2% goat serum, 0.7% cold water fish skin gelatin, and 0.2% Triton-X-100. Slices were then incubated overnight with polyclonal anti-cleaved caspase 3 antibody (1:100) or polyclonal anti-Ki67 (1:100) at 4°C. Slices were rinsed in PBS-Tx and incubated for 1 h with Alexa Fluor 488-conjugated anti-rabbit IgG at room temperature. F-actin was visualized by staining with Alexa Fluor 633-conjugated phallotoxin (Invitrogen, Carlsbad, CA) for 30 min at room temperature. Following several washes, stained slices were mounted on glass slides using Prolong mounting medium and stored at 4°C.

The labeling of apoptotic cells was performed using the APO-bromodeoxyuridine (BrDu) TdT-mediated dUTP nick-end labeling (TUNEL) assay kit as described by the manufacturer (Invitrogen, Carlsbad, CA). Briefly, parotid slices were fixed with 4% paraformaldehyde overnight at 4°C. Slices were added to the DNA-labeling solution consisting of TdT enzyme, BrdUTP, and reaction buffer for 1 h at 37°C. At the end of incubation, slices were rinsed and placed into the antibody staining solution consisting of Alexa Fluor 488-labeled anti-BrDu antibody for 30 min, and then 500 μl of propidium iodide-RNase A was added to each sample for an additional 30 min before slices were placed on coverslips.

All images were captured with a Leica TCS SP5 broadband confocal microscope and visualized as projected images.

**Field stimulation of parotid slices.** Agarose-embedded slices were loaded by incubation with 5 μM fura 2-AM. The slice was restrained on a coverslip by use of nylon mesh. The coverslip formed the base of a perfusion chamber. The slice was constantly superfused at a rate of 1 ml/min. Field potential stimulation of endogenous nerves in the slice was accomplished by placing platinum electrodes connected to a
Grass or A-M Systems stimulator (pulse protocol as indicated) into the superfusion chamber. The changes in fluorescence or optical density were recorded via a Till Imaging system as previously described.

**Viral infection of parotid slices.** Agarose-embedded slices were incubated in 35-mm tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 0.5% FBS and penicillin-streptomycin. Recombinant adenovirus encoding green fluorescent protein-tagged ribonuclease was added to the dishes at a concentration of 1–2 X 10^8 plaque-forming units/ml. The infected slices were incubated at 37°C and 5% CO2 for 10–16 h then washed in HEPES-buffered saline solution prior to confocal imaging.

**RESULTS**

Organotypic slices provide tractable model preparations to study complex physiological processes. For example, brain slices have been extensively used to image Ca^{2+} signaling and morphological dynamics in delicate structures such as dendrites (27, 34). Slice preparations have also been used successfully to monitor Ca^{2+} signaling in lung (26), exocytotic and ion channel activity in adrenal gland (7), and perinatal development and function of the pancreas (19).

Recently, we developed a mouse parotid gland slice preparation. By this procedure, slices can be imaged both by transmitted light and by fluorescence following incubation with indicator using a standard inverted fluorescence or confocal/multiphoton microscope.

This preparation has practical advantages over studying isolated cells. For example, no enzymes were used to dissociate the tissue into isolated cells and, thus, the complement of cell surface receptors remained largely unaffected. Additionally, the structural integrity of the tissue was maintained such that cells retained normal cell-cell associations. A parotid slice shown in Fig. 1 demonstrates that the relationships between cells retained normal cell-cell associations. A parotid slice shown in Fig. 1 demonstrates that the relationships between isolated cells. For example, no enzymes were used to dissociate the tissue into isolated cells and, thus, the complement of cell surface receptors remained largely unaffected. Additionally, the structural integrity of the tissue was maintained such that cells retained normal cell-cell associations. A parotid slice shown in Fig. 1 demonstrates that the relationships between acinar, ductal structures, and innervating nerve terminals are maintained.

Maintenance of associations between acinar clusters may be of particular importance because loss of junctional complexes following dissociation may alter the distribution of membrane proteins, such as receptors in individual cells. Consistent with this notion, we found that acinar cells in slices, in contrast to enzymatically dispersed acini, retained polarized morphology over hours to days. Transmitted light images of whole lobules as well as individual acinar units using DIC optics are shown in Fig. 2, a–d. In freshly prepared or 24-h cultured slices (Fig. 2, c and d, respectively), zymogen-containing secretory granules were found mainly at apical and basolateral regions of cells in tightly coupled acinar clusters. Although on average there was some expansion of the area occupied by secretory granules in slices maintained in culture for 24 h, subsets of acini were visually indistinguishable from acini of freshly prepared slices.

The maintenance of polarized distribution of granules was also observed at the ultrastructural level. Figure 2, e–h compares electron micrographs of acinar clusters from freshly prepared slices and slices cultured for 24 or 48 h (j). Luminal structure was also largely preserved at 24 h, as shown by presence of tight junctions.

Another feature indicative of acinar cell polarity is an elaborate subluminal F-actin network. This network has been previously shown to be critical for maintaining secretory function and signaling in exocrine tissue. To test whether normal F-actin distribution was preserved, we treated slices with fluorescently labeled phalloidin, a fungal toxin that selectivity binds to F-actin, and used confocal microscopy to localize F-actin distribution. As shown in Fig. 2, the F-actin signal following 24 h in culture (j) was similar to that for freshly prepared slices (i) with only minor expansion of the lumen and a slightly elevated background fluorescence compared with fresh slices. However, we observed a time-dependent reorganization of this signal. Thus, although a subluminal F-actin signal was still evident, significant luminal dilation and redistribution of F-actin to basal regions of acinar cells was evident in slices cultured for greater than 48 h (data not shown).

Because of the loss of normal morphology of slices over an extended period in culture, we evaluated tissue viability at 0-, 24- and 48-h time points using markers of cell proliferation and apoptosis. To assess whether there were short-term changes in the proliferative capacity of organotypic slices, we used immunofluorescence localization with antibody to Ki67, a nuclear antigen present in all non-G0 cell cycle phases. As shown in Fig. 3, a–c, proliferating cells were rarely observed in freshly isolated or 24-h cultured slices. In contrast, there were many more proliferating cells observed in cultured slices at the 48-h time point. In a parallel set of experiments, TUNEL assays were performed to determine the numbers of apoptotic cells in slices prior to and following culturing. As shown in Fig. 3, d–f, there was a time-dependent increase in the numbers of apoptotic cells compared with total cell number indicated by propidium iodide staining. Similar data were obtained by use of anti-cleaved-caspase 3 antibody (Fig. 3, g–i).

These data suggest that, although there is substantial increase in both apoptotic cells and in the number of unidentified...
proliferative cells in tissue with longer-term culture, there are only modest differences in the turnover of cells within the first 24 h of culture.

**Short-Term Culture of Parotid Gland Slices Preserves Physiological Function**

Having demonstrated that short-term culture of parotid gland organotypic slices retained markers of normal acinar cell morphology, we determined whether function was also preserved. The primary function of this exocrine gland is to secrete fluid and protein in response to autonomic neural input. Secretory activity is controlled by parasympathetic and sympathetic neuron-mediated increases in cytosolic levels of Ca\(^{2+}\) and cAMP. Therefore, to assay functional integrity, we tested the effectiveness of cholinergic and/or adrenergic agonists to induce Ca\(^{2+}\) signals or exocytotic secretory activity using freshly prepared or cultured slices.

**Carbacholamine-Evoked Calcium Dynamics**

To assess Ca\(^{2+}\) signaling, slices were loaded with fura 2-AM and transferred onto glass bottom dishes. The tissue slices were perfused by local application of physiological saline with or without agonist using a small glass tube positioned above the slice and an air pressure driven reservoir system. Fluorescence intensity changes were monitored by live-cell digital fluorescence imaging methods. Although attempts were made to place regions of interest on each cell in a field of view, it was often difficult to accurately distinguish individual cells because they were not always at the same focal plane. In addition, other than avoiding obvious ductal structures, no specific effort was made to conclusively identify specific fura 2-loaded cell types. However, the majority of cells in the slices are serous acinar cells.

It should be noted that for the present study we used an inverted microscope to record signals from cells adjacent to the coverslip while superfusing from the top or side of the slice. This configuration was the same for fresh or cultured slices. Because slices were maintained in minimal volume, bath solutions could be readily exchanged. To address concerns regarding accessibility of agonists, we generally focused on groups of acini that were near the periphery of the slice.

To load slices with a sufficient amount of indicator we found it necessary to adjust our standard loading protocol and treat with higher levels of fura 2-AM (2–5 \(\mu M\)) and for longer time periods (1 h) than that previously demonstrated to effectively load enzymatically dispersed acini (1 \(\mu M, 30\) min). Despite these adjustments and a generally lower level of absolute fluorescence compared with isolated acini, agonist-induced Ca\(^{2+}\) signals were found to be qualitatively similar to signals evoked in enzymatically dispersed acini. To assess whether Ca\(^{2+}\) signaling was preserved in slices maintained in short-term culture, we compared the resting levels and agonist-evoked changes in Ca\(^{2+}\) following maintenance in culture for 24 h. On average, the resting (prestimulus) ratio values of individual cells for freshly prepared and 24-h cultured cells were 0.387 \(\pm 0.006\) ratio units \((n = 238)\) and 0.414 \(\pm 0.005\) ratio units \((n = 346)\), respectively. These values indicated that on average there was a significant elevation of resting Ca\(^{2+}\)
levels after 24 h in culture, perhaps suggesting a diminishment of slice health. An alternative explanation was that the elevated averaged resting Ca\(^{2+}\) level reflected the contribution of a subpopulation of acini that were damaged by slicing and developed altered Ca\(^{2+}\) homeostasis over a period of many hours. We therefore examined the distribution of ratio values by plotting the data in 0.01-unit bins and fit the resulting histograms using a peak detection algorithm (IgorPro, WaveMetrics, Lake Oswego, OR). As shown in the histogram in Fig. 4a, the majority of cells in freshly prepared and in cultured slices exhibited resting ratio values less than 0.4 ratio units and were distributed with Gaussian peak values of 0.330 and 0.350 ratio units, respectively. This indicated that in both fresh and cultured slices the majority of the cells retained the ability to maintain Ca\(^{2+}\) homeostasis. However, following 24 h in culture, a substantial additional number of cells showed elevated resting ratio values distributed around 0.540 ratio units, indicating the emergence of a population of cells that exhibited compromised Ca\(^{2+}\) homeostasis following short-term culture. It was unlikely that time-dependent changes in autofluorescence accounted for the elevated resting ratios. Although there was minimal autofluorescence in both fresh and unloaded slices, we observed on average about a 25% increase in F\(_{380}\) gray value levels at 24 h that remained stable out to 48 h, without significant changes in F\(_{340}\) values. This increase would have little substantive effect to account for the increased resting ratio values because the contribution to the fura signal was minimal and an increase in autofluorescent signal induced by F\(_{380}\) would actually diminish the ratio value.

Next, we characterized the Ca\(^{2+}\) responses induced in slices by the application of the acetylcholine receptor agonist, carbacholamine (CCh). Although there was found to be a greater number of unresponsive cells, or cells that exhibited altered signaling in 24-h cultured slices compared with fresh slices, many of the cells showed functional indexes that were nearly indistinguishable from those of freshly prepared slices. Treatment with acetylcholine or CCh is known to evoke rapid apical-to-basal Ca\(^{2+}\) responses in parotid acinar cells that typically consist of rapid sinusoidal oscillations on an elevated steady-state or “plateau” level. Similarly, in both freshly prepared and cultured slices, the increase in cytosolic Ca\(^{2+}\) concentration of 0.058 ± 0.009, 0.128 ± 0.014, and 0.162 ± 0.016 ratio units, respectively (12 ≤ n ≤ 18). Corresponding responses for slices maintained for 24 h in culture were 0.083 ± 0.012, 0.117 ± 0.010, and 0.191 ± 0.016 ratio units, respectively (15 ≤ n ≤ 18).
To assess more thoroughly the consequences on Ca\textsuperscript{2+}/H1\textsubscript{1001} dynamics of culturing slices, we placed a region of interest on each cell observable in the fields of view to monitor their Ca\textsuperscript{2+}/H1\textsubscript{1001} responses. We then compared both the steady-state (plateau) amplitude of the changes in Ca\textsuperscript{2+}/H1\textsubscript{1001} as well as the Ca\textsuperscript{2+}/H1\textsubscript{1001} oscillation frequencies, in response to application of 10\textsuperscript{-9}M CCh for freshly prepared and 24-h cultured slices. Representative fura 2 traces from individual acinar cells in freshly prepared slices or 24-h cultured slices following continuous bath application of CCh are shown in Fig. 4c.

The average steady-state amplitude evoked in cells of freshly prepared slices was 0.129 ± 0.005 ratio units (n = 179). In comparison, the average amplitude evoked in cultured slices was 0.162 ± 0.004 ratio units (n = 204). On average, there was a significant elevation in the evoked change in Ca\textsuperscript{2+} in slices maintained in culture. As outlined above, the data were plotted as histograms to determine the distribution of responses. The plateau values for fresh and cultured slices were distributed with peaks at 0.110 and 0.130 ratio units, respectively, as shown in Fig. 4d. This analysis revealed that many of the cells in cultured slices retained the plateau characteristics we observed in freshly prepared slices. Thus, despite a broadening in the distribution profile for 24-h cultured slices, there was substantial overlap of the range of plateau values between freshly prepared and cultured slices. As shown in the inset, there was no obvious relationship between resting level and the plateau amplitude for either fresh or 24-h cultured slices.

The frequency of oscillations, which has previously been shown in pancreatic acinar cells to be highly dependent on integrity of the acinar cell cytoskeleton and gap junction communication, was also used as an index of acinar cell function (32). The oscillation frequency in response to application of 1 \mu M CCh was compared between freshly prepared and 24-h cultures slices. The average frequencies of oscillations were 10 ± 0.5 oscillations/min (n = 179) and 7 ± 0.4 oscillations/min (n = 204), respectively. These data indicated a significant reduction in the frequency of oscillations in slices maintained in short-term culture. When the responses from individual cells were plotted as histograms it was revealed that a greater number of cells in cultured slices failed to exhibit oscillations in response to agonist treatment (Fig. 2e). For cells that responded, however, the distribution of oscillation fre-
quencies for fresh and cultured slices generally overlapped. The overlap indicated that a substantial portion of acinar cells responded with oscillatory signatures that were similar to those induced in freshly prepared slices.

Our data indicated that a substantial number of acinar cells in slices could retain functionally intact Ca\(^{2+}\) signaling machinery for nearly 2 days. Consistent with the principle that Ca\(^{2+}\) release is the dominant signal for fluid secretion, we also observed robust apparent volume changes in acini in freshly prepared and cultured slices. Relative changes in acinar area were estimated directly from the transmitted light images. This was achieved by circumscribing the basal border of an acinus in freshly prepared slices. Acini with a high overlap indicated that a substantial portion of acinar cells were damaged and were excluded from subsequent analysis. Compared to granule-rich apical and lateral regions of individual acinar cell clusters, typically comprised of five to six cells, were imaged and exocytotic activity was monitored over a 6- to 8-min time period. The total numbers of exocytotic events were counted frame by frame prior to and during continuous application of cholinergic and/or adrenergic agonist. An example of an acinar cluster imaged in this manner is shown in Fig. 5a. In general, there was little exocytotic activity in slices that were not stimulated. The rate of exocytosis prior to application of agonist for freshly prepared and cultured slices was 0.74 ± 0.13 events·cell\(^{-1}\)·min\(^{-1}\) (n = 30) and 0.42 ± 0.17 events·cell\(^{-1}\)·min\(^{-1}\) (n = 34), respectively. The data indicated that the resting rates of exocytosis were not significantly different between freshly prepared and cultured slices. Acini with a high basal rate of activity (>3 events·cell\(^{-1}\)·min\(^{-1}\)) were assumed to be damaged and were excluded from subsequent analysis.

Application of CCh, Iso, or CCh/Iso induced robust exocytotic responses in freshly prepared slices. As shown by plot in Fig. 5b, the average rates of exocytosis evoked by these challenges were 3.25 ± 0.78, 2.49 ± 0.52, and 5.62 ± 1.20 events·cell\(^{-1}\)·min\(^{-1}\), respectively (6 ≤ n ≤ 9). When tested on slices that were cultured for 24 h, agonist treatment revealed that exocytotic function was largely retained but that activity was on average diminished. The rates of exocytosis evoked by CCh, Iso, or CCh/Iso challenges were 1.38 ± 0.39, 1.58 ± 0.22, and 3.50 ± 0.69 events·cell\(^{-1}\)·min\(^{-1}\), respectively (5 ≤ n ≤ 9). However, the scatter of the data indicated that despite the reduced rate of exocytotic activity on average, a subpopulation of individual acini or acinar cells in cultured slices responded to agonist treatment with exocytotic rates and profiles that were indistinguishable from those of freshly prepared slices. As example, Fig. 6 shows that analysis of the spatial and kinetic profiles of fusion events in representative acini following cotreatment with 1 μM CCh and Iso yielded similar results. Typically, the majority of the exocytotic fusion events mapped to granule-rich apical and lateral regions of individual acinar cells in both fresh and cultured slices. This is shown in Fig. 6, a and b, where masks indicating the sites of fusion events were
placed on tracings of acinar clusters to produce exocytotic maps. Typically, agonist application induced an initial exocytotic “burst” followed by a phase of persistent activity. This burst was not universally evident, since individual cells of acini did not always activate in a synchronous fashion or display this characteristic. Figure 6d gives an example of the kinetic profile of an individual cell (marked by asterisk) of the acinus shown in b. This cell displayed the typical kinetic profile observed in fresh slices. Events per 20 image frames were binned and plotted as histograms.

Two-Photon Excitation Imaging of Exocytosis in Parotid Gland Slices

Sequential compound exocytosis has been visualized in real-time in rat pancreas (22) and guinea pig nasal gland acini (23) by two-photon microscopy. The deeper penetration and reduced scatter associated with two-photon microscopy has an advantage over conventional confocal microscopy in visualizing apical membrane structure in acini. Therefore, we used two-photon microscopy as an alternative method to monitor individual exocytotic events in 200- to 400-μm-thick parotid slices. Following immersion in physiological saline containing 0.5 mM SRB, a fluorescent fluid phase marker, detailed images of acinar structure in slices were readily made apparent. As shown in Fig. 7a, two-photon excitation at 830 nm revealed SRB fluorescence labeling of intercellular spaces. In most slices the fluorescent image remained stable over a time period of 10–20 min. Following stimulation by addition of 10 μM CCh to the bath solution, the formation of primary, secondary, and tertiary exocytotic fusions was visualized. This was achieved by rapid diffusional equilibrium of SRB with the lumen of secretory granules that fused to the plasma membrane or the membrane of previously fused granules. Granule fusions produced stable, long-lived structures that decorated the apical

Fig. 6. Spatial and temporal dynamics of exocytosis using transmitted light microscopy and time-differentiated imaging analysis. DIC images of acini from freshly prepared (a) or 24-h cultured slices (b) were collected at 2 Hz. c: Corresponding maps and histograms of fusion events evoked by continuous application of 1 μM CCh coapplied with 1 μM Iso at 50 image frames are shown. d: Example of the kinetic profile of an individual cell (marked by white asterisk) of the acinus shown in b. This cell displayed the typical kinetic profile observed in fresh slices. Events per 20 image frames were binned and plotted as histograms.

Fig. 7. Spatial and temporal dynamics of exocytotic activity were visualized by 2-photon microscopy. Exocytotic events in parotid acini imaged by 2-photon excitation of the fluid phase dye marker, sulforhodamine B (SRB), added to the bath solution. SRB fluorescence was evident at intercellular spaces and solution surrounding acinar clusters. Granule fusions were visualized as bright long-lived fluorescent spots primarily at apical and lateral borders of acinar cells. Images revealed exocytotic activity at a single image plane of a 400 μM thick slice.
and lateral borders of acinar cells, consistent with that observed in pancreatic acini (31) and our previous data using time-differentiated imaging analysis (8). Example of these structures are shown in Fig. 7b (arrows).

Field Stimulation-Evoked Ca^{2+} Signals in Slices

An advantage of the organotypic slice preparation is preservation of diversity and integration of signaling and function of the parotid gland at the supracellular level. Maintenance of the integrative relationships between specific tissue types in the organ allowed us to the study secretory function in a situation that more completely resembled the gland in vivo. For example, secretory activity in the parotid gland is largely regulated by autonomic nervous system control. Sympathetic and parasympathetic innervations via superior cervical and otic ganglia, respectively, terminate on the basal regions of the acinar cells. Although cholinergic stimulation is the primary signal for fluid secretion, a variety of studies have demonstrated that both sympathetic and parasympathetic stimulation can induce substantial protein secretion.

We presumed that these connections were preserved in the slice. To demonstrate this we visualized the nerve fibers in the slice and gauged their spatial relationship to acini and ducts using confocal microscopy. As shown in Fig. 8, a–c, in a slice costained with TUJ, an antibody against neuron specific β-tubulin (red signal) and Alexa Fluor-488 phallotoxin to visualize F-actin (green signal), there is extensive neural input within the slice preparation. The overlay of fluorescent images revealed that autonomic innervations essentially encapsulate each individual acinus. Innervation of the slice was comprised of sympathetic (Fig. 8d) and parasympathetic (Fig. 8e) input in roughly equal proportions as indicated by labeling with specific antisera for VACHT or TH as markers, respectively.

The preservation of the neural structures suggested that it would be possible to induce neurotransmitter release and subsequent activation of acinar and ductal cell receptor signaling. Field stimulation of slices (stimulus application at 20 Hz unless otherwise indicated) was used to test whether the remnant fibers were functional. As illustrated in Fig. 9, field stimulation of endogenous nerves with platinum electrodes placed in a perfusion chamber evoked a robust Ca^{2+} signal in the acinar cells of the slice. The temporal trace in Fig. 9d shows the profile of the Ca^{2+} signal from an individual acinar cell. These signals were largely inhibited by pretreatment with 10 mM atropine, indicating that they occurred as a result of muscarinic receptor stimulation (data not shown). These data are consistent with activation of parasympathetic input to the gland. We also demonstrated that field stimulation could be used to induce exocytotic activity in slices. An example is shown in Fig. 10.

Adenovirus Infection in Slices

Genetic studies to probe salivary gland function have largely been advanced through the use of transgenic animals or injection of adenovirus vectors into the parotid duct to introduce genes of interest. However, these experiments are relatively labor, time, and cost intensive. Overexpression, knockdown, or knockin studies are potentially useful approaches, but their use has been limited because exocrine acinar cells rapidly lose function when isolated in primary culture. Thus an in vitro system in which one could utilize optical and electrophysiological approaches in combination with genetic manipulation would provide a valuable tool for investigating salivary gland function. Thus these approaches are not suitable to maintain acinar cells in culture long enough for expression of potential genes of interest.

![Image](image-url)
The use of parotid slices, which can be cultured for up to 48 h and retain viability, potentially provides a system where exogenous genes of interest can be introduced and expressed to probe the molecular underpinnings of salivary gland physiology. To test this possibility, we used adenovirus infection to introduce a green fluorescent protein (GFP)-ribonuclease fusion protein construct known to target expression of fluorescent cargo to secretory granules. As shown in Fig. 11, slices can be infected with adenovirus to produce robust expression of exogenous protein within 16 h. As indicated by the fluorescent signal (b) and the overlay with the transmitted light image (c), the fusion protein appeared localized to secretory granules, indicating that synthesis and sorting of granule protein is maintained in cultured organotypic slices. The bright fluorescence indicated that the GFP signal was not appreciably quenched and suggested that the labeled intracellular compartments were not strongly acidic. (The fluorescence of GFP is stable from pH 6 to 10 but decreases at pH < 6; Ref. 25). The moderate acidity of zymogen secretory granules is not unexpected. Whereas the secretory granules of neuroendocrine cells such as chromaffin cells or pancreatic beta cells have an intragranule acidity ranging from pH 5 to 5.5 (13, 14), the intragranule acidity of zymogen granules, in contrast, is estimated at pH 6.5 (1). Thus we are confident that the observation of GFP fluorescence in zymogen granules in situ is not an indication of loss of intragranule pH homeostasis or unhealthy slices.

These data underscore the potential usefulness of this preparation to study salivary gland function by overexpression methods.

**DISCUSSION**

In this study, we demonstrated that the organotypic slice of the adult mouse parotid gland is a useful preparation to study acinar cell morphology and secretory function using a variety of optical approaches. There are several advantages of this approach over the enzymatic digestion of tissue and preparation of isolated cells.

First, tissue slicing is relatively rapid, taking only tens of minutes following gland isolation, and obviates the need for time- and labor-intensive treatment with enzymes such as collagenase (with activity that can vary depending on lot) and mechanical dissociation of the tissue. Second, slicing not only preserves much of the normal three-dimensional associations between acinar, ductal, and myoepithelial cells that comprise the exocrine tissue, but associated connective tissue, autonomic nerve fibers, and microvasculature as well. In addition, 40- to 100-μm-thick slices are well suited for imaging with standard bench-top microscopy techniques because they are typically one to four cell layers thick.

Furthermore, the semi-intact nature of the organotypic slice allows access to study supracellular signaling and complex integrative functions over a variety of cell types in a more physiological context. For example, in slices acinar tissue is exposed as the connective tissue capsule that normally sheaths the tissue is removed en face. This enables efficient loading of cell-permeable dyes and access for fluid phase markers, drugs, or agonists. Importantly, slicing allows for the free exchange of gases and nutrients. Consistent with this notion, we observed that the polarized cell mor-
Phology of thin slices was maintained in short-term culture better than thick slices (>200 μm). We reasoned that acinar cell function in thin slices in short-term culture might also be preserved.

A significant advantage and step forward through the use of the slice preparation was the ability to maintain polarized morphology and secretory function of acinar cells out to 24 to 48 h in short-term organotypic culture. Modifications of the slice culture methods will likely extend slice viability and functionality. Although we did not optimize cell viability, there are a variety of strategies that can potentially improve long-term slice viability. Necrosis might be reduced through the use of a vibratome equipped with diamond knife rather than the featherweight razor blades used for the present study. This would likely reduce tearing or mechanical damage of the tissue and improve cell viability. In some cases, transient heat shock of the tissue following slicing has also been shown to help maintain slice health. Culture conditions may be modified to reduce apoptosis (17). For example, toxins that promote activation of apoptosis such as ammonia or oxidants can be reduced by placing slices on substrate such as a permeable filter membrane, agitating medium, and addition of antioxidants to the medium. Similarly, addition of growth factors or low level, periodic electrical stimulation or addition of factors mimicking neural maintenance of the tissue could be included. We are currently testing these possibilities, as well as the application of this technique to other exocrine tissues.

Fig. 10. Exocytotic secretory dynamics evoked by field stimulation of parotid slice. a: Transmitted light images demonstrating rapid and transient volume changes of an acinus in a parotid slice induced by field stimulus application (10 V, 5 ms, 20 Hz) as indicated by arrows. b: Examples of individual exocytotic events (circles) captured by time-differentiated imaging. c: Individual events were counted, binned, and plotted as a histogram.

Fig. 11. Adenoviral infection of a parotid slice. Transmitted light (a) and fluorescence images (b) of a slice 16 h postinfection with GFP-ribonuclease fusion I protein construct that localizes to zymogen secretory granules. c: Overlay of fluorescence image with transmitted light image demonstrating that a punctate fluorescent signal is primarily seen in apical regions of acini.
example, we recently prepared viable thin slices of mouse submandibular gland and pancreas with only minimal modifications of the slice preparation methods.

The ability to combine optical and electrophysiological methods with genetic manipulation in an in vitro model system should greatly facilitate investigations of fluid and protein secretion in the parotid and other exocrine glands.

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