Role of gap junctions in fluid secretion of lacrimal glands

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Walcott, Benjamin, Leon C. Moore, Aija Birzgalis, Nidia Claros, Virginijus Valiunas, Thomas Ott, Klaus Willecke, and Peter R. Brink. Role of gap junctions in fluid secretion of lacrimal glands. Am J Physiol Cell Physiol 282: C501–C507, 2002. First published October 24, 2001; 10.1152/ajpcell.00004.2001.—In glands such as the liver and pancreas, gap junctions containing connexin 26 and 32 (Cx26 and Cx32, respectively) couple the secretory cells. Uncoupling these junctions compromises the secretory function of these glands. Lacrimal glands also contain extensive arrays of gap junctions consisting of Cx26 and Cx32. We wanted to determine the role of these junctions in fluid secretion. In Cx32-deficient mice, immunocytochemistry showed that, in the male lacrimal gland, the remaining Cx26 was found evenly distributed in the membrane whereas there was little in the membranes of female glands. Western blot analysis of Cx26 showed that female Cx32-deficient mice expressed Cx26.

Patch-clamp analyses of acinar cell coupling showed that the cell pairs from male glands were coupled whereas those from female glands were not. Stimulated fluid production by the glands from Cx32-deficient mice was abnormally low in female glands compared with controls at low topical doses of carbachol. The protein secretory response to different doses of carbachol was the same in all animals. These data suggest that gap junctions are essential for optimal fluid secretion in lacrimal glands.

IN EXOCRINE GLANDS, THE ACINAR cells that produce the secretory product are coupled by gap junctions (10). Gap junction channels, which link the cytoplasm of adjacent cells, are made up of membrane-spanning proteins, the connexins. In most glands, the major connexins are 32 and 26 (Cx32 and Cx26, respectively). The integrity of these junctions is apparently necessary for normal glandular secretory function. In the liver, for example, Cx32-deficient mice showed a 78% reduction in glucose mobilization on neuronal stimulation (12). Blocking liver gap junctions with 18-glycyrrhetinic acid reduced hormonal control of bile secretion (11). In Cx32-deficient mice, circulating levels of amy-
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**Immunocytochemistry.** Animals were killed with halothane, and the glands were removed. The tissue was immersion fixed for 3 h in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C. The tissue was rinsed in 30% sucrose in 0.1 M phosphate buffer and then stored overnight in fresh sucrose buffer at 4°C. Cryostat sections were cut at 14 μm, mounted on slides, and stored at 4°C until they were stained. Commercial rabbit polyclonal antibodies to Cx32, Cx26, and synaptophysin, a protein found at neurotransmitter release sites, were obtained from Zymed Laboratories (San Francisco, CA). Sections were hydrated in 0.1 M phosphate buffer and then exposed to normal goat serum diluted 1:75 for 1 h. The goat serum was drained off, and the primary antisera, diluted 1:200 with the phosphate buffer, were applied to the sections overnight at room temperature in a humidified chamber. Control slides were always prepared in parallel and consisted of sections exposed to the normal goat serum overnight instead of the primary antibody. Slides were then washed three times in phosphate buffer and then exposed to FITC-coupled goat anti-rabbit secondary antisera for 1 h in the dark at room temperature. The slides were then washed in phosphate buffer for 1 h in the dark and then cover slipped with use of Vectorshield mounting medium. Sections were examined by using a Zeiss epifluorescence microscope equipped with a Sony DKC 5000 camera attached to a personal computer. Images were captured by using Photoshop software and were printed on a digital Sony printer (UPD 8800).

**Western blot.** Fresh gland tissue was blotted dry, weighed, and then homogenized in cold sample buffer (0.1 M Tris-HCl, pH 6.8) with 2% SDS and 2 β-mercaptoethanol to a constant gland weight to buffer volume of 0.1 g/ml. The protein concentration of each sample was determined by using the method of Peterson (15). Sufficient volumes of sample were added to each well so that 90 μg of each sample were loaded into the wells of a 2–20% polyacrylamide gradient gel (Bio-Rad, Hercules, CA) with pretrained molecular weight standards (SeeBlue Plus; Novex, CA) being placed in the two outer wells. The gel was transferred to polivinylidene difluoride protein transfer membrane (Schleicher and Schuell, Keene, NH) and blocked with 1% milk powder in buffer. The membrane was exposed to a 1:1,000 dilution of a mouse monoclonal antibody to Cx26 (Zymed Laboratories) overnight. The membrane was then washed and exposition to a goat anti-mouse antibody coupled to alkaline phosphatase (Bio-Rad). Finally the membrane was treated with a chemiluminescence system (Immuno-Star chemiluminescent protein detection system, Bio-Rad) and exposed to X-ray film for 3 min.

**Protein secretion.** Glands were removed as described and were weighed before being cut into 0.5-mm slices with a scalpel blade. The slices from a gland were placed in 5 ml of an artificial saline solution (in mM: 116 NaCl, 5.4 KCl, 1.8 CaCl2, 0.81 MgCl2, 1.01 Na2HPO4, 26.2 NaHCO3, 5.6 dextrose, and 1.0 β-hydroxybutyric acid, pH 7.4) that was maintained at 37°C and was vigorously aerated with 95% O2 and 5% CO2 for 10 min. The solution was changed three times and discarded. The gland fragments were then placed in 5 ml of medium, and, after 10 min, 1 ml of medium was removed and replaced with fresh medium. This was repeated three times, and the 1 ml of the collected medium after each of the 10-min incubations was saved. In the last exchange, the medium that was added to replace that removed contained sufficient carbachol to make the final concentration in the bath either 0.1, 1, or 10 μM. After 10 min, 1 ml was removed. All four 1-ml samples were analyzed for their total protein content by using a Coomassie colorimetric system (Pierce, Rockford, IL). BSA was used as a standard protein, and standards were run with each batch of experiments. Unknown protein concentrations were determined from the standard curves measured with each experiment.

**Patch-clamp studies.** For patch-clamp analysis, acinar cells were isolated from freshly dissected glands by using a method developed for rat lacrimal glands (6). The mice were anesthetized with halothane and then decapitated. The glands were removed, placed in soybean trypsin inhibitor (0.1 mg/ml, Sigma Chemical) and cut into small pieces by using two sharp sterile scalpel blades. The pieces were then washed with Hanks’ balanced salt solution and incubated at 37°C for 15 min. After being washed again with soybean trypsin inhibitor, the cells were incubated at 37°C in an agitated mixture of collagenase (225 U/ml, Gibco), DNase (10 U/ml, Sigma Chemical), and hyaluronidase (600–700 U/ml, Sigma Chemical) in DMEM for 25 min. The resulting mixture was centrifuged at 1,200 rpm for 5 min, and the pellet was resuspended in medium. The suspension was filtered through sterile mesh to remove the large fragments and debris, and the resulting cell suspension was centrifuged again. The pellet was resuspended in medium, and 0.5-ml aliquots were plated on small sterile dishes that had been coated with Matrigel. The plates were incubated overnight to allow the cells to settle and were used the next day.

For patch clamping, the dishes were drained of medium, and the cells were covered with saline (in mM: 110 NaCl, 1 MgCl2, 1 CaCl2, and 10 HEPES, pH 7.2). Electrodes were produced on a P-57 Sutter puller, and an Axopatch 200 series patch amplifier was employed for recording. The pipette solution contained (in mM) 110 KCl, 0.9 EGTA, 0.1 CaCl2, 0.1 MgCl2, and 10 HEPES at pH 7.05. Cell pairs were patched in a whole cell configuration, and coupling was measured by use of conventional methods.

**Fluid secretion.** To measure the fluid production of the exorbital lacrimal gland, we anesthetized mice with 120–130 mg/kg Inactin (RBI, Natick, MA) and 120–130 mg/kg ketamine. The animal was placed on a heated surgical table; rectal temperature was maintained at ~37°C. A tracheal cannula was inserted to avoid aspiration of saliva, the flow of which can rise during carbachol (acetylcholine agonist) stimulation. A catheter was inserted into the left jugular vein for infusion of 0.5 ml·h⁻¹·100 g body wt⁻¹ isotonic saline during surgery and measurements. Another catheter was placed in the right femoral artery for arterial pressure monitoring.

The animal was placed on its side, and its head was immobilized with nonpenetrating steel pins and rods fixed to the surgical table. The exorbital lacrimal gland was exposed, and its sheath of connective tissue was dissected away via blunt dissection under a dissecting microscope. Care was taken not to injure nearby nerves and blood vessels. The lacrimal duct was cut at a point where 50% of the duct remained attached to the gland. After bleeding from the severed periductal vessels had stopped, the proximal end of the duct was placed into a glass constriction pipette (fabricated in the lab on a microforge) made from constant-bore capillary tubing (2- or 5-μl Microcaps, Drummond). The diameter of the constriction was matched to the diameter of the duct so that flow would not be impeded. The capillary was held in a horizontal position with a micromanipulator; the capillary was positioned so that it did not touch the surface of the experimental field. Throughout the experiment, the gland was covered with a thin plastic film to prevent desiccation of the tissue.

After a 30-min recovery period, the last 10 min of which were used as a measure of baseline, nonstimulated fluid flow, a fresh capillary was mounted, the duct was inserted, and 2...
µl of carbachol (0.12 mM) were topically applied to the glandular surface under the plastic film. Fluid collection occurred spontaneously into the capillaries, and this amount of carbachol was sufficient in all control animals to elicit a prompt rise in fluid secretion that could be monitored by observing the movement of the fluid meniscus through a stereomicroscope. After 2 min, the position of the meniscus was marked. A fine absorbent wick was then applied to the base of the lacrimal duct to verify that fluid was not being collected from the surface, and the fluid collection continued for ~15 min. If the gland did not respond to the initial challenge or if the fluid flow was very low, up to two additional 2-µl aliquots of the low dose (0.12 mM) of carbachol were applied after resectioning of the duct and reinsertion into a fresh capillary. This was done to verify that zero or low secretion rates were not the result of blockage of the duct, and the highest resultant flow was taken to reflect the secretory response of the gland. After ~30 min, 2 µl of a higher dose of carbachol (6.0 mM) were topically applied, and the flow response was measured, as above. The volume of fluid collected was measured optically by determining the length of the fluid column up to the 2-min mark and multiplying by a factor expressing the volume per millimeter of capillary length.

**Statistics.** Differences between group means were tested with ANOVA, either one- or two-way, as appropriate, and Tukey’s multiple comparison test. Differences between proportions were tested with the z-test. A significance level of 0.05 was assumed.

**RESULTS**

**Immunocytochemistry.** We examined the Cx32 and Cx26 immunoreactivity in exorbital lacrimal gland tissue from control animals. The pattern of staining seen with both antisera was similar (Fig. 1) in males and females, consisting of numbers of small patches of immunoreactivity associated with the secretory acinar cells. Many of the patches were linearly arranged as if associated with the plasma membranes of adjacent cells. In addition, we found immunoreactivity above control levels that appeared to be localized within the cells. The duct cells, distinguished by their obvious

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**Fig. 1.** A: control male (C57BL/6) gland section stained with antiserum to connexin 26 (Cx26). Note the linear arrangement of patchlike staining associated with acinar cells. Ducts (d) do not show the patchlike staining and have little cytoplasmic immunoreactivity. B: control male gland section stained with antiserum to connexin 32 (Cx32). C: control male gland section stained with antiserum to Cx26. D: control female section stained with antiserum to Cx32. E: control section of male gland in which the tissue was not exposed to the primary antiserum but otherwise was treated the same. Scale in A–E is 25 µm.
lumen and more cuboidal shape (see Fig. 1A), showed no positive staining for either of the connexins.

When we examined the lacrimal glands of Cx32-deficient animals, male or female, we found that there was no staining for Cx32, as expected (Fig. 2, B and D). However, we found that there was a difference in the Cx26 staining pattern between male and female Cx32-deficient animals. In the male animals, there was faint but clear Cx26 staining associated with the membranes of the acinar cells (Fig. 2C). The staining pattern was, however, different from that seen in control animals in that it was more linear than patchlike. In the female animals, there were no patches but there was also much less membrane staining than in the male. There was mainly a diffuse background staining of the acinar cell cytoplasm (Fig. 2A).

**Western blot.** Figure 3 shows a Western blot stained with a monoclonal antibody to Cx26. This blot compares the glands from a male and a female C57BL/6 control mouse and from a male and a female Cx32-deficient animal. In all cases, there is a major band at ~30,000 Da that shows positive immunoreactivity. Additional blots on glands pooled from four different animals of each type and sex showed similar results, with Cx26 being present in both male and female glands of Cx32-deficient animals as well as control animals.

**Protein secretion.** Figure 4 shows the amount of protein released by gland fragments in response to three different concentrations of the acetylcholine agonist carbachol. The data show that there is dose-dependent protein secretion in response to carbachol in glands from both male and female Cx32-deficient animals as.
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Fig. 3. Western blot of lacrimal gland tissue. Lane 1 is C57BL/6 male, lane 2 is C57BL/6 female, lane 3 is Cx32-deficient male, and lane 4 is Cx32-deficient female. Note that there is expression of Cx26 in the female Cx32-deficient glands even though these cells do not appear to be electrically coupled. Each lane of this blot is from the glands of 2 individual animals. A similar result was seen when glands were pooled from an additional 4 animals of each group.

well as control strain (Swiss Webster) female glands. At the lowest dose of carbachol (0.1 µM), the Cx32-deficient female gland fragments secreted significantly more protein than fragments from the other glands (t-test, P < 0.05). At 1 µM carbachol, there was no significant difference, whereas at 10 µM again the female Cx32-deficient gland fragments secreted more protein than the others (P < 0.05). The protein secretion from the female Cx32-deficient gland fragments was dose dependent and robust.

Cell coupling. Patch-clamp analysis of cell pairs from control glands showed that the majority of cells were coupled (Fig. 5), as has been previously reported (14). The majority of cell pairs from Cx32-deficient female glands, however, were not coupled, and those that were coupled had a much lower average conductance (1.8 ± 1.2 vs. 10.0 ± 2.5 ps) than cell pairs from control animals. Cell pairs from Cx32-deficient males were coupled and showed a conductance similar to that of control animals.

Fluid secretion. As described in METHODS, the secretory responses with no stimulation and then with topical carbachol at low (2–6 µL at 0.12 mM) and high (2–4 µL at 6.0 mM) doses were measured. In all animals, our methods were not able to measure significant amounts of basal, nonstimulated flow. In control female glands, the peak flow rate induced by the lower dose of carbachol was significant but not as high as that induced by the higher dose (Fig. 6). The carbachol diffused at least some distance into the gland because there was a relatively rapid (within 1 min) drop in mean arterial pressure after the application of the dose of carbachol. In each group (n = 6 in all groups), the dose response to carbachol was significant (P < 0.05 ANOVA). In both the control and Cx32-deficient groups, the response to carbachol was significantly higher in males than in females (P < 0.001, two-way ANOVA). This is likely a reflection of the larger gland size in male mice. However, deficiency of Cx32 compromised flow responses only in female mice and, in particular, only at the low topical dose of carbachol (P < 0.05, two-way ANOVA, Tukey’s test). Application of the high dose of carbachol induced a flow rate in Cx32-deficient female glands indistinguishable from that of the control female glands.

DISCUSSION

The data presented here show that the mouse exorbital lacrimal gland acinar cells are highly coupled by gap junctions and that these junctions consist of Cx32 and Cx26. Such junctions have been shown to exist in the rat exorbital lacrimal gland (10), where they develop in number and size as the gland increases its secretory ability with growth and development (7). Also in the rat lacrimal gland, several patch-clamp studies have shown that the acinar cells are highly coupled and that the conductance of these junctions is modulated by acetylcholine independently of the internal calcium levels (13). This regulation of junctional conductance seems to require activation of protein kinase C (16). The rat salivary glands also have Cx32 and Cx26 junctions between acinar cells, and the conductance of these junctions is also affected by acetylcholine.
Fig. 6. Fluid production by lacrimal glands in response to topical carbachol application. Nonstimulated flow was too low to measure (labeled NM). Carbachol at low dose and then at a higher dose (as indicated on the figure) was applied to 1 gland, the lacrimal duct of which was inserted into a microcapillary tube for flow measurement. In each group, flow increased in response to carbachol \((P<0.05,\text{ ANOVA})\). In both controls and Cx32-deficient (\(\text{def}\)) groups, flow response in male mice exceeded that in female mice \((P<0.001,\text{ 2-way ANOVA})\). Flow responses of control and Cx32-deficient male mice did not differ significantly, whereas the responses in the female groups did \((P<0.025,\text{ 2-way ANOVA})\). In particular, female Cx32-deficient glands showed a significantly \((P<0.05)\) reduced flow in response to the low dose compared with controls. At high concentrations of carbachol, there was no difference between Cx32-deficient and control glands. *NM, flow too low to be measured with our methods and assumed to be zero; ns, not significant.

via a protein kinase C system \((8,9)\). Although it is clear that gap junctions are present and can be regulated, there are no data on either mouse or rat lacrimal or salivary gland systems to show the role of gap junctions in the secretory process itself. We have found that the Cx32-deficient female mice showed a reduced fluid secretion in response to low doses of topically applied carbachol. Only one of seven animals (see Fig. 6) had any significant fluid flow, whereas the others had little to no response. This is in contrast to control animals as well as Cx32-deficient males, in which there was always fluid secretion in response to topically applied low doses of carbachol. These results correlate with the coupling data, which showed that female Cx32-deficient lacrimal gland acinar cell pairs were electrically uncoupled, whereas those from male Cx32-deficient cell pairs were coupled, as were cell pairs from control animals. The observation that topical applications of high doses of carbachol induced normal fluid flow in all animals suggests that, in the Cx32-deficient female glands, the acetylcholine receptor second messenger system is not significantly different from that in control glands. The integrity of the muscarinic acetylcholine-mediated second messenger system is further supported by the observation that protein secretion, which is also induced by acetylcholine, was similar in gland fragments from all glands. A simple interpretation of these data would be that gap junctions are necessary for the spread of excitation from the surface cells to deeper cells within the gland. When low doses of topical carbachol are used, the carbachol does not diffuse at a high enough concentration to cause excitation of many of the deeper cells within the gland tissue. In glands where the cells are coupled, excitation can be spread through the tissue via the gap junctions. In Cx32-deficient females, the junctions are defective, so perhaps there is minimal excitation of deep cells.

In other secretory tissues such as liver and pancreas, loss of Cx32 or blockage of the gap junction channels has a profound effect on the regulation of the secretory ability of the glands \((10,12)\). The livers of Cx32-deficient mice, for example, secreted less glucose in response to glucagon or norepinephrine stimulation when half-maximum doses were used \((17)\). However, secretion of glucose in response to saturating doses was the same between Cx32-deficient and normal animals. This behavior is similar to what we observed on the lacrimal gland fluid secretion. The liver is also controlled by sympathetic neurons. In Cx32-deficient animals, stimulation of the sympathetic innervation to the liver resulted in a significantly reduced \((78\%)\) production of glucose \((12)\). Thus reduction of coupling between cells in some glands affects the ability of those glands to respond to either endocrine or neural stimulation.

The immunocytochemical data (Figs. 1 and 2) showed that there was a difference in the morphology of the Cx26 immunoreactivity in the lacrimal glands between the control and Cx32-deficient mice. In control mice, the pattern of staining was predominantly patchlike, often in rows along the cell margins, although there was some diffuse staining in the cytoplasm. In the Cx32-deficient animals, Cx26 was expressed, but its distribution was different between males and female animals. In Cx32-deficient males, the staining pattern observed was of a uniform staining on the plasma membranes as well as diffuse cytoplasmic staining. In the female Cx32-deficient glands, there was little membrane-associated Cx26 immunoreactivity, but there was diffuse cytoplasmic staining. The Western blot data (Fig. 3) showed that there was little difference in the expression of Cx26 between male and female control mouse glands and that the female Cx32-deficient glands contained Cx26 as well even though most cell pairs isolated from these glands showed no coupling. The male Cx32-deficient glands, whose cells were still electrically coupled, also showed an expression of Cx26. These data can be explained by the following hypotheses: either the deletion of the Cx32
gene could affect Cx26 expression and thus its ability to normally insert in the membrane, or expression of both Cx32 and Cx26 may be needed for the normal insertion or retention of Cx26 in the membrane. Alternatively, the difference in the placement of the Cx26 in male and female Cx32-deficient animals may be due to androgens that could play a role in the normal expression and insertion of these connexins into the membrane.

Androgens affect the lacrimal glands in several ways. In many species, including mice and humans, the lacrimal gland acini of males are larger in area than those from females (4). Studies of the activity of certain enzymes in the lacrimal glands of rabbits show that significant sex-related changes occurred as the animals matured, which indicated that the glands were influenced by the androgens and estrogens of the sexually maturing animals (1). Lacrimal gland acinar cells have androgen-binding sites in their nuclei (18). Glandular fluid secretion is dependent on the presence of androgens (2), as ovariectomy of female rabbits, for example, reduced blood androgen levels and resulted in a reduced ability of the glands from those animals to secrete fluid (20). These studies show that androgens have a significant role in the maintenance and function of lacrimal glands. It is therefore likely that the difference in Cx26 placement between the male and female Cx32-deficient animals could be due to the different androgen levels in these animals. The mechanism by which the androgens exert their influence on the gap junction channel proteins remains to be determined.

Our results on Cx32-deficient mice suggest that gap junctions play an important role in the excitation secretion process of lacrimal glands. The determination of whether older Cx32-deficient females have drier eyes than males, however, is not a simple task. The ocular fluid is derived from a number of sources such as the accessory glands, Harderian glands, cornea, and conjunctiva, all secreting different types of fluids (19). To grossly measure the total fluid on the surface by means of Schirmer’s strips, for example, would not necessarily show any difference. However, there could well be differences in the amounts of lacrimal gland-specific proteins, such as peroxidase or lactoferrin, that reach the ocular surface because it is likely that fluid flow is necessary to move these proteins down the lumen of the acini and the ducts. Uncoupling of acinar cells due to either reduced expression, failure to insert the connexin channels in the membrane, and/or reduced permeability due to regulatory effects could result in reduced fluid flow from the lacrimal glands and hence dry eye and/or could affect the composition of the tear fluid.

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