PHYSIOLOGICAL AND MORPHOLOGICAL EVIDENCE FOR COUPLING IN MOUSE SALIVARY GLAND ACINAR CELLS

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ABSTRACT

Three experimental techniques were employed to examine coupling between acinar cells of the mouse salivary gland. Passage of DC current pulses via intracellular microelectrodes between neighboring cells showed that small ions could be directly passed from one cell to another. Intracellular iontophoresis of the dye Lucifer Yellow CH into a single cell indicated that small molecules could spread by means of intercellular cytoplasmic bridges throughout an acinus and, occasionally, into cells of adjacent acini. Freeze-fracture replicas of acinar cell membranes indicated the presence of gap junctions which were correlated with both electrical and dye coupling experiments. Suggestions are made for the function of direct intercellular exchange in salivary secretory cells. The role of electrical coupling in coordination of the activity of different secretory cell types is discussed as one possible function.

KEY WORDS salivary glands ionic coupling dye coupling gap junctions intercellular communication electrical coupling

Coordinated functioning of systems composed of heterogeneous subunits in the form of different cell types depends upon the degree of communication between such components. Neural systems frequently act to coordinate ensembles of effector cells as in, for example, the well-known “motor unit”. This model is also applicable to other effector systems such as those involved in secretion. Exocrine glands are innervated, but the specifics of functional coordination are not so well worked out as for muscle.

Direct intercellular coupling is another means of coordinating the composite functioning of systems. Prominent here is coupling through gap junctions which has variously been demonstrated to coordinate both electrical (2) and biochemical events (19). Such coupling, in addition to its well-established role in neural systems (2), is frequently observed in epithelia. Electrical coupling between epithelial cells of many types has been reported, including the salivary gland of insects (11, 22, 23), the salivary gland of the snail (18), mammalian liver (28, 6), thyroid gland (14), pancreas (30), and vertebrate stomach (16). Many of these tissues are coordinated exogenously by neural input or endocrine control as well as endogenously coordinated via direct intercellular coupling.

Mammalian salivary glands are thus far known to have only neural input although Petersen (29) has assumed that electrical coupling exists between acinar cells of this gland.

At least three types of evidence may be used to prove the existence of direct exchange of small molecules between adjacent cells. First, electrical coupling, in which DC current is injected into one cell via an intracellular microelectrode and recorded as a voltage deflection in a neighboring cell, implies that small ions can move freely from one cell to another. A second kind of evidence
involves the injection of a marker, often a small molecular weight dye, into one cell and direct observation of its spread to adjacent cells. The third type of evidence is a morphological correlate of coupling. Specialized cell junctions, nexuses or gap junctions, have been correlated with electrotone and dye coupling and are believed to provide the pathway for transmission of small molecules between cells (15, 10, 3). Gap junctions have been observed in acinar cells of the rat salivary gland (8), but they have not been correlated with coupling studies in this gland. We describe here the use of all three techniques to examine coupling in the mouse submaxillary gland.

MATERIALS AND METHODS

Animals used in this study were inbred female mice descended from breeding stocks of the strains, C57BL/ Sn10, AKR, and C3H, obtained from the Jackson Laboratory (Bar Harbor, Maine). They were raised in a controlled environment (temperature, 22°C, and light:dark cycle, 14:10) with food and water available ad lib.

The submaxillary glands were removed after anesthetization with sodium pentobarbital. Small lobes of tissue, ~2 mm in diameter, were gently teased from the gland and pinned to a Sylgard pad. The pad was inserted into a lucite recording chamber similar to that used by Thomas (37) for continuous flow of physiological saline. Saline entered the chamber via a gravity flow system and was removed by aspiration. Both dissections and electrophysiological recordings were performed in physiological saline containing: NaCl, 136.9 mM; KCl, 2.68 mM; CaCl₂, 1.84 mM; MgCl₂, 1.03 mM; NaHCO₃, 11.91 mM; NaH₂PO₄, 0.435 mM; and dextrose, 1 g/l, gassed with a mixture of 95% O₂-5% CO₂. For electrophysiological recordings, the preparation was maintained at 37°C ± 2°C by heating the saline with a nichrome wire coil wrapped around the inlet of the recording chamber. The recording bath temperature was monitored with a thermometer system. Current in the heating coil was manually varied to maintain the bath temperature at 37°C.

Conventional electrophysiological recording, stimulation, and display techniques were used. Glass fiber-filled micropipettes (2 mm outside diameter of stock tubing) were filled with 3 M potassium acetate or KCl (DC resistance = 15 to 50 MΩ). These electrodes were connected with a silver-silver chloride wire to a dual-channel, high input impedance, capacity compensated, unity gain amplifier equipped with a bridge circuit for injecting current. The output of the amplifier was displayed and photographed on an oscilloscope or recorded on a Brush 220 chart recorder (Gould Inc., Minneapolis, Minn.). Electrode penetrations were made under visual control by use of a Wild dissection microscope. Current was monitored using a virtual ground system (25). For critical electrode bridge balance, such as that needed for electrical coupling measurements, the bridge was balanced with the electrode inside a cell. Injection of brief current pulses (<1 ms) of too short a duration to charge the membrane allowed only electrode impedance to be nulled. This method is useful because the time constants of the cell are considerably longer than that of the electrode system. The process was repeated for each value of current injected.

Evidence for the direct passage of a small molecule between cells was obtained by the use of a new fluorescent dye, Lucifer Yellow CH, developed by Walter Stewart of the National Institutes of Health (36). This dye is similar to Procion yellow (L.C.I./Organics/Inc., Providence, R. I.) in characteristics but has a much higher fluorescence yield. Stewart (36) has demonstrated by intracellular staining on both molluscan central nervous system neurons and vertebrate retinal cells that Lucifer does not readily pass through cell membranes but that it will pass between coupled cells in the retina and the crayfish septate axon. Recently, this dye has been used in work on vertebrate central neurons (26) as well as in electrically coupled embryonic cells (4). Bennett et al. (5) have found that Lucifer compares favorably with fluorescein for looking at permeability of intercellular junctions. Intracellular staining in salivary gland cells was obtained by injecting Lucifer Yellow according to the method described for Procion yellow by Kater and Nicholson (17). 20-s DC current pulses of 10 nA were used for iontophoresis of the dye. After dye injection, the gland tissue either was viewed without fixation under a Zeiss compound microscope, or was fixed for 5 min in 4% formaldehyde in phosphate buffer at pH 7.4 and then returned to the physiological saline. Formaldehyde has previously been demonstrated to bind Lucifer to cellular components (36). A Zeiss ICM inverted microscope with epi-illumination from a mercury light source and FITC filter system was used to view and photograph injected glands.

For electron microscopy, the gland tissue was fixed for 1 h in 1.5% glutaraldehyde in 0.1 M cacodylate buffer. After fixation, the tissue was infiltrated with a solution of 20% glycerol in distilled water to prevent the formation of ice crystals during the freezing. Tissue was frozen in Freon 22 (E. I. duPont de Nemours & Co., Wilmington, Del.), cooled and liquefied with liquid nitrogen, and then placed in the vacuum chamber of a Balzers freeze-fracture apparatus (BAF 301) (Balzers High Vacuum Corp., Santa Ana, Calif.) and fractured at a temperature of ~110°C. Replicas of the fractured surfaces were produced by evaporating a thin layer of platinum on the tissue at an angle, giving a shadowing effect, and reinforcing with a layer of carbon evaporated perpendicularly. The replicas were removed from the tissue surface, mounted on copper grids, and viewed through an RCA EMU-4 transmission electron microscope.

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RESULTS

Physiological Evidence for Coupling

Microelectrode penetration into a secretory cell of the salivary glands was signaled by a rapid, negative-going shift in potential. The mean resting potential, calculated from a total of 103 cells in six different preparations, was \(-50 \pm 0.75\) mV (SE). This value for resting potential in salivary gland cells is consistent with those reported by other experimenters (27, 34). Unless a potential was held for at least 30 s, it was not tabulated. It was often the case that potentials \(<-35\) mV showed progressive decrease over the first few seconds of penetration and usually signaled poor impalements of the cells. The most consistent impalements were made when the microelectrode was placed on the surface of a single cell by visual inspection and lightly tapped into that particular cell. Visual impalement of cells also greatly facilitated paired microelectrode impalement of neighboring secretory cells.

Ongoing spontaneous transient fluctuations in membrane potential were frequently observed throughout our study. Of primary importance for the present communication is the fact that when two cells within a single acinus were impaled with separate microelectrodes, spontaneous transient voltage fluctuations were nearly identical in both cells (Fig. 1). Such near simultaneity of activity has been accounted for in other systems (e.g., salivary glands of Helisoma [18]) by synchronous neural inputs to each cell, tight electrical coupling between gland cells, or a combination of these two. Direct innervation of individual salivary secretory cells is reported in the cat submandibular gland (7). Thus, synchronous neural inputs could be responsible for the simultaneous spontaneous activity observed, and direct electrical coupling might serve to augment this effect.

A typical set of records showing the passage of DC current between cells is shown in Fig. 2. This record was obtained by paired microelectrode impalements of salivary gland cells within a single acinus of the mouse submaxillary gland. Intracellular current injection by a bridge circuit produced voltage deflections both in the cell into which current was injected as well as in adjacent cells or even distant cells within the same acinus. Due to the small size of salivary gland cells, paired double microelectrode impalement of individual cells was not possible; therefore, quantitative values expressed in this study have the inherent errors of bridge techniques. Qualitatively, on the other hand, there are several consistent features of this coupling. The most notable of these were that coupling appeared to be restricted to a single acinus and that coupling values decreased with distance from the site of current injection as is characteristic of electrotonic potentials. With careful bridge balancing, adjacent cells showed coupling coefficients \((V_2/V_1)\) as high as 1.00. Within the limits of our technology, no rectification was found in this coupling.

Another feature which precluded precise quantitative analysis of coupling between cells was the
fact that the input impedance of cells seemed to fluctuate with time. This problem was compounded by the fact that, apparently due to secretory material within the cells, the resistance of microelectrodes used for current injection changed considerably even over a period of a few minutes. This shift in electrode resistance while recording intracellularly has been noted in other secretory cells studied in this laboratory. Nonetheless, input impedance values for these cells were similar to those reported elsewhere (29). Again, due to error inherent in bridge technology, quantitative statements are difficult to make. Observed values for input impedance ranged from 3 to 16 MΩ.

Spontaneous voltage fluctuations like those in Fig. 1 were correlated with observations of the type in Fig. 2. Whenever synchronous transients were observed, DC current could be passed between the two impaled cells. Conversely, when nonsynchrony of activity was apparent in a pair of cells, we were never able to observe the passage of DC current from one to the other.

**Tracer Evidence for Coupling between Salivary Gland Cells**

It is generally recognized that DC current is carried between electrically coupled cells by the movement of small ions (2). Several studies have employed tracer dyes to provide another measurement of the movement of molecules between cells (3, 21). We used the dye Lucifer Yellow to support the electrical coupling experiments described above. As previously mentioned, the extent of dye movement throughout gland cells was determined in both living cells and those fixed after injection. Observations on living material, permitting visualization of dye diffusion, were made by rapid transfer of tissue from the injection station to a nearby compound fluorescence microscope. Because dye movement was very rapid, there were usually two or three cells already stained by the time visualization could be achieved. We could then see the dye gradually diffuse into neighboring cells with movement being restricted to a cluster of cells (~15) which appeared to represent a single acinus.

Permanent records of dye movement were best obtained using fixed tissues as shown in Fig. 3. Epi-illumination provided best results for photography due to the thickness of the tissue. Cells filled at different depths in the tissue; however, photographs provide a limited view of any particular dye injection because of limitations in depth of focus. In the example on the right of Fig. 3, only one cluster of cells was filled with dye after a single cell was injected, a situation that was most often observed. The other photograph depicts the somewhat rarer condition in which as many as two or three clusters (likely different acini) were filled with the dye. Also evident in this picture is the more intense staining of nuclei by Lucifer Yellow after fixation, as previously reported for Procion

![Figure 3](image-url)
yellow (17). In all cases, the dye was restricted to intracellular compartments. It is interesting to note that where more than one acinus appeared to be stained, the intensity of the dye was considerably lower, as would be expected if it were partitioned throughout a larger volume. Essentially identical observations were made on living and fixed material, and no problems associated with fixation artifacts were apparent. These results were entirely in accord with the notion of electrical coupling via intercellular channels.

Putative Morphological Basis for Electrical and Dye Coupling

In essentially all previously reported cases of electrical and dye coupling, gap junctions have been implicated as the ultrastructural correlate (24, 15, 10, 3). We have surveyed the salivary glands of the mouse for the presence of gap junctions by freeze-fracture techniques. Fig. 4 provides four examples of the sorts of gap junctions seen in these glands. The area of membrane covered by junctional particles varied from one junctional example to another. One large area (Fig. 4a) was observed where particles appeared to be more loosely packed than in the other, smaller gap junctions.

In addition to variation in area of membrane covered by a single junction, we noticed some variability in particle size (ranging from 6 to 9 nm in diameter) from one example to another. Epstein and Gilula (9) reported a homogeneity of particle size in gap junctions from cultured mammalian cell lines; however, direct comparisons with the present material would require more quantitative determinations.

DISCUSSION

The physiological and morphological evidence presented here demonstrates that acinar cells of the mouse submaxillary gland are tightly coupled, at least within a single acinus. Dye injection experiments further indicate that some amount of coupling may exist between cells of separate acini. These experiments extend the occurrence of coupling in salivary glands to include not only invertebrates (e.g., references 22, 11, 18) but also mammalian salivary glands.

Input impedances measured here were similar to the values reported by other experimenters (29). The close coupling we demonstrated between acinar cells of the mouse submaxillary gland puts these values in a new light. Junctions between cells would be expected to serve as shunt pathways for test current pulses so that the resultant voltage developed is not representative of the majority of the nonjunctional membrane. Input impedance values obtained from cells of intact glands are therefore expected to be considerably lower than, say, those which might be recorded from isolated, individual cells.

The physiological role of coupling in salivary secretory cells has not been established although a number of possible functions may be suggested. Coupling could provide a means for electrotonic spread of a secretory potential evoked in only one cell (12). Alternatively, Kater et al. (18) have suggested that it could serve to coordinate the activity of various secretory cell types to produce a mixed saliva with components in the appropriate proportions. Either of these functions would seem more likely if there were no means of achieving synchronous neural input to acinar cells; however, in the well-studied case of the cat salivary gland, every acinar cell seems to have its own neural input (7). The innervation pattern is very complex. Every cell seems to receive input from the parasympathetic nervous system, whereas the sympathetic nervous system does not seem to have synaptic endings on each acinar cell (33). Thus electrical coupling between acinar cells might play a different role depending on whether the parasympathetic or sympathetic innervation is active in any particular gland. In a recent study by Iwatsuki and Petersen (13), electrical coupling in mouse and rat pancreatic acinar cells was lowered by extracellular iontophoresis of ACh. This finding is consistent with the notion that coupling could serve the function of spreading excitation from the sympathetic nervous system but might not be so important for stimulation via ACh from parasympathetic nerve endings.

Coupling in salivary glands might serve functions other than the electrotonic spread of secretory potentials. For instance, the channels between cells could provide a pathway for exchange of metabolic products. One molecule that might pass between cells is cyclic AMP, which is known to be the intracellular mediator of the response to adrenergic stimulation (31, 32) and is known to pass between coupled heart cells (38). Cyclic AMP is also known to cause secretion of amylase from rat parotid gland (1). Lawrence et al. (19) showed for cultured cells that the response to a specific hormonal stimulus, mediated intracellular-
larly by cyclic AMP, could be transmitted from the usual target cells to cells receptive to a different hormonal stimulus, but also mediated by cyclic AMP, when the two target tissues were in contact and electrically coupled. Again, the possibility that cyclic AMP passes directly between cells indicates that coupling may be important for the distribution and synchronization of sympathetic stimulation of salivary gland acinar cells.

Another function that has been proposed for
direct cellular communication is that it could allow cell-to-cell passage of small molecules that might regulate activities such as growth and differentiation (3, 20, 35). Further research is needed to establish a relationship between any of these proposed roles of coupling and the direct communication that we have demonstrated in salivary gland acinar cells.

The studies presented here represent a qualitative analysis of coupling via gap junctions; however, both dye injection results and freeze-fracture analyses of gap junctions suggested that quantitative variation might occur in coupling of mouse submaxillary acinar cells. The use of inbred strains of mice to investigate coupling between salivary gland cells makes possible the comparison of coupling characteristics between animals of different genotype. We plan to examine genetic variability in salivary cell coupling in future experiments.

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Note added in proof: Since this article was submitted for publication, a study by M. G. Hammer and J. D. Sheridan (1978. J. Physiol. (Lond.). 275:495) demonstrating coupling in rat salivary gland has appeared.

REFERENCES

1. BOOGAH, A., and M. SCHRAMM. 1965. The function of 3'5' cyclic AMP in enzyme secretion. Biochem. Biophys. Res. Commun. 18:452-463.
2. BESSELL, M. V. L. 1966. Physiology of electrotonic junctions. Annu. N. Y. Acad. Sci. 137:509-530.
3. BESSELL, M. V. L. 1973. Function of electrotonic junctions in embryonic and adult tissues. Fed. Proc. 32:65-75.
4. BESSELL, M. V. L., M. E. Sxia, and D. C. SPRAY. 1978. Permeability of gap junctions between embryonic cells. J. Cell Biol. 75:644 (Abstr. 1).
5. BESSELL, M. V. L., M. E. Sxia, and D. C. SPRAY. 1978. Permeability of gap junctions between embryonic cells of Fundulus, a reevaluation. Dev. Biol. In press.
6. BOREK, C., S. HAGIWARA, W. R. LOWENSTEIN. 1969. Intercellular communication and tissue growth. IV. Conductance of the membrane junctions of normal and cancerous cells in culture. J. Membr. Biol. 1:274-293.
7. CREED, R., E., and J. A. F. WILSON. 1969. The latency of response of acinar cells to nerve stimulation in the submandibular gland of the cat. Acta J. Biol. Sci. 47:135-144.
8. DEWET, M. M., and L. BARK. 1964. A study of the structure and distribution of the nexus. J. Cell Biol. 23:553-585.
9. EPSTEIN, M. S., and N. B. GLIHA. 1971. A study of communication specificity between cells in culture. J. Cell Biol. 56:707-707.
10. GIHLA, N. B., O. R. REEVES, and A. STEINBACH. 1972. Metabolic coupling, ionic coupling, and cell contacts. Nature (Lond.). 235:262-265.
11. GINSKOVSK, B., S. R. H. HOUSE, and E. M. SILKESLY. 1974. Conductance changes associated with the secretory potential in the cockroach salivary gland. J. Physiol. (Lond.). 236:727-731.
12. HOUSE, C. R. 1975. Intracellular recording of secretory potentials in a "mixed" salivary gland. Experientia (Basel). 31:904-906.
13. IWAI, N., and O. H. PETERSEN. 1978. Pancreatic acinar cells: acetylcholine-evoked electrical uncoupling and its ionic dependency. J. Physiol. (Lond.). 274:81-96.
14. JAMAKRANKOVA, A., and W. R. LOWENSTEIN. 1968. Intracellular communication and tissue growth. Ill. Thyroid cancer. J. Cell Biol. 38:556-560.
15. JOHNSON, R., and J. D. SHERIDAN. 1971. Junctions between cancer cells in culture. Ultrastructure and permeability. Science (Wash. D. C.). 174:717-719.
16. KANN, Y., and Y. MATSU. 1968. Cellular uncoupling in cancerous stomach epithelium. Nature (Lond.). 218:755-757.
17. KATER, S. B., and C. NICHOLSON. 1973. In Intracellular Staining in Neurobiology. Springer-Verlag, New York. 307-325.
18. KATER, S. B., J. R. REED, and A. D. MURPHY. 1978. Propagation of action potentials through electrotonic junctions in the salivary glands of the pulmonate mollusc, Helisoma trivincula. J. Exp. Biol. 72:77-90.
19. LAWRENCE, T. S., W. H. BEERS, and N. B. GIHLA. 1978. Transmission of hormonal stimulation by cell-to-cell communication. Nature (Lond.). 272:501-506.
20. LOWENSTEIN, W. R. 1968. Emergence of order in cells and tissues. Communication through cell junctions. Implications in growth control and differentiation. Dev. Biol. 19(suppl. 2):151-183.
21. LOWENSTEIN, W. R. 1972. Permeable junctions: permeability, formation, and genetic aspects. In The Basic Neurosciences. Raven Press, New York. 1419-1436.
22. LOWENSTEIN, W. R., and Y. KANNO. 1964. Studies on epithelial (gland) cell junctions. I. Modifications of surface membrane permeability. J. Cell Biol. 22:545-586.
23. LOWENSTEIN, W. R., J. L. SULLIVAN, S. HAGIWARA, Y. KANNO, and N. DAVIESON. 1965. Intercellular communication: renal, urinary, bladder, sensory, and salivary gland cells. Science (Wash. D. C.). 149:295-296.
24. MCINTYRE, N. S., and R. S. WEINSTEIN. 1973. Membrane ultrastructure at mammalian intercellular junctions. Prog. Biophys. Mol. Biol. 26:47-101.
25. MOORE, J. W. 1971. Voltage clamp methods. In Biophysics and Physiology of Excitable Membranes. W. J. Adelman, Jr., editor. Van Nostrand Reinhold Co., New York. 143-167.
26. NICHOLSON, C., S. B. KATER, and W. W. STEWART. 1977. Neuroend and glial elements of cerebellum visualized with new intracellular stain. Society for Neuroscience Abstracts. 3:60.
27. NISHIMURA, A., and O. H. PETERSEN. 1974. Membrane potential and resistance measurement in acinar cells from salivary glands in vivo: effect of acetylcholine. J. Physiol. (Lond.). 242:173-180.
28. PEUS, R. D. 1966. Ionic communication between liver cells. J. Cell Biol. 30:171-174.
29. PETERSEN, O. H., and A. TENENHOUSE. 1968. Cyclic AMP. 236:727-731.
30. PETERSEN, O. H. 1976. Electrophysiology of mammalian gland cells. Physiol. Rev. 56(3):535-577.
31. PETERSEN, O. H., and N. UEDA. 1976. Pancreatic acinar cells: the role of calcium in stimulus-secretion coupling. J. Physiol. (Lond.). 254:683-690.
32. RASMUSSEN, H., and A. TENENHOUSE. 1968. Cyclic adenosine monophosphate, Cab, and membranes. Proc. Nat. Acad. Sci. U. S. A. 59:1364-1370.
33. ROBINSON, G. A., R. W. BUTCHER, and E. W. SUTHERLAND. 1968. Cyclic AMP. Annu. Rev. Biochem. 37:149-168.
34. SCHNEIDER, L. H., and N. EMMELUSD. 1974. Salivary secretion. In International Review of Physiology: Gastrointestinal Physiology, E. D. Jacobson and L. L. Shanbour, editors. University Park Press, Baltimore. 4:183-226.
35. SCHNEIDER, L. H., and C. A. SCHNEIDER. 1965. Membrane potentials of salivary gland cells of rat. Am. J. Physiol. 219:1300-1310.
36. SHERIDAN, J. D. 1968. Electrophysiological evidence for low-resistance intercellular junctions in the early chick embryo. J. Cell Biol. 37:650-659.
37. SHERIDAN, J. D. 1968. Electrophysiological evidence for low-resistance intercellular junctions in the early chick embryo. J. Cell Biol. 37:650-659.
38. STEWART, W. W. 1978. Intracellular marking of neurons with a highly fluorescent naphthalimide dye. Cell. In press.
39. THOMAS, R. C. 1972. Intracellular sodium activity and the sodium pump in small neurons. J. Physiol. (Lond.). 230:55-71.
40. TRLS, R., W., and R. WARGAFT. 1976. Intraocular effects of cyclic AMP in cali muscular studied by a cut end method. J. Physiol. (Lond.). 280:117-141.