Increase in Gap Junctions in Frog Skin Epithelium

James T. Blankemeyer and Sharif H. Shahin*

Department of Zoology, Oklahoma State University, Stillwater, OK 74078
*Present address: Department of Physiology, College of Veterinary Medicine, Hama, Syria.

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Abdominal frog skin, mounted on an Ussing chamber, actively transports sodium from the outer or pond side of the epithelium to the serosal side. The outermost layer of the skin is composed of cornified cells and does not participate in active transport. Pondside sodium enters through the first living layer of cells, the *stratum granulosum*. Electron microprobe studies demonstrated that the *stratum granulosum* and the more basal strata were in the route of sodium active transport. Previously we have shown, using frog skin processed immediately after euthanasia, that the *stratum granulosum* had significantly fewer gap junctions per unit length of membrane than the other strata. In this study we examined a time series of frog skins, from zero min to four h. Using an electron microscopic counting technique, we then determined the number of gap junctions and desmosomes in each stratum. The density of gap junctions (number/micrometer of membrane) in the *stratum granulosum* rose from 0.04/μm to 0.13/μm 30 min after chamber mounting. During this period, the density of gap junctions in the other strata was nearly constant. We conclude that the number of gap junctions in frog skin changes during chamber mounting.

INTRODUCTION

The isolated abdominal skin of the frog actively transports sodium from the pond side of the skin through the epithelial cells to the serosa. The epithelium is composed of four layers. Nearest the outer surface is a layer of cornified dead cells (*stratum corneum);* next is the granular cell layer (*stratum granulosum*); next the pentagonal cell layer (the *stratum spinosum*); and finally the undifferentiated cells *stratum germinativum*. Interspersed in the epithelium are mitochondria-rich cells (clear cells, flask cells) associated with chloride transport (*1*). Ussing and Windhager (*2*) proposed a sodium-active transport model in which all the epithelial cells are connected into a syncytium. Frog-skin active-transport studies (*2,3-5*) use excised skins mounted on Ussing chambers, bathed in frog Ringer's solution, and short-circuited. Short-circuiting is a technique to maintain the electrical potential difference at 0 V by using an external current source. Rick and collaborators (*3-5*), using the electron microprobe to analyze intracellular sodium in chamber-mounted and short-circuited frog skins, showed that sodium from the pond side is present in all the layers of the epithelium except the *stratum germinativum* and mitochondria-rich cells.

We recently demonstrated and quantified gap junctions in all the living strata of the frog skin epithelium (*6*). Using skins quickly excised and immediately fixed, we found that the density of gap junctions in the *stratum granulosum* was significantly lower than in the other strata (*6*). The lower density of gap junctions in the *stratum granulosum* raised the possibility that the cells in that layer did not exchange ions with the neighboring strata. Since the electron microprobe studies were performed with Ussing chambers and under short-circuit conditions and our junction counting study was performed with fresh skin, we became interested in comparing the density of gap junctions between freshly excised frog skins and skins that had been mounted on an Ussing chamber and short-circuited for varying periods of time. In this study we measured the effect of mounting the skin on an Ussing chamber on the density of gap junctions in the frog skin epithelium. We also examined the effect of chamber mounting on the density of desmosomes as a control on any systematic counting errors.

MATERIALS and METHODS

Leopard frogs, *Rana pipiens,* were obtained commercially (Wm. Lemberger, Oshkosh, WI). Frogs arrived during the week prior to the experiment and were kept unfed in running tap water prior to

experiment. All experiments were completed within one week after frogs were received from the supplier. They were anesthetized with 10% urethane injected into the dorsal lymph sac and then decapitated. The abdominal skin was removed and mounted on an Ussing chamber. After exposure to frog Ringer's solution and short-circuiting for time periods from 0 to 240 min, the solution was drained from the chamber and the skin quickly removed and fixed. All skins were short-circuited using an external automatic voltage clamp. Skins were bathed in frog Ringer's solution (110.0 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 5.0 mM Tris, pH 8.3). The bath solution was stirred with gas-lift pumps using hydrated air.

After exposure for the selected time periods (0, 30, 60, 120, and 240 min) the skin was removed and processed for electron microscopy as previously described (*6*). Abdominal skin samples were removed from the chamber and immediately immersed in fixative solution. A solution of 2% tannic acid in Na cacodylate buffer (pH 7.4) was prepared at 50 °C. Two and one-half ml of the tannic acid solution was mixed with an equal volume of 5% glutaraldehyde in 0.2 M Na cacodylate buffer to produce a final concentration of 2% tannic acid with 2.5% glutaraldehyde. The abdominal skin was immersed in this fixative for two h. Postfixation was performed with 2% osmium tetroxide in 0.1 M cacodylate buffer. The skin was then immersed in 5% uranyl acetate with 5% sucrose for two h. Fixed tissues were quickly dehydrated in graded ethanol and embedded in polybed resin. Silver to gray sections were cut with diamond knives on a Sorvall MT-2 ultramicrotome. Sections were collected on carbon-coated grids, stained with lead citrate and aqueous uranyl acetate and examined with a JEOL 100 CX II electron microscope (*6*) (Table 1).

Density of junctions was determined by counting the number of junctions per unit length of membrane by using the grid intersection method of Garfield et al. (*7*). The cellular membrane length of each section was determined by superimposing a lined transparent grid over the photograph. The total length of membrane was calculated by counting the intersections between the cellular membranes and the grid lines. The membrane length was calculated by using the formula: $B=(\pi/2)(I/L)(A \times M)$, wherein: B is the length of cell membrane in the photograph; I is the number of intersection points; L is the total length of grid lines; A is the area of the photograph; and M is the magnification factor.

Twenty frogs were used, four for each time period. Statistical analysis was performed with Systat (Evanston, IL) using the MGLH module ANOVA procedure with Bonferroni adjustment.

RESULTS

Figure 1a displays the gap junction density (as defined) for the *stratum granulosum*, the most apical living stratum in the epithelium. At zero time, the density of gap junctions was 0.041 ± 0.013. As illustrated in Fig. 1a, between the time of mounting the skin on the chamber (0 min) and 30 min, the density of gap junctions changed from 0.041 to 0.128 ± 0.016 and remained at or above the 30-min value through the 240-min point. Anova analysis of the data (using a model MSE of 0.003 with 85 degrees of freedom) showed that the zero-time density of gap junctions (denoted with an 'a' on Fig. 1a) was significantly different from all the other time points ($p \leq 0.002$). Although the gap junction density rose from 0 min to the 60-min point and then declined to near the 30-min value by 240 min, there were no significant differences among the 30- to 240-min densities at the 95% significance level.

TABLE 1. Number of blocks:sections for each time-point

	Time/min				
Area	0	30	60	120	240
S. granulosum	2:12	3:16	3:24	3:15	2:23
S. spinosum	2:15	3:19	3:19	2:17	2:12
S. germinativum	2:12	2:06	2:07	3:11	2:10

The *stratum spinosum* gap junction densities are presented in Fig. 1b. At 0 min exposure, we found a gap junction density of 0.162 ± 0.018. The highest density was at 60 min followed by a decline to a low point at 240 min. The gap junction density at 240 min was significantly different from the density at 60 and 120 min ($p \leq 0.012$); the 30-min point was significantly differrent from the 60-min point ($p \leq 0.038$).

The density of gap junctions in the

TABLE 2. Density[a] of desmosomes

Area	Time/min 0	30	60	120	240
s. granulosum	0.42(0.12)	0.32(0.08)	0.37(0.08)	0.26(0.05)	0.24(0.06)
s. spinosum	0.28(0.14)	0.33(0.06)	0.28(0.10)	0.25(0.06)	0.21(0.06)
s. germinativum	0.07(0.05)	0.21(0.04)	0.07(0.04)	0.11(0.10)	0.09(0.05)

[a] All entries represent the mean density (number per micrometer), with the standard deviation in parentheses.

stratum germinativum is represented in Fig. lc. The gap junction density at 0 min was 0.21 ±0.03. The maximum density was found at 60 min (0.312±0.053). After the 60-min point the density declined to a low at 240 min (0.154±0.015). The gap junction density at 240 min differed significantly from the 60 min value(p=0.024). No other statistically significant differences were found at the 95% level. There was an increase in density from 0 to 60 min but this was followed by a decrease to a low at 240 min.

We also measured the density of desmosomes in all the living strata at the same time points and in the same sections as for the gap junctions. In the *stratum granulosum* and *stratum spinosum*, the density of desmosomes decreased with time. The *stratum germinativum* desmosome density was scattered and produced no clear trend. The desmosome results are summarized in Table 2.

DISCUSSION

Our previous study (6) demonstrated that gap junctions were found in all the strata of the freshly excised frog skin except the cornified cells of the *stratum corneum*, the outermost layer of cells. However, the number of gap junctions per micrometer of membrane (density of gap junctions) was significantly lower in the *stratum granulosum*, the layer of living cells nearest the pond side of the frog skin, than in the other strata of the skin.

The zero-time gap junction density of the *stratum granulosum* found in this study (0.041±0.013) is not significantly different from the gap junction density (0.04±0.018) found previously (6). Analysis of the gap junction density data demonstrates that the density increased significantly during the first 30 min of chamber mounting in the most apical layer of living cells, the *stratum granulosum*. After 30 min, no statistically significant difference was seen among the other data points. Although there was no

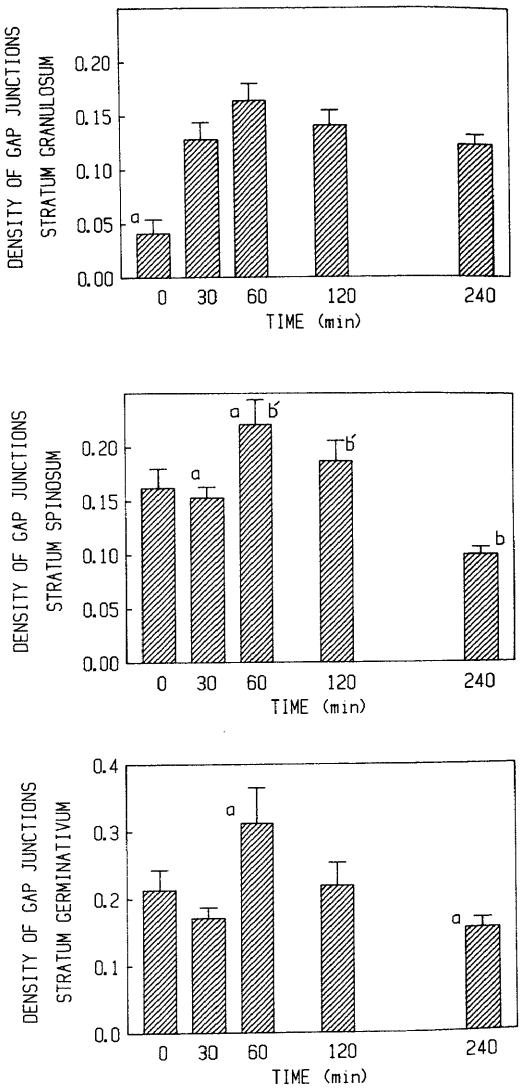

Figure 1. Time-course representation of the density of gap junctions in (a) *stratum granulosum*, (b) *stratum spinosum*, and (c) *stratum germinativum*. Density is number of gap junctions per micrometer of membrane. Each point is the mean of data from four frogs; error bar is standard error of the mean. At the 95% probability level: in (a), point 'a' is significantly different from all other data points; in (b), points 'a' differ significantly, and point 'b' differs significantly from points 'b'"; in (c), points 'a' differ significantly.

statistically meaningful change in density after 60 min, the trend of the data is toward a lower density of gap junctions.

In the *stratum spinosum*, the density of gap junctions increased to a maximum at 60 min, then declined to a minimum at 240 min. In this stratum, the 240-min gap density was significantly different from the 60- and 120-min densities. Also the 30-min density was significantly different from the 60-min density. In the *stratum germinativum* the gap junction density rose to a maximum at 60 min, then declined to a minimum at 240 min. The 240-min point was significantly different from the 60- and 120-min gap junction density.

The frequency of desmosomes declined (*stratum granulosum*, *stratum spinosum*) or stayed constant (*stratum germinativum*) during the 240-min time course of the experiment. The decline of desmosome density during the same time period while the gap junction density increased and then decreased suggests that no systematic counting errors occurred.

The data suggest two conclusions. The first is that mounting the frog skin on the Ussing chamber, exposing the skin to Ringer's solution, and short-circuiting the frog skin causes the density of gap junctions in the *stratum granulosum* to increase to a value consistent with that in the other strata in the frog skin. This increase occurs during the first 30 min of exposure to Ringer's solution. The second is that there is a decrease in the number of junctions in all living strata from a maximum at 60 min to a minimum at 240 min.

Both the membrane potential differences, affected by short-circuit conditions and intracellular calcium activity, alter the conductivity of gap junctions (*8*). We can speculate then that short-circuit conditions or bath calcium may also affect the number of gap junctions.

Our data indicate that the frog skin undergoes fundamental changes during the initial periods of mounting on an Ussing chamber. This early period (ca. the first 240 min) is known as an "equilibration period" during which the frog skin adapts to new ion concentrations in the bath solutions and to the electrical changes caused by short-circuit conditions. Probably there are internal morphological changes during the adaptation that are more than physiological adaptations. If the actual cell-to-cell coupling changes during the equilibration period, the characteristics of sodium transport and the model derived from those characteristics would describe a chamber-mounted frog skin rather than intact frog skin. There are some suggestions that the *stratum granulosum* can be uncoupled from the other strata. Vouté and collaborators(*9, 10*), in studies now out of favor, hypothesized that only the *stratum granulosum* was involved in active transport of sodium. The other literature suggestion is the study of Rick et al. (*5*) in which arginine vasopressin and low serosal sodium uncoupled the *stratum granulosum* from the other strata in the epithelium. Our attempt to correlate the active transport of sodium with the structural data gave only confusing results. The rate of active transport increases from the time of mounting to about 60 min after mounting, corresponding to the increase in gap junction density found in this study. After 60 min the trend in the short-circuit current is unpredictable although it usually levels out after about four h. Whether the structural changes we have found alter the physiology of transport during the equilibration period is yet to be determined. We are now proceeding to measure intracellular calcium, intracellular sodium and intercellular pH during the equilibration period.

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