Basal lamina of ovarian follicle regulates an inward Cl\(^-\) current in differentiated granulosa cells

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Qin, Wuxuan, Stanley G. Rane, and Elikplimi K. Asem. Basal lamina of ovarian follicle regulates an inward Cl\(^-\) current in differentiated granulosa cells. Am J Physiol Cell Physiol 282: C34–C48, 2002.—Patch-clamp experiments were conducted to study the effects of basal lamina (basement membrane) of preovulatory chicken ovarian follicle on transmembrane ion transport is required for steroid hormone synthesis in granulosa cells. Therefore, experiments were conducted to study the effects of basal lamina (as a unit) on transmembrane ion transport in granulosa cells. The combination of basal lamina and granulosa cells isolated from the preovulatory follicle of the chicken ovary was used as a model system to study the effects of basal lamina on granulosa cells.
in a homologous system. This homologous system was made possible by the anatomical structure of the avian ovarian follicle. In the mature avian ovarian follicle, the granulosa cell layer (membrana granulosa) consists of a single layer of cells located between the basal lamina and perivitelline layer (11, 13, 41, 42, 52); this arrangement made possible the isolation of intact basal lamina in hypotonic solution (4, 7).

MATERIALS AND METHODS

Chemicals. HEPES, collagenase type IV, soybean trypsin inhibitor, BSA (fraction V), penicillin G, streptomycin, N-methyl-d-glucamine, fungizone, Trizma base, 4,4’-diisothio-cyanostilbene-2,2’-disulfonic disodium salt (DIDS), tetra-ethylammonium chloride (TEA), nifedipine, actinomycin D, and cycloheximide were purchased from Sigma Chemical (St. Louis, MO). Medium 199 containing Hanks’ salts was from GIBCO-BRL (Grand Island, NY). 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was purchased from Research Biochemicals International (Natick, MA). Flurazinc was obtained from Calbiochem (San Diego, CA).

Solutions. The currents were recorded under quasi-physiological conditions (high K+ in pipette and Na+ in bath solution). Unless noted otherwise, the pipette solution contained (in mM) 139 KCl, 5 NaCl, 3 ATPMg, 0.5 GTP, 10 HEPES, 0.1 EGTA, and 1 MgCl2 (pH 7.2). The bath solution was modified Hanks’ balanced salt solution containing (in mM) 134.3 NaCl, 5.4 KCl, 2.5 CaCl2, 1.1 MgCl2, 5.6 glucose, and 10 HEPES (pH 7.4). In some cases, the bath solution contained 10 mM barium chloride instead of 2.5 mM CaCl2. Where required, Cs+ was substituted for K+ in the pipette solution and 10 mM TEA was included in the bath solution.

Animals. Single Comb White Leghorn hens obtained from Purdue University Poultry Research Farms (West Lafayette, IN) in their first year of reproductive activity were caged individually in a windowless, air-conditioned room with a 14:10-h light-darkness cycle. They had free access to a layer ration and tap water. The time of egg lay of each bird in the colony was noted to the nearest 30 min (daily). Animals were injected with ketamine (50 mg/kg body wt) 10 min before being killed by cervical dislocation (~10–12 h before the expected time of ovulation of the largest preovulatory follicle (F1)). The largest preovulatory follicle was removed and placed in ice-cold Hanks’ salt solution containing (in mM) 140 NaCl, 5 KCl, 1.1 MgCl2, 2.5 CaCl2, 10 HEPES, and 5.6 glucose (pH 7.4). The theca and granulosa cell layers (membrana granulosa) were separated by the method of Gilbert et al. (25).

Isolation of intact basal lamina. Basal lamina was isolated as previously described (4, 7). Briefly, the granulosa cell layer obtained from the largest preovulatory follicle (F1) was placed in a hypotonic solution containing 10 mM Tris-HCl (pH 7.4), 0.5 mg/l leupeptin, 1 mM EDTA-Na2, 0.7 mg/l pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride in a petri dish. The granulosa cells, sandwiched between the basal lamina and perivitelline layer, were lysed hypsomotically, and the theca lamina and perivitelline layer were separated. This basal lamina of the avian ovarian follicle preparation is an intact and complete basal lamina. The side of basal lamina that was in contact with granulosa cells in situ was designated as the “granulosa side,” and the side in contact with theca tissue was designated as the “theca side.”

Solubilization of basal lamina. Basal laminae were solubilized in one step. They were placed in a microfuge tube and solubilization buffer containing 6 M guanidine hydrochloride and 50 mM Tris-HCl (pH 7.4), and 5% β-mercaptoethanol was added (100 µl basal lamina−1·follicle−1). After shaking for 60 min at 4°C, the entire basal lamina was solubilized (referred to as “total fraction”). Exclusion of β-mercaptoethanol from the solubilization buffer led to incomplete solubilization of the basal lamina (fragments remained). The solubilized material was placed in a 3-kDa cutoff dialysis membrane and dialyzed against 150 mM NaCl and 50 mM Tris-HCl, pH 7.4, at 4°C overnight; it turned cloudy because of precipitation of proteins.

Preparation of intact basal lamina-containing dishes for cell culture. For experiments designed to study the effects of intact basal lamina on membrane currents, pieces of the intact basal lamina (2–3 cm²) were spread in 35-mm tissue culture dishes (Corning) and allowed to dry in a laminar flow hood. Blank dishes served as controls. The experiments were conducted with cells incubated on the granulosa side of the intact basal lamina. The intact basal lamina-containing dishes were either used immediately or wrapped in aluminum foil and stored at 4°C. Tissue isolation and preparation of culture dishes were carried out under sterile conditions.

Preparation of solubilized basal lamina-coated coverslips for cell culture. The solubilized basal lamina (total fraction) was diluted with deionized water, and aliquots of 100–200 µl containing 5–50 µg of protein were spread on a 12-mm round glass coverslip (Warner Instrument, Hamden, CT) and allowed to dry under a tissue culture hood ( precoated coverslips). Coverslips that received vehicle only served as controls. The precoated coverslips were either used immediately or wrapped in aluminum foil and stored at 4°C. Tissue isolation, solubilization, dialysis, and preparation of coverslips were carried out under sterile conditions.

Enzymatic isolation of granulosa cells. Granulosa cells were dispersed in medium 199 containing 350 mg/l NaHCO3, 10 mM HEPES, 500,000 U/l collagenase, and 200 mg/l trypsin inhibitor at pH 7.4 (39). Cell viability, determined by the trypan blue exclusion method, was routinely >95%.

Cell culture. Collagenase-dispersed chicken granulosa cells were plated in intact basal lamina-containing dishes or in 35-mm dishes containing coverslips that were precoated with a total fraction of solubilized basal lamina. The cells were incubated for 24 h at 37°C in serum-free medium 199 containing 0.1% (wt/vol) BSA and 10 mM HEPES (pH 7.4; see Ref. 39).

Voltage clamp. The conventional whole cell recording method was used in this study. Recording pipettes were fabricated from borosilicate filament glass (Warner Instrument). Electrode resistance was between 3 and 9 MΩ in the bath solution. Unless otherwise noted, the linear (leak) component of the total membrane current was subtracted by extrapolating the linear currents obtained during voltage steps in more negative potential regions (~100 to −80 mV), where no voltage-activated currents were seen. Current amplitudes were small enough that the series resistance error was <5 mV. Junction potentials were corrected manually on the amplifier. The bath solution and the reference electrode were connected with a 3 M KCl-containing agar bridge. This permitted recordings to be made without readjusting the junction potential after the changing of bath solution. Analog compensation was applied to attenuate capacitive current transients and to estimate cell capacitances, a measure of the total cell plasma membrane area. Membrane currents were measured with an Axopatch 1-D patch clamp (Axon Instruments, Foster City, CA) and filtered at 1 kHz. The currents were digitized and stored directly to disk (DigiData 1200 Interface; Axon Instruments) and were analyzed with pCLAMP 6.0.3 software (Axon Instruments).
In the conventional whole cell mode, the membrane potential was held at −80 mV. Membrane currents were elicited by test pulses of 333 ms in duration from −60 to +80 mV in 20-mV steps. In some experiments, the membrane currents were elicited with a 10.5-s test pulse at a −40-mV voltage step. All experiments were conducted at room temperature (21–23°C). Cells were perfused such that complete bath exchange is accomplished within 1 min. All bath solutions were at pH 7.4.

For experiments designed to study the effects of solubilized basal lamina, the granulosa cells incubated on coverslips were transferred to a perfusion chamber (Warner Instrument) containing bath solution (see above). The volume of the chamber is 180 μL.

Data analysis. For current traces that were obtained with application of a 333-ms test pulse, the amplitudes of both inward and outward currents were measured at the end of the command pulse and used to plot current-voltage relationship curves. In experiments in which a 10.5-s test pulse was applied, the amplitude of the inward current was measured 250–800 ms after the onset of the command pulse. A t-test (2-tailed) was performed to compare the differences among treatment means and control values. Differences at P < 0.05 were considered significant.

RESULTS

Freshly isolated differentiated granulosa cells (obtained from the largest preovulatory chicken ovarian follicle) were incubated in serum-free medium 199 for 24 h on plastic or glass coverslips and studied with the conventional whole cell patch-clamp technique. With a holding potential of −80 mV, depolarizing voltage steps between −60 and +80 mV activated outward and inward currents. In very few cells (~3%) only a rapidly activating and inactivating inward current (transient current) was activated (Fig. 1). In ~14% of cells, in addition to the transient inward current, a large slowly activating and inactivating inward current (slow current) could be activated as well (Fig. 2). In the majority of cells, one large inward current that appeared to have two components was observed (Fig. 3).

Examples of current traces recorded with a 333-ms command pulse, holding potential of −80 mV, and depolarizing voltage steps between −60 and +80 mV are shown in Figs. 1A, 2A, and 3A. In Figs. 1–3, the insets show sections of current traces obtained with a −40-mV voltage step presented on an expanded scale. Figure 3A, inset, shows a current trace that has two components, suggesting that the tracing is the result of combinations of transient and slowly activating inward currents. To determine the inactivation characteristics of the slowly activating/inactivating current, the cells were activated with longer command pulses (10.5-s command pulse from a holding potential of −80 mV to a depolarizing voltage step of −40 mV), and the results are shown in Figs. 1B, 2B, and 3B. The current traces shown in Figs. 1B, 2B, and 3B were recorded in cells from which currents in Figs. 1A, 2A, and 3A were recorded.

When observed together, the amplitude of the transient inward current was always smaller than that of the slowly activating/inactivating current. The larger of the two inward currents could be elicited at potentials between −60 and −50 mV, and it required 250–800 ms to reach peak amplitude and 2–3 s to fully inactivate (Fig. 2, A and B), whereas the fast-activating transient inward current required 15–30 ms to reach a peak (Fig. 1A, inset). The outward current was elicited at test potentials positive to −20 mV and displayed outward rectification. It was slowly activating and showed no sign of inactivation during the depolarizing test pulses.

Ionic basis of the membrane currents. The granulosa cells incubated on plastic in serum-free medium expressed a fast-activating/inactivating (transient) inward current or a slowly activating/inactivating inward (slow) current in combination with the transient current or a single large inward current that has two components. These inward currents could result from
The outflow of Cl\textsuperscript{−} or inflow of Ca\textsuperscript{2+} among other possibilities. Experiments were conducted to determine the carrier of the inward currents in cells that expressed only the large inward current. In such cells, the reduction of Cl\textsuperscript{−} content of the external solution resulted in a shift of the reversal potential of the slowly activating inward current toward the Cl\textsuperscript{−} equilibrium (Fig. 4, A–C, n = 4 cells). In addition, the slowly activating inward current was suppressed by the Cl\textsuperscript{−} channel blockers DIDS (0.5 mM; n = 4 cells; Fig. 4D) and NPPB (20 μM; n = 3 cells, data not shown) in granulosa cells incubated on plastic, suggesting that the slow inward current is a Cl\textsuperscript{−} current (caused by outflow of Cl\textsuperscript{−}). A residual transient current remained after the application of the Cl\textsuperscript{−} channel blocker (Fig. 4D, inset). The slowly activating inward current was also eliminated completely in Ca\textsuperscript{2+}-deficient bath solution in cells that expressed only one inward current (n = 5 cells; Fig. 5, A–C), and the general Ca\textsuperscript{2+} antagonist, cobalt, suppressed the slowly activating inward current reversibly (n = 4 cells; Fig. 5D). In addition, a combination of the T-type Ca\textsuperscript{2+} channel inhibitor (flunarizine) and L-type Ca\textsuperscript{2+} channel inhibitor (nifedipine) suppressed the slowly activating inward current reversibly (n = 4 cells; Fig. 5E). The result shown in Fig. 5 indicates that the slowly activating inward current, carried by Cl\textsuperscript{−}, was dependent on extracellular Ca\textsuperscript{2+}. It was hypothesized that the large inward current is made up of a Ca\textsuperscript{2+}-carried transient current and a Ca\textsuperscript{2+}-dependent slowly activating Cl\textsuperscript{−} current. To test this hypothesis, experiments were conducted with cells that expressed only one inward current in Ba\textsuperscript{2+}-containing Ca\textsuperscript{2+}-deficient solution; the transient inward current persisted; however, the larger component of the inward current was eliminated (Fig. 6, A–C), proving that the single large inward current is composed of the fast and slowly activating and inactivating currents. This result also showed that the fast-activating inward current was carried by Ca\textsuperscript{2+}. (The persistence of the transient inward current was caused by the conductance of Ba\textsuperscript{2+} through specific Ca\textsuperscript{2+} channels, whereas the component of the inward current, which required the availability of external Ca\textsuperscript{2+}, was eliminated.) To substantiate this finding, additional experiments were conducted with cells that expressed only one inward current in Ba\textsuperscript{2+}-containing Ca\textsuperscript{2+}-deficient solution; the transient inward current persisted; however, the larger component of the inward current was eliminated (Fig. 6, A–C), proving that the single large inward current is composed of the fast and slowly activating and inactivating currents.
conducted with cells that expressed both fast-activating (transient) and slowly activating inward currents in Ba\(^{2+}\)-containing Ca\(^{2+}\)-deficient solution (Fig. 6, D-F). The substitution of Ba\(^{2+}\) for Ca\(^{2+}\) in the bath solution resulted in the persistence of the fast-activating transient inward current and the loss of the slowly activating/inactivating current in a reversible manner, proving that the fast-activating/inactivating current was carried by Ca\(^{2+}\). Experiments were also conducted to determine the effect of chelation of intracellular Ca\(^{2+}\) on the inward current. The addition of 5 mM EGTA to the pipette solution had no appreciable effect on the activation of the Ca\(^{2+}\)-dependent Cl\(^{-}\) current \((n = 3, \text{ data not shown})\). This result is consistent with extensive literature showing that the kinetics of Ca\(^{2+}\) buffering by EGTA is slow relative to the kinetics of Ca\(^{2+}\) binding to many Ca\(^{2+}\)-activated channels.

The outward current was eliminated when Cs\(^{+}\) was substituted for K\(^{+}\) in the pipette solution (Fig. 7), indicating that the outward current was carried by K\(^{+}\).
Thus the prominent currents expressed in granulosa cells incubated on plastic in serum-free medium are the following: a fast-activating/inactivating (transient) inward Ca\(^{2+}\) current; a slowly activating/inactivating (slow) inward Ca\(^{2+}\)-dependent Cl\(^{-}\) current; and a slowly activating outward K\(^{+}\) current.

**Effect of intact basal lamina on membrane currents and capacitance.** Differentiated granulosa cells incubated on intact basal lamina for 24 h in serum-free medium 199 assumed a spherical shape (with reduced plasma membrane area), whereas those incubated on plastic in control dishes became highly flattened with increased total plasma membrane area (data not shown). The cells were stimulated with a 333-ms test pulse from a holding potential of −80 mV to depolarizing voltage steps between −60 and +80 mV. The amplitudes of both inward and outward currents were measured at the end of the step potentials. The slow inward current (Ca\(^{2+}\)-dependent Cl\(^{-}\) current) was suppressed in differentiated granulosa cells incubated on basal lamina for 24 h (Fig. 8, compare A and B). The cells were also stimulated with a 10.5-s command pulse from a holding potential of −80 to −40 mV, and the peak of the inward current was regarded as the amplitude of the slow (Cl\(^{-}\)) inward current (it was measured 250–800 ms after the onset of the command pulse to exclude the amplitude of the transient current). The peak amplitude of the slow inward current was significantly (P < 0.001) greater for cells grown on plastic than for cells grown on basal lamina (Fig. 8, compare C and D). When the current amplitude was normalized to plasma membrane area, the current density of granulosa cells incubated on basal lamina (n = 43 cells) was significantly (P < 0.001) smaller than that for cells grown on plastic (n = 37 cells; Fig. 9, A and B). Basal lamina had no significant effect on the outward K\(^{+}\) current under conditions in which it suppressed the slow inward current (Fig. 8, A and B; also Fig. 9, C and D). In the data shown in Fig. 9, C and D, the amplitudes of the currents were measured at the end of the +80-mV step (with a 333-ms stimulus). Membrane capacitance, an indirect measure of the total plasma membrane area, was significantly (P < 0.01) smaller for cells cultured on intact basal lamina (n = 43 cells) than for the control cells incubated on plastic (n = 37 cells; Fig. 9E). The greater the total plasma membrane area, the larger the membrane capacitance.

**Influence of storage on the effect of intact basal lamina on membrane capacitance and currents.** Membrane capacitance and currents were recorded in differentiated granulosa cells incubated in intact basal lamina-containing culture dishes that had been stored for 12 mo or longer, and the results are shown in Fig. 10. The membrane capacitance of cells incubated on intact basal lamina stored for 12 mo (n = 11 cells) was significantly (P < 0.01) smaller than that in the cells incubated on plastic (n = 7 cells; Fig. 10A). Also, the density of the inward Ca\(^{2+}\)-dependent Cl\(^{-}\) current in control cells (n = 7 cells) was significantly (P < 0.05) larger than that of cells incubated on the basal lamina stored for 12 mo (n = 11 cells; Fig. 10B).

**Relationship between membrane capacitance (cell shape) and current.** Because of the apparent consistent relationship between the inward Ca\(^{2+}\)-dependent Cl\(^{-}\) current and membrane capacitance in response to basal lamina, correlative decreases in these two parameters in individual cells were estimated. The amplitudes or densities of the inward current of granulosa
cells incubated on plastic and basal lamina were plotted vs. their respective membrane capacitances (Fig. 11). The slopes of the linear regression were not different from zero ($P > 0.05$), suggesting that no correlation existed between the amplitudes or densities of the inward current and the membrane capacitance (cell shape) in granulosa cells incubated on plastic or on basal lamina. Moreover, the relative change in ampli-

Fig. 7. Isolation of membrane currents. Holding potential was $-80$ mV, and test potentials were between $-60$ and $+80$ mV with a 333-ms command pulse. The amplitudes of both $K^+$ and $Cl^-$ currents were measured at the end of the command pulse. A: $Cs^+$ was substituted for $K^+$ in pipette solution, and the bath solution contained 10 mM TEA to block $K^+$ current (inward $Cl^-$ current was isolated). B: $Ca^{2+}$ was excluded from bath solution to eliminate inward $Ca^{2+}$-dependent $Cl^-$ current (outward $K^+$ current was isolated). C: current-voltage curve of the isolated inward $Cl^-$ and outward $K^+$ currents (the inward $Cl^-$ currents were recorded under conditions described in A, and outward $K^+$ currents were recorded under conditions described in B). Each point is the mean $\pm$ SE ($n = 7$ for both $Cl^-$ and $K^+$ currents).

Fig. 8. Effect of freshly prepared intact basal lamina on membrane currents in differentiated granulosa cells. Granulosa cells isolated from the largest preovulatory follicle of hen ovary were incubated on plastic or intact basal lamina in serum-free medium 199 for 24 h. The granulosa cells were stimulated from a holding potential of $-80$ mV to test potentials between $-60$ and $+80$ mV in 20-mV steps for 333-ms duration (A and B). The amplitudes of both inward and outward currents were measured at the end of the step potentials, and data were used to construct the current-voltage relationship curves (E). C and D: cells were stimulated from a holding potential of $-80$ to a $-40$-mV test potential, with 10.5-s command pulse. A and C: cells incubated on plastic (control). B and D: cells incubated on basal lamina. E: current-voltage relationship of whole cell currents. Each point is the mean $\pm$ SE of 26 cells (control) and 16 cells (basal lamina).
Fig. 9. Effect of freshly prepared intact basal lamina on cell membrane capacitance, whole cell current amplitude, and current density. Granulosa cells isolated from the largest preovulatory follicle of hen ovary were incubated on plastic or intact basal lamina in serum-free medium 199 for 24 h. A: cells were stimulated from a holding potential of −80 mV to a voltage step of +40 mV test potential with 10.5-s command pulse. The peak amplitude of the slow (Cl<sup>−</sup>) inward current was measured 250–800 ms after the onset of the command pulse (to exclude the amplitude of the transient current). B: inward current density (amplitude normalized to capacitance). C: cells were stimulated with a 333-ms test pulse from a holding potential of −80 mV to a voltage step of +80 mV. The amplitude of the outward current was measured at the end of the step potential. D: outward current density (amplitude normalized to capacitance). E: cell membrane capacitance. Each point is the mean ± SE (n = 37 cells for control; n = 43 cells for basal lamina). *P < 0.05 vs. respective control.

tudes of the inward current (70–85%) in cells incubated on basal lamina was about threefold that of the change in membrane capacitance (−22%; see Fig. 9, A and E). These results suggest that changes in cell shape alone cannot account for the observed effects of basal lamina on the inward current.

Time course of effect of intact basal lamina on membrane currents. Time course experiments revealed that it required >9 h of incubation of differentiated granulosa cells on plastic to observe the slowly activating/inactivating inward current (Fig. 12). The current was not detectable after 1 h of incubation on plastic; it was expressed modestly after 6 and 9 h of incubation. The inward current was highly expressed after 24 h of incubation on plastic. The current was attenuated in granulosa cells incubated on basal lamina at all time points tested (Fig. 12). Thus basal lamina appeared to either suppress the upregulation of the slowly activating/inactivating inward current or to inhibit the formation of the current-conducting channels.
Effect of solubilized basal lamina on membrane currents. In additional experiments, basal lamina was solubilized to enable the assessment of different amounts of the matrix material on membrane currents. Differentiated granulosa cells were incubated on coverslips precoated with different amounts (5 and 15 μg/cm²) of solubilized basal lamina (see MATERIALS AND METHODS). Similar to observations made for granulosa cells incubated on intact basal lamina, granulosa cells incubated on coverslips precoated with solubilized basal lamina have reduced membrane capacitance and inward current density (Fig. 13). The effects of solubilized basal lamina were concentration dependent; the current density was reduced 70 and 98%, respectively, by 5 and 15 μg/cm² of solubilized basal lamina.

Effects of cycloheximide and actinomycin D on the slow inward current. The effects of the protein synthesis inhibitor cycloheximide and transcription inhibitor actinomycin D on the slow inward current were tested. Differentiated granulosa cells were incubated on plastic and intact basal lamina for 24 h, and membrane currents were recorded. The cells were stimulated with

Fig. 10. Effect of intact basal lamina stored for 12 mo on cell membrane capacitance and whole cell current density. Granulosa cells isolated from the largest preovulatory follicle of hen ovary were incubated on plastic or intact basal lamina that had been stored at 4°C for 12 mo in serum-free medium 199 for 24 h. Cells were stimulated from holding potential of −80 mV to a −40-mV test potential with 10.5-s command pulse. The peak amplitude of the slow (Cl⁻) inward current was measured 250–800 ms after the onset of the command pulse (to exclude the amplitude of the transient current). A: cell membrane capacitance. B: inward Cl⁻ current density. Each point is the mean ± SE (n = 7 cells for control; n = 11 cells for basal lamina). *P < 0.05 vs. respective control.

Fig. 11. Relationship between the amplitude or density of the inward Cl⁻ current and membrane capacitance of cells incubated on either control or basal lamina. Granulosa cells isolated from the largest preovulatory follicle of hen ovary were incubated on plastic or intact basal lamina in serum-free medium 199 for 24 h. Cells were stimulated from a holding potential of −80 mV to a −40-mV test potential with 10.5-s command pulse. The peak amplitude of the slow (Cl⁻) inward current was measured 250–800 ms after the onset of the command pulse (to exclude the amplitude of the transient current). A: current amplitude vs. membrane capacitance. B: current density vs. membrane capacitance. Each point is datum from a single cell. The slope of the linear regression for cells incubated on either control or basal lamina was not significantly different from zero.
a 10.5-s command pulse from a holding potential of −80 to −40 mV, and the peak amplitude of the slow (Cl⁻) inward current was measured 250–800 ms after the onset of the command pulse (to exclude the amplitude of the transient current). Both cycloheximide (Fig. 14) and actinomycin D (Fig. 15) inhibited the expression of the slow inward Cl⁻ current in cells incubated on plastic. Both agents also augmented the inhibitory effects of basal lamina on the slow inward Cl⁻ current (Figs. 14 and 15). It is noteworthy that cycloheximide or actinomycin did not inhibit the expression of the transient inward (Ca²⁺) current in granulosa cells incubated in the absence or presence of basal lamina (Fig. 14, B and D, and Fig. 15, B and D).

DISCUSSION

The results of the present study show that basal lamina of the preovulatory follicle regulates transmembrane ion transport in differentiated chicken granulosa cells in a homologous system. Differentiated chicken granulosa cells expressed inward and outward whole cell currents. The Cl⁻ channel inhibitors DIDS and NPPB suppressed the inward current, suggesting that a major component of this current was carried by Cl⁻. The activation of this putative inward Cl⁻ current appears to be dependent on Ca²⁺ because the removal of the divalent cation from the external medium also resulted in the suppression of the inward current.

Results of the present study confirm the earlier report of Mealing et al. (36) that differentiated chicken granulosa cells express an inward Ca²⁺-dependent Cl⁻ whole cell current. The present results also confirm the earlier observation that differentiated chicken granulosa cells express a transient inward current characteristic of T-type Ca²⁺ current (16, 36, 49). Furthermore, the outward current observed in the present study has the characteristics of previously described delayed-rectifier K⁺ current in chicken (16, 43, 46) and swine (29) granulosa cells.

In a recent study, a transient outward Cl⁻ current could be activated in differentiated chicken granulosa cells exposed to Ca²⁺-deficient solution with step potentials between +80 and +120 mV from a holding potential of −80 mV (43). The transient outward Cl⁻ current was not observed in the present study, presumably because different step voltage protocols were used; step potentials between −60 and +80 mV from a holding potential of −80 mV were employed in the present study.

The observations that membrane capacitance (an indirect measure of total plasma membrane area) was smaller for granulosa cells incubated on basal lamina support the results of a previous study in which morphometric measurements (of cell area, perimeter, and circularity) were used to demonstrate the effects of basal lamina (intact and solubilized) on the morphology of granulosa cells (4); the present and previous studies (4) confirm the observation that basal lamina causes granulosa cells to become rounded. It was demonstrated that shape per se can regulate cellular functions in certain instances (15, 26). For example, cell shape determined whether capillary endothelial cells from human and bovine origin would undergo apoptosis (15). In addition, cell shape modulated the control of cell cycle progression in human capillary endothelial cells (26). This raised the possibility that the observed effects of basal lamina on inward Ca²⁺-dependent Cl⁻ current in this study are consequences of cell shape or that changes in shape or reduction in membrane area of granulosa cells can account for the observed effects of basal lamina on the amplitude of the inward current.

Because granulosa cells are exposed to the complex intact basal lamina as a unit in vivo (but not individual components in isolation), the effect of basal lamina was examined as a unit. It is presumed that the action of basal lamina monitored here is the result of the combined effects of different components of the basal lamina. The nature of the components of basal lamina that regulated the inward currents and their mechanisms of action are yet to be determined. Some of the components are likely to stimulate cellular processes that other components suppress, although a few components would be without effect. Therefore, the actions of the individual components of basal lamina may be synergistic, additive, antagonistic, or neutral, as the case may be. It was shown recently that the basal lamina of the chicken ovarian follicle reacted positively to antibodies raised against extracellular matrix proteins (type IV collagen, laminin, entactin, heparan sulfate proteoglycan, and fibronectin), growth factors (epidermal growth factor, platelet-derived growth factor, basic and acidic fibroblast growth factor, insulin-
like growth factor, transforming growth factor-α, transforming growth factor-β, cytokines, matrix metalloproteinases, and their tissue inhibitors (8). The possibility remains that these bioactive molecules acted concomitantly to regulate the Cl\(^{−}\)-carried inward currents in the present studies.

The inhibitory actions of intact basal lamina under the present experimental conditions were exerted on the inwardly directed Ca\(^{2+}\)-dependent Cl\(^{−}\) current. The ability of the basal lamina to influence the membrane current was not affected by solubilization because the total fraction of solubilized basal lamina (containing all components with molecular mass >3 kDa) also suppressed the Ca\(^{2+}\)-dependent Cl\(^{−}\) whole cell current in granulosa cells in a dose-dependent manner. The concentrations of solubilized basal lamina used in the present study were shown to significantly regulate progesterone production in avian granulosa cells (7).

The observations that cycloheximide and actinomycin D suppressed the Ca\(^{2+}\)-dependent Cl\(^{−}\) inward current in control cells suggest that the slow inward (Cl\(^{−}\)) currents in cells incubated on plastic were upregulated by processes that involved gene transcription and protein synthesis. Because cycloheximide or actinomycin D had little or no effect on the transient inward current, the actions of the two inhibitors may not be the result of general inhibition of the cellular processes. The requirement of at least a 12-h exposure of granulosa cells to the basal lamina preparations to result in the inhibition of the slow inward Cl\(^{−}\) current suggests that the action of basal lamina is the result of a long-term (several hours to days) effect of the matrix material. Although an acute (μs to a few min) effect of basal lamina on the inward Cl\(^{−}\) current should not be ruled out, the action of basal lamina in the present study is consistent with a long-term effect. The possibility remains that basal lamina reduced the number of Cl\(^{−}\) channel proteins. Indeed, cycloheximide and actinomycin D blocked the slow inward Cl\(^{−}\) current in the presence of basal lamina. Although these results do not prove that basal lamina has a direct effect on the

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**Fig. 13.** Effect of solubilized basal lamina on cell membrane capacitance and inward current density. Granulosa cells isolated from the largest preovulatory follicle of hen ovary were incubated on glass coverslips precoated with solubilized basal lamina in serum-free medium 199 for 24 h. Cells incubated on bare coverslips served as controls. Cells were stimulated from a holding potential of −80 mV to a −40-mV test potential with 10.5-s command pulse. The peak amplitude of the slow (Cl\(^{−}\)) inward current was measured 250–800 ms after the onset of the command pulse (to exclude the amplitude of the transient current). Each point is the mean ± SE. A and B: 5 μg/cm\(^{2}\) basal lamina protein (n = 13 cells for control, n = 12 cells for basal lamina). C and D: 15 μg/cm\(^{2}\) basal lamina protein (n = 11 cells for control, n = 11 cells for basal lamina). *P < 0.05 vs. respective control.
synthesis of channel proteins, they do not negate the possibility that basal lamina reduced the number of Cl⁻ channel proteins. The mechanisms that subserve the actions of basal lamina are yet to be determined.

The results of a recent study showed that both intact and solubilized basal lamina regulated the shape of differentiated chicken granulosa cells (4). In additional studies, solubilized basal lamina (5 and 15 μg/cm²) regulated progesterone production by differentiated chicken granulosa cells in a dose-dependent fashion (7). The previous and present results indicate that the effects of basal lamina on metabolic functions of granulosa cells are associated with the regulation of the shape and transmembrane ion transport of these cells. An interesting finding of the present study is that the storage of basal lamina for 12 or more months at 4°C did not affect its ability to regulate the inward Cl⁻ current. In relation to this observation is the previous finding that basal lamina stored for 18 mo or longer regulated the shape of granulosa cells similar to freshly prepared basal lamina (4).

How do the present observations fit in the known metabolic functions of differentiated avian granulosa cells? The granulosa cells used in the present study are differentiated ones isolated from the largest (F₁) pre-ovulatory follicles of hen ovary. These cells are known to produce large amounts of progesterone (a differentiation marker in avian granulosa cells; see Refs. 7 and 39). Because solubilized basal lamina suppressed progesterone synthesis in differentiated granulosa cells (7) and because Cl⁻ was shown to be required for progesterone synthesis in chicken granulosa cells (37), it is possible that the regulation of transmembrane Cl⁻ transport is a component of the mechanisms whereby basal lamina suppressed progesterone production in differentiated granulosa cells (7). It is noteworthy that the suppression of steroidogenesis in differentiated granulosa cells exposed to solid basement membrane protein matrixes is not limited to avian granulosa cells. Notably, Aten et al. (10) observed that rat granulosa cells incubated on matrigel matrix, basement membrane reconstituted from extracts of Engelbreth-Holm-Swarm tumor, produced less progesterone than those incubated on plastic. In addition, progesterone production by rat granulosa cells in laminin-coated wells was decreased according to Aharoni et al. (1). In other studies, when human granulosa cells retrieved from patients undergoing in vitro fertilization were cultured in laminin-coated wells, steroidogenesis was suppressed (22). Similarly, human chorionic gonadotropin-induced progesterone synthesis was attenuated in laminin-coated dishes (22). Therefore, reports in the literature indicate that the incubation of granulosa cells in dishes precoated with reconstituted or components of basement membranes or basal lamina results in the reduction of steroid hormone synthesis.

![Fig. 14. Effects of cycloheximide on the Ca²⁺-dependent Cl⁻ current density. Differentiated chicken granulosa cells were incubated on plastic or intact basal lamina in serum-free medium 199 for 24 h in the absence and presence of 5 μg/ml cycloheximide. Membrane potential was held at −80 mV and stepped to −40 mV with a 10.5-s command pulse. The voltage protocol is shown above the tracings. A: control. B: cycloheximide. C: basal lamina. D: cycloheximide and basal lamina. E: peak amplitude of the slow (Cl⁻) inward current measured 250–800 ms after the onset of the command pulse. Each point is the mean ± SE (n = 9 for control, n = 9 for cycloheximide; n = 7 for basal lamina, n = 6 for basal lamina + cycloheximide).](http://ajpcell.physiology.org/)

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present data support the hypothesis that Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} currents are low in differentiated granulosa cells in vivo. The data also support the hypothesis that differentiated granulosa cells that are not in contact with basal lamina or extracellular matrix in vivo express Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} currents, most likely in association with aberrant metabolic functions.

Because in the present study the Cl\textsuperscript{−}-carried inward current was expressed in granulosa cells that were spread/stretching on plastic, it could be argued that the extensive spreading/stretching was responsible for the observed increased transmembrane ion transport in granulosa cells incubated on plastic; perhaps the expression of the inward Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} current in granulosa cells is a stretch-sensitive or a stretch-associated phenomenon. It was shown that solid elements of the extracellular matrix exert mechanical stress on cells (28, 51). The nature or type of response to stress is known to be influenced by the source of stress; for example, the response of a cell to stress induced by osmotic, hydrostatic pressures and shear and gravity forces would be expected to be different from that caused by the extracellular matrix. It follows that the response of granulosa cells to stress caused by spreading on plastic would be different from that caused by extracellular matrix, such as basal lamina (51). Although mechanical stress imposed by extracellular matrix results in the regulation of cell function, presumably via tensegrity (tensional integrity) architecture (18, 28), the nature of the mechanoreceptors that convert the mechanical stress to intracellular signals is not well defined. Mechanical stress from the extracellular matrix can be transmitted via integrins coupled to cytoskeleton but not through nonadhesion receptors (50, 51). [Integrins are a large family of heterodimeric transmembrane proteins with different \(\alpha\)- and \(\beta\)-subunits that function as extracellular matrix adhesion receptors (27).] In addition, stretch-sensitive ion channels located in the plasma membrane are among the initial sites of action of mechanical stress (30, 40). Therefore, integrins and ion channels may serve as transducers of mechanical stress (mechanotransducers) imposed by different means.

In a tensionally integrated cytoskeleton, the transfer of external mechanical force (mechanotransduction) can cause rearrangements of the cytoskeleton simultaneously at multiple sites in the cell to enable rapid transmission of the mechanical signal, resulting in changes in cellular morphology and function (50). Thus the initial perception of mechanical stress at the cell surface is transmitted to the components of the cytoskeletal system and then to other signaling effectors. Stress-specific Ca\textsuperscript{2+} channels were activated by stretch in vascular endothelial cells (30). Mechanical force activated stress-sensitive K\textsuperscript{+} currents in vascular endothelial cells (40), and stretch also activated the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in cardiac cells (53).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig15}
\caption{Effects of actinomycin D on Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} current density. Differentiated chicken granulosa cells were incubated on plastic or intact basal lamina in serum-free medium 199 for 24 h in the absence and presence of 8 \(\mu\)g/ml actinomycin D. Membrane potential was held at \(-80\) mV and stepped to \(-40\) mV with a 10.5-s command pulse. The voltage protocol is shown above the tracings. A: control. B: actinomycin D. C: basal lamina. D: actinomycin D and basal lamina. E: peak amplitude of the slow (Cl\textsuperscript{−}) inward current measured 250–500 ms after the onset of the command pulse. Each point is the mean \(\pm\) SE (\(n = 11\) for control, \(n = 5\) for actinomycin D, \(n = 9\) for basal lamina, \(n = 5\) for basal lamina + actinomycin D).
}
\end{figure}
Extracellular matrix proteins have been shown to regulate ion transport in different types of cells. For example, inward whole cell Na⁺ currents were reduced significantly in rat adult alveolar type II cells cultured on fibronectin-coated coverslips for 48 h (54). Also, rat adult alveolar type II cells cultured on human amniotic basement membrane had smaller short-circuit currents than cells cultured on collagen substrate (19). Furthermore, matrigel (reconstituted basement membrane) modulated K⁺ current density in fetal rat alveolar type II cells (34).

In summary, the unique anatomic structure of the avian ovarian follicle enabled the isolation of pure and intact basal lamina (basement membrane) and its associated granulosa cells and provided the opportunity for the study of the effect of basal lamina on membrane currents in the granulosa cells in a homologous system. Under the quasiphysiological conditions applied, basal lamina regulated the membrane ion transport in differentiated granulosa cells. It suppressed a stretch-sensitive Ca²⁺-dependent Cl⁻ whole cell current. The observed effects of basal lamina may be a component of the mechanisms that subserve the reported actions of basement membranes or other matrix materials on the metabolic functions of differentiated avian, mammalian, and human granulosa cells.

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