Molecular Basis of Voltage-Dependent Potassium Currents in Porcine Granulosa Cells

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ABSTRACT

The major objective of this study was to elucidate the molecular bases for K⁺ current diversity in porcine granulosa cells (GC). Two delayed rectifier K⁺ currents with distinct electrophysiological and pharmacological properties were recorded from porcine GC by using whole-cell patch clamp: 1) a slowly activating, noninactivating current (Iₛ) antagonized by clofilium, 293B, L-735,821, and L-768,673; and 2) an ultrarapidly activating, slowly inactivating current (Iₚₛ) antagonized completely by clofilium and 4-aminopyridine and partially by tetraethylammonium, charybdotoxin, dendrotoxin, and kalitoxin. The molecular identity of the K⁺ channel genes underlying Iₛ and Iₚₛ was examined using reverse transcription-polymerase chain reaction and immunoblotting to detect K⁺ channel transcripts and proteins. We found that GC could express multiple voltage-dependent K⁺ (Kv) channel subunits, including KCNQ1, KCNE1, Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv1.6, Kvβ1.3, and Kvβ2. Coimmunoprecipitation was used to establish the hetero-oligomeric nature of granulosa cell Kv channels. KCNE1 and KCNQ1 were coassiated in GC, and their expression coincided with the expression of Iₛ. Extensive coassociation of the various Kv α- and β-subunits was also documented, suggesting that the diverse electrophysiological and pharmacological properties of Iₛ currents may reflect variation in the composition and stoichiometry of the channel assemblies, as well as differences in post-translational modification of contributing Kv channel subunits. Our findings provide an essential background for experimental definition of granulosa K⁺ channel function(s). It will be critical to define the functional roles of specific GC K⁺ channels, because these proteins may represent either novel targets for assisted reproduction or potential sites of drug toxicity.

Granulosa cells (GC) surround the oocyte within the ovarian follicle and play an essential role in creating the conditions required for follicular development, ovulation, fertilization, and implantation (Salustri et al., 1993). During folliculogenesis, GC undergo a series of mitotic divisions (proliferation) then acquire gonadotropin receptors and enhanced steroidogenic activity (differentiation). Autocrine-paracrine and endocrine regulation of the granulosa cell maturation have been extensively investigated and much is known about the specific roles of various growth factors, hormones, transmembrane receptors, and second messengers (Steele and Leung, 1993). In contrast, the functional significance of voltage-dependent ion channels in GC is far from understood.

It has been reported that GC can generate action potentials (Mealing et al., 1994), and indirect evidence suggests that modulation of granulosa cell electrical activity may provide a means to regulate cell function (Mattioi et al., 1990, 1991, 1993; Kusaka et al., 1993). For example, granulosa cell depolarization has been described as a consistent feature of oocyte maturation in different experimental systems (Mattioi et al., 1990). Voltage-gated potassium currents with distinct electrophysiological and pharmacological properties have been described in both acutely isolated and cultured GC, and have been shown to regulate granulosa cell resting membrane potentials.
membrane potential (Mattioli et al., 1991, 1993; Kusaka et al., 1993). Delayed rectifier currents with both slow and rapid activation and inactivation kinetics have been described in porcine GC (Mattioli et al., 1991, 1993; Kusaka et al., 1993). However, the molecular correlates of these currents have not been identified.

The major objective of this study was to elucidate the molecular bases for $K^+$ current diversity in porcine GC. Specific antibodies were used to demonstrate directly the existence in GC of Kv1 (KCNA) and KCNQ1 (KvLQT1) channel proteins, as well as Kv6 (KCNAB) and KCNE1 (minK or IsK) auxiliary subunits. Coimmunoprecipitation was used to establish the hetero-oligomeric nature of granulosa cell $K_v$ channels. Whole-cell patch-clamp techniques were used to record granulosa cell $K^+$ currents and to document the specific effects of a variety of $K^+$ channel antagonists.

We report not only that a variety of voltage-gated $K^+$ channel $\alpha$- (pore-forming) and $\beta$- (accessory) subunits are present in freshly isolated GC but also that their expression is temporally regulated as GC spontaneously differentiate (luteinize) in culture. Our data suggest that the diverse electrophysiological and pharmacological properties of native granulosa cell $K^+$ currents, described here and elsewhere (Mattioli et al., 1991, 1993; Kusaka et al., 1993), may reflect variation in the composition and stoichiometry of the hetero-oligomeric channel complexes, as well as differences in post-translational modification of contributing channel subunits. The documented potential for dynamic interchange of various associated $K^+$ channel subunits as a function of the cells’ metabolic status suggests that these ion channels may participate in control of granulosa cell proliferation or differentiation.

Materials and Methods

Reagents. Cell culture media, supplements, and sera were obtained from Invitrogen (Carlsbad, CA), unless stated otherwise. Chemicals were obtained from Sigma Chemical (St. Louis, MO), unless stated otherwise. Regular pork insulin and LY-97241 were obtained from Merck Research Laboratories (Westpoint, PA). HEK-293 cells, and cultured at 37°C for 5 min. Cells were washed twice with a 1:1 mixture of Ham’s F-10 nutrient medium and Dulbecco’s modified Eagle’s medium (DMEM) containing HEPES (25 mM), penicillin (50 U/ml), streptomycin (50 $\mu$g/ml), Gentamicin (57 ng/ml), and amphotericin (2.5 $\mu$g/ml). Freshly isolated GC were plated on collagen-coated 24-well plates at a density of 2 to 3 $\times$ 10^5 cells, and cultured at 37°C in humidified atmosphere containing 5% CO_2. Culture media were changed 16 to 20 h after plating, and replaced at 24-h intervals thereafter. In one series of experiments, GC were incubated for 4 h in methionine-free DMEM containing 100 $\mu$Ci/ml [35S]methionine (PerkinElmer Life Sciences, Boston, MA) before harvest.

Electrophysiological Recordings. Membrane currents were recorded using standard patch-clamp procedures in the whole-cell configuration. Voltage-clamp protocols and solutions for measuring slow (I_Ks) and ultrarrapid (I_Kur) cardiac delayed rectifier currents under whole-cell recording conditions have been described in detail previously (Arena and Kass, 1988; Nattel et al., 1999); similar protocols were used to elicit granulosa cell currents. Briefly, recording pipettes were pulled to resistances of 2.5 to 6 M$$\Omega$ when filled with intracellular (pipette) solution containing 110 mM potassium aspartate, 1 mM MgCl_2, 11 mM EGTA, 1 mM CaCl_2, 10 mM HEPES, 10 mM K_2ATP, pH 7.3, attained by addition of 1 N KOH to bring the final potassium concentration to 140 mM. Extracellular recording (bath) solution consisted of 132 mM NaCl, 1.2 mM MgCl_2, 1 mM CaCl_2; 5 mM glucose; 0.0, 0.5, or 4.8 mM KCl; 10 mM HEPES, pH adjusted to 7.4 with NaOH. The reference electrode was an Ag/AgCl half-cell immersed in the pipette solution and connected to the bath via a 3 M KCl-agar salt bridge. Tip potentials were zeroed before seal formation. All recordings were performed at room temperature (22–24°C) from a Plexiglas chamber mounted on an inverted microscope (Nikon Diaphot 300; Nikon, Tokyo, Japan). Data acquisition and analysis were accomplished using an IBM compatible computer interfaced to an Axopatch 200-A amplifier driven by pClamp software (Axon Instruments, Union City, CA). The standard voltage-clamp protocol for activation of I_Ks consisted of a series of 1- to 4-s depolarizing test pulses from a holding potential of −40 mV to test potentials (V_h) ranging from −40 to +60 mV at an interpulse interval of 14 s. I_Kur was elicited from holding potentials of either −80 to −40 mV by 80- to 1000-ms depolarizing test pulses to V_h between −40 and +60 mV.

The resting membrane potential of freshly isolated porcine GC averaged −38.4 ± 4.3 mV ($n = 8$). Granulosa cell membrane capacitance, calculated as the time integral of the capacitive response to a 5-mV hyperpolarizing step from a −40-mV holding potential was 16.1 ± 2.8 pF ($n = 8$). Series resistance, estimated by dividing the time constant of the decay phase of the uncompensated capacitive transient by the calculated membrane capacitance, was 2.6 ± 0.9 M$$\Omega$ and was electrically compensated to minimize the duration of the capacity transient (>90%). Peak currents used to derive activation curves did not exceed 300 pA; therefore, the voltage errors associated with uncompensated series resistance never exceeded 1 mV.

I_Kur was measured as the current level at the end of a depolarizing pulse relative to the zero current level. I_Ks amplitude was similarly determined from the amplitudes of the tail currents recorded on
return to the holding potential. The voltage dependence of channel activation was determined by calculating normalized peak conductance values from the peak current amplitudes at different potentials, and fitting the data with a Boltzmann distribution of the following form: $G/G_{\text{max}} = 1/(1 + e^{(V_{1/2} - V_i)/k})$, where $V_{1/2}$ is the half-activation voltage and $k$ is the slope factor for the activation curve.

Reverse Transcription-Polymerase Chain Reaction. mRNA was isolated from fresh GC by using a Micro-Fast Track kit (Invitrogen). Reverse transcription was performed using random hexamers and enhanced avian reverse transcriptase (Sigma Chemical) under recommended conditions. PCR detection of KCNE1 was performed using gene-specific oligonucleotide primers designed to amplify a 170-bp fragment of sense: 5'-ACCTGGCCCATCCTGCTGAGT-3'; antisense: 5'-TGGCCGGCTGTCTTTCAATGAC-3'. Four additional primers were used for nested PCR designed to amplify an ~180-bp KCNE1 product for the outer reaction [5'-AGACTGGAACCACCTAC-3'; 5'-CTCCAGAACCAGGGCCTG-3'], and an ~110-bp KCNE1 product for the inner reaction [5'- GGTTTCTCACCCTGCGGC-3'; 5'- CTTTCCTGGCAGGCTT-3']. The PCR reaction mixtures (25 μl) contained 0.4 mM each 5' and 3' primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP, and 1 unit of REDTaq DNA polymerase (Sigma). These PCR reactions were amplified for 30 cycles consisting of denaturation for 30 s at 94°C, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The PCR product was cloned into Invitrogen cells by using an Original TA Cloning kit (Invitrogen) and sequenced using T7 and M13 primers.

A similar PCR protocol was used to detect mRNA for Kv β-subunits. Primer sets were identical to those used previously to detect a 150-bp fragment of Kvβ1.1, a 141-bp fragment of Kvβ2, and a 178-bp fragment of Kvβ3 (Yuan et al., 1998).

Preparation of Granulosa Cell Lysates and Membrane Proteins. Whole-cell lysates were made from GC monolayers by standard techniques with a lysis buffer consisting of phosphate-buffered saline with 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (1:500, P8340; Sigma Chemical). Lysis buffer was added to the culture dish after washing with cold phosphate-buffered saline three times. The culture dishes were scraped and the lysate was aspirated into a syringe with a 21-gauge needle to shear DNA. The lysates were rocked in the cold for 1 h and centrifuged for 10 min at 10,000g.

GC membranes were prepared by homogenizing pellets of freshly isolated GC in cold (4°C) HEPES-buffered saline (10 mM HEPES, 83 mM NaCl, and 1 mM MgCl₂, pH 7.9) containing protease inhibitor cocktail (1:500). A crude membrane fraction was obtained by differential centrifugation (1000g, 1 min; 100,000g, 30 min) and solubilized in cold (4°C) radioimmunoprecipitation assay buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, and 1% deoxycholate; pH 7.9) containing protease inhibitor cocktail (1:500).

Protein concentrations of GC lysates and membrane proteins were determined by the bicinchoninic acid method (Micro BCA protein assay; Pierce Chemical, Rockford, IL). Lysates and solubilized membrane proteins were used for immunoblotting and immunoprecipitation as indicated.

Immunoblotting. Solubilized proteins were separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes by the semidy transfer method. The membranes were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS; 100 mM Tris, 0.9% NaCl, pH 7.5) containing 0.1% Tween 20 then incubated overnight at 4°C with primary antibody diluted in the blocking solution. Primary antibody dilutions were α-Kv1.1 (1:1000), α-Kv1.2 (1:1000), α-Kv1.3 (1:500), α-Kv1.4 (1:500), α-Kv1.5 (1:500), α-Kv1.6 (1:500), α-KvLQT1 (1:500), α-miK (1:500), and α-HERG (1:500). Unless otherwise stated, after three washes with 0.1% Tween 20/TBS, membranes were incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody diluted 1:1500 in 0.1% Tween 20/TBS. After four additional washes with 0.1% Tween 20/TBS, bound primary antibodies were visualized using an ECL detection system (Amersham Biosciences, Inc.) and recorded on radiographic film. Densitometric analysis was performed using Scion Image beta version 4.02 (Scion Corporation, Frederick, MD). Blots that were reprobed with antiphosphotyrosine were stripped with IgG elution buffer (Pierce Chemical) for 30 min then washed 3 × 10 min with TBS. Blots were then treated with ECL reagent as described for Western blots to confirm that antibodies were stripped completely. The blots were then reprobed with HRP-linked antiphosphotyrosine (1:500) and developed as described above.

Deglycosylation. The product of a Kv1.3 immunoprecipitation reaction was subjected to enzymatic deglycosylation by using peptide-N-glycosidase F (PNGase-F) according to the manufacturer’s instructions (Glyko Deglycosylation Plus kit; Glyko, Inc., Novato, CA). Briefly, the glycoprotein was denatured before digestion by using SDS and β-mercaptoethanol. Samples were heated to 100°C for 5 min then cooled on ice. Excess Nonidet P-40 was added to the cooled reaction to complex the SDS then PNGase-F was added (0.2 U/ml) and the sample was incubated overnight at 37°C. A control reaction containing buffer instead of enzyme was incubated in parallel. After incubation, sample buffer was added, and the reactions were analyzed by SDS-PAGE.

Immunoprecipitation. To preclude nonspecific binding, protein G-agarose beads (Immunopure; Pierce Chemical) were incubated with samples for 2 h at 4°C with constant gentle agitation then pelletted by centrifugation (700g). Antibodies directed against channel proteins were subsequently added to the supernatant and mixed overnight at 4°C. Immune complexes were then immobilized onto protein G-agarose beads, washed three times with cold radioimmunoprecipitation assay buffer, and eluted from the beads with SDS buffer containing 5% β-mercaptoethanol. Immunoprecipitated proteins and supernatant fractions were analyzed by immunoblotting as described above.

Statistical Analysis. Data are expressed as mean ± S.E.M. Significant differences between treatment groups were identified by analysis of variance with appropriate general linear models. Multiple comparisons were made using least significant difference procedure (Statistix; Analytical Software, Tallahassee, FL). Differences were considered significant when $p < 0.05$.

Results

Electrophysiological Differentiation of Delayed Rectifier K⁺ Currents in Porcine Granulosa Cells. Two different outward potassium currents with distinct activation and inactivation kinetics could be elicited by a series of depolarizing test pulses from a −40-mV holding potential: a slowly activating, nonactivating current (Fig. 1) and an ultrarrapidly activating, slowly inactivating current (Fig. 2). A discrete rapidly activating and inactivating (A-type) current was rarely seen when a similar voltage protocol was combined with a −80-mV holding potential (2/50 cells); this current was not further characterized.

The slowly activating, nonactivating K⁺ current was present in 67% of freshly isolated GC and absent completely from GC cultured for more than 48 h. The basic electrophysiological properties and expression pattern of this current have been described previously by Mattioli et al. (1993). We refer to this current as granulosa cell IKs, because of its overt similarity to the extensively characterized IKs present in cardiac myocytes (Sanguinetti and Jurkiewicz, 1990) and other cells that coexpress KCNE1 (minK) and KCNQ1 (KvLQT1) channel proteins (Barhanin et al., 1996; Sanguinetti et al., 1996).

Granulosa cell IKs could be measured both in the presence
Fig. 1. A, left, slowly inactivating, nonactivating delayed rectifier K⁺ current (I_{Ks}) elicited by a series of 4-s depolarizing test pulses (0 to +60 mV, step 20), followed by return to the −40-mV holding potential [K⁺]_{out} = 0 mM, 22°C. Right, normalized isochronal (4-s) activation curve for I_{Ks} determined from tail currents (n = 9). Continuous curve is a Boltzmann relationship fit to the mean at each test potential. The half-activation voltage (V_{1/2}) and slope factor (k) are 12.8 and 16.9 mV, respectively. B, left, granulosa I_{Ks} recorded at 40 mV by using the protocol described above, in the presence of increasing concentrations of the 3R,4S-enantiomer of chromanol 293B. Right, isochronal activation curves recorded in the absence and presence of 10 μM racemic 293B (n = 3). Data were normalized to the maximal control (drug-free) conductance. Continuous curves are Boltzmann relationships fit to the mean data. C, left, granulosa I_{Ks} recorded at +40 mV by using the protocol described above, before and after addition of 10 nM L-735,821. Right, granulosa I_{Ks} recorded at +40 mV by using the protocol described above, before and after addition of 20 nM L-768,673.
and absence of extracellular potassium (Fig. 1); decreasing extracellular potassium enhanced current amplitude in a manner consistent with the change in driving force (data not shown). Granulosa cell $I_{\text{Ks}}$ activated with a half-activation voltage ($V_{1/2}$) and a slope factor ($k$) of 12.8 and 16.9 mV, respectively. The time course of $I_{\text{Ks}}$ activation was described using a double exponential function, whereas that of deactivation was described by a single exponential. The fast and slow time constants of activation associated with a +60-mV test pulse were $394.7 \pm 156.3$ and $1370.3 \pm 165.3$ ms, respectively ($n = 7$). The deactivation time constant associated with subsequent return to −40 mV was $1028.3 \pm 94.3$ ms. Thus, the potassium-, time-, and voltage-dependence of GC $I_{\text{Ks}}$, the failure to inactivate, and the decay kinetics of the tail currents are typical of native cardiac and heterologously expressed $I_{\text{Ks}}$ (Sanguinetti and Jurkiewicz, 1990; Barhanin et al., 1996; Sanguinetti et al., 1996). Moreover, the amplitude of GC $I_{\text{Ks}}$ is substantially diminished by drugs described as specific $I_{\text{Ks}}$ antagonists (Busch et al., 1996; Salata et al., 1996; Selnick et al., 1997), not only the chromanol 293B (Fig. 1B, right) and its more potent enantiomer (3R,4S)-293B (Fig. 1B, left) but also two potent benzodiazepine blockers of $I_{\text{Ks}}$, L735,821 (Fig. 1C, left) and L768,673 (Fig. 1C, right). Granulosa cell $I_{\text{Ks}}$ was also inhibited by the class III antiarrhythmic drug clofilium and its p-nitro tertiary amine analog LY97241. The amplitude of GC $I_{\text{Ks}}$ was reduced by $>80\%$ in the presence of these compounds at concentrations between 25 and 100 μM. For example, clofilium (50 μM) reduced $I_{\text{Ks}}$ tail current associated with +60-mV test pulse by 91.4 ± 2.9% ($n = 3$). As reported previously for the cardiac slow delayed rectifier K$^+$ current (Arena and Kass, 1988), the LY97241-induced inhibition of GC $I_{\text{Ks}}$ was reversible, whereas the clofilium-induced block was not. Thus, the electrophysiological and pharmacological properties of GC $I_{\text{Ks}}$ are virtually identical to those of native and recombinant delayed rectifier currents associated with K$^+$ channels formed by KCNQ1 in combination with KCNE1. There was no MK-499-sensitive current in GC ($n = 5$).

\begin{figure}
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\includegraphics[width=\textwidth]{fig2}
\caption{A, two examples of $I_{\text{Ks}}$ recorded from granulosa cells maintained in monolayer culture for 48 h before patch-clamp recording. The current traces in the left and center were elicited by an identical series of 1-s depolarizing test pulses (0 to +40 mV, step 20), followed by return to the −40-mV holding potential. Scale bars, 250 ms, 25 pA. Right, current density plotted as a function of test potential for the traces on the left (●, solid line) and center (◆, dash-dot line). B, frequency distributions for $I_{\text{Ks}}$ half-activation voltage (left, $n = 22$), inactivation time constant at +40 mV (center, $n = 18$), and ratio of peak to sustained current at +40 mV (right, $n = 23$).}
\end{figure}
The ultrarapidly activating, slowly inactivating delayed rectifier K⁺ current (Fig. 2) will be referred to as GC Iₖᵤᵣ, because of its kinetic similarity to cardiac ultrarapid delayed rectifier currents described previously (Nattel et al., 1999). In contrast to Iₖᵤᵣ, Iₖₛ could be measured in the presence, but not the absence of extracellular potassium. Furthermore, Iₖᵤᵣ was present in both freshly isolated GC (6/16) and GC maintained in culture for up to 72 h (28/34 cells). Currents were elicited using a series of depolarizing test pulses applied from either a −40- or −80-mV potential in 30 of 34 GC exhibiting Iₖᵤᵣ, and elicited only from a −80-mV holding potential in three additional cells.

Table 1 shows the basic electrophysiological properties of Iₖᵤᵣ recorded in response to 1-s test pulses from a −40-mV holding potential, from GC that expressed Iₖᵤᵣ in the absence of Iₖₛ, after 0 to 24, 25 to 48, or 49 to 72 h of monolayer culture in serum-supplemented (10% fetal bovine serum) media. The current density, extent of time-dependent inactivation, and voltage dependence of activation of Iₖᵤᵣ varied substantially between cells on all days of culture, as illustrated by the obvious difference in the voltage dependence of activation of the current traces in Fig. 2A, and the magnitude of the standard errors in Table 1. There was no significant effect of day of culture on any parameter (Table 1). The extent of cumulative inactivation was also highly variable. For example, after 96 h of culture, there were cells with currents that showed either substantial (3/6) or no (3/6) use-dependent inactivation (Fig. 3); pulse-dependent potentiation was not seen.

The pharmacological profile of GC Iₖᵤᵣ was complex and variable. Iₖᵤᵣ amplitude was diminished (<2% change, n = 3) by 293B (100 μM), L-735,821 (100 nM), or L-768,673 (100 nM). However, Iₖᵤᵣ was antagonized by three nonspecific Kv1 channel antagonists (Po et al., 1993; Grissmer et al., 1994; Yamagishi et al., 1995; Sobko et al., 1998; Rolf et al., 2000): clofilium (25–100 μM, n = 5), 4-AP (0.2–1 mM, n = 4), and verapamil (20 μM, n = 2) (Fig. 4C). Antagonism of GC Iₖᵤᵣ by both clofilium and verapamil was associated with an accelerated time course of decay (Fig. 4C), as described previously for these drugs’ open channel block of Kv1.2 and Kv1.5 (Rampe et al., 1993; Malayev et al., 1995; Yamagishi et al., 1995). Components of whole-cell Iₖᵤᵣ were also sensitive to external TEA (Fig. 4A), as well as to the Kv1 subunit-specific toxins CTX (Fig. 4B), DTX (Fig. 4B), KTX (Fig. 4B), and MTX (data not shown). Peak Iₖᵤᵣ measured at +40 mV was diminished on average (n = 3): 47 ± 1% by CTX (10 nM), 25.3 ± 14% by DTX (20 nM), and 67 ± 4% by KTX (50 nM).

Although no component of Iₖᵤᵣ in any cell tested (n = 5) was sensitive to 100 μM TEA, a 100-fold higher TEA concentration (10 mM) reduced peak current at +40 mV by 11 to 35%. The component of Iₖᵤᵣ that exhibited cumulative inactivation was sensitive to 10 mM TEA. Furthermore, the TEA-sensitive component of Iₖᵤᵣ activated over a more negative voltage range than the TEA-insensitive component; for example, the half-activation voltages (millivolts) determined by Boltzmann analysis of the conductance-voltage relationships for the control, TEA-insensitive, and TEA-sensitive currents shown in Fig. 4A were, respectively, −15.8, −9.4, and −20.1.

The electrophysiological and pharmacological properties of GC Iₖᵤᵣ are inconsistent with expression of any single population of Kv α-subunit homotetramers. For example, homomeric Kv1.1 channels are blocked by micromolar concentrations of TEA (Grissmer et al., 1994), whereas GC Iₖᵤᵣ is not. In contrast to the GC current, homomeric Kv1.2 channels show pulse-dependent potentiation and lack KTX sensitivity (Grissmer et al., 1994). If not coassembled with Kv1.6, Kv1.4 channels exhibit fast, N-type inactivation not typical of granulosa cell Iₖᵤᵣ (Po et al., 1993; Roeper et al., 1998). Heterologously expressed Kv1.3 currents exhibit cumulative inactivation, less sensitivity to DTX (IC₅₀ = 250 nM) than either CTX (IC₅₀ = 2.6 nM) and KTX (IC₅₀ = 0.65 nM), and moderate TEA sensitivity; thus, Kv1.3 could account for only one component of granulosa Iₖᵤᵣ (Grissmer et al., 1994). Kv1.5 channels are not only relatively insensitive to all three toxins tested plus TEA but also lack cumulative inactivation (Grissmer et al., 1994); hence, Kv1.5 currents resemble another component of granulosa Iₖᵤᵣ. Kv1.6 channels are sensitive to DTX and TEA but insensitive to CTX and MTX (Kirsch et al., 1991; Koschak et al., 1998). On this basis, homomeric Kv1.6 channels are also unable to account completely for GC Iₖᵤᵣ.

On the basis of the data presented above, we hypothesized that GC Iₖᵤᵣ reflects heterogeneous expression of multiple Kv α-subunits, assembled as homo- and heterotetramers, sometimes associated with regulatory β-subunits. It would
have been impossible to adequately test this hypothesis by measuring only electrophysiological and pharmacological characteristics of \( I_{\text{Kur}} \), because 1) the voltage dependence, gating kinetics, and toxin sensitivity of heteromeric \( K^+ \) channels are not easily predicted and vary considerably with expression environment (Hopkins, 1998; Petersen and Nerbonne, 1999); and 2) there are no specific antagonists of \( \text{Kv1.5} \). Accordingly, we performed additional experiments with standard molecular and biochemical techniques, as detailed below.

**Potassium Channel Transcripts and Proteins Expressed in Porcine Granulosa Cells.** Our goal was not only to increase knowledge about the electrophysiological and pharmacological properties of GC delayed rectifier \( K^+ \) currents but also to determine which potassium channel subunits might contribute to GC \( K^+ \) channels. To this end, we used qualitative RT-PCR and Western analysis to examine \( K^+ \) channel protein expression at the mRNA and protein levels, respectively. To address directly the issue of molecular diversity in GC \( K^+ \) channel assembly, we used sequential coimmunoprecipitation and immunoblotting to document not only the presence of but also the potential for heteromultimer formation by channel subunits from the ERG (\( \text{KCNH} \)), KCNQ, KCNE, Kv1, and \( \text{Kv}\beta \) families.

Immunoblots with either N- and C-terminal anti-HERG antibodies described by Pond et al. (2000), or a commercially available anti-HERG antibody (Alomone Laboratories), failed to reveal expression of ERG protein in membranes prepared from freshly isolated porcine GC, although these antibodies detected ERG1 (\( \text{KCNH2} \)) successfully in transiently transfected CHO cells, stably transfected HEK-293 cells, and guinea pig and horse heart (Fig. 5). These data are consistent with the absence in GC of an MK-499–sensitive \( K^+ \) current similar to cardiac \( I_{\text{Kr}} \), the rapid delayed rectifier \( K^+ \) current (Sanguinetti and Jurkiewicz, 1990).

On the basis of its electrophysiological and pharmacological properties, we hypothesized that granulosa \( I_{\text{Ks}} \) was associated with channels formed by combination of the six transmembrane domain, pore-forming, \( \alpha \)-subunit \( \text{KCNQ1} \) with the \( \beta \)-subunit \( \text{KCNE1} \). Gene-specific primers based on highly conserved regions of KCNE1-amplified RT-PCR products of the expected sizes (Fig. 6, top); sequencing confirmed the presence of transcripts encoding a protein with high homology to previously cloned \( \text{KCNE1} \) (Fig. 6, bottom). Immunoblotting was used to confirm expression of \( \text{KCNNE1} \) protein and demonstrate expression of \( \text{KCNQ1} \) protein; coimmunoprecipitation studies suggest strongly that these channel subunits associate in porcine GC (Fig. 7). Mattioli et al. (1993) reported, and we have confirmed, that expression of \( I_{\text{Ks}} \) by GC in monolayer culture is progressively diminished and ultimately lost over a period of 24 to 48 h. Here, we extend these findings to show that expression of the \( K^+ \) channel subunits \( \text{KCNQ1} \) and \( \text{KCNE1} \) is diminished in GC after 24 h in primary culture (Fig. 8).

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**Fig. 4.** A, \( I_{\text{Kur}} \) recorded from granulosa cell maintained in monolayer culture for 72 h before patch-clamp recording has a moderately TEA-sensitive component. Left, control current recorded in the absence of TEA. Middle, TEA-resistant current recorded 5 min after application of 10 mM TEA (extracellular solution with 10 mM sodium replaced by 10 mM TEA). Right, TEA-sensitive current obtained by subtracting TEA-resistant current from control. Scale bars, 250 ms, 50 pA. B, peptide toxins inhibit components of \( I_{\text{Kur}} \). Left, inhibition by CTX (10 nM) of \( I_{\text{Kur}} \) recorded from a granulosa cell after 48 h in culture. Middle, inhibition by DTX (20 nM) of \( I_{\text{Kur}} \) recorded from a granulosa cell after 48 h in culture. Right, inhibition by KTX (50 nM) of \( I_{\text{Kur}} \) recorded from a granulosa cell after 72 h in culture. Scale bars, 50 pA. C, effects of nonspecific \( K^+ \) channel antagonists on \( I_{\text{Kur}} \) recorded from granulosa cells maintained in monolayer culture for 48 h before patch-clamp recording. Effects of clofilium, 4-AP, and verapamil are shown in the left, middle, and right, respectively. Similar drug effects were observed in granulosa cells cultured for 72 or 96 h. Scale bars, 250 ms, 50 pA.
We hypothesized that the variable electrophysiological and pharmacological properties of granulosa I Kur reflected the presence and coassociation of multiple Kv channel subunits. This hypothesis was confirmed using the experimental approach illustrated in Figs. 8 to 11. Summary data are presented in Table 2.

As shown in Fig. 9A, antibody to Kv1.5 immunoprecipitated from CHO cells stably transfected with Kv1.5 a band of the expected molecular mass (~75 kDa, based on immunoblotting), and one additional unidentified band (~48 kDa). In contrast, the same antibody pulled down from GC not only a band spanning 68 to 80 kDa but also bands at ~60 and ~110 kDa. The molecular masses of the 35S-labeled bands immunoprecipitated from GC are consistent with the reported molecular mass of Kv1 subunits. Indeed, additional immunoblotting experiments revealed that freshly isolated GC and primary cultures expressed multiple Kv1 channel proteins, including Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, and Kv1.6 (Figs. 8 and 9).

As illustrated in Fig. 9B for Kv1.5, the specificity of the Kv1 antibodies used was verified by comparing immunoblotting results obtained under standard conditions to those obtained in the presence of excess antigen (either fusion protein or synthetic peptide); only bands blocked by antigen are reported. Immunoblots of GC membranes and lysates were compared with positive and negative controls for additional verification of antibody specificity. Positive controls included lysates of Jurkat cells (positive control for Kv1.3) and CHO cells stably transfected with Kv1.5 (positive control for Kv1.5 shown in Fig. 9), as well as membranes prepared from rat brain (positive control for Kv1.1, 1.2, 1.4, and 1.6). Untransfected CHO cells and HEK-293 cells transfected with HERG served as negative controls.

On immunoblots of GC lysates and membranes, antibodies specifically recognized high- and low-molecular-mass forms of Kv1.1 (60 and 84 kDa), Kv1.2 (65 and 80 kDa), Kv1.3 (64 kDa) from CHO cells stably transfected with Kv1.5 a band of the expected molecular mass (~75 kDa, based on immunoblotting), and one additional unidentified band (~48 kDa). In contrast, the same antibody pulled down from GC not only a band spanning 68 to 80 kDa but also bands at ~60 and ~110 kDa. The molecular masses of the 35S-labeled bands immunoprecipitated from GC are consistent with the reported molecular mass of Kv1 subunits. Indeed, additional immunoblotting experiments revealed that freshly isolated GC and primary cultures expressed multiple Kv1 channel proteins, including Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, and Kv1.6 (Figs. 8 and 9).

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On immunoblots of GC lysates and membranes, antibodies specifically recognized high- and low-molecular-mass forms of Kv1.1 (60 and 84 kDa), Kv1.2 (65 and 80 kDa), Kv1.3 (64 kDa)
membranes were prepared using granulosa cells harvested from the small and medium follicles on 150 to 300 pig ovaries.

Fig. 7. KCNQ1 and KCNE1 expression in freshly isolated porcine GC. Top, KCNQ1 immunoreactivity in membranes (10 μg) prepared from GC, guinea pig heart, and untransfected CHO cells. KCNQ1 antibody (α-KCNQ1) was applied to the membrane directly (−) or after preincubation (+) with 10 μg/ml of peptide against which the antibody was generated. Bottom, left, KCNE1 immunoreactivity in membranes (20 μg) from GC, CHO cells transfected with KCNQ1 (CHO-Q1), and CHO cells cotransfected with KCNQ1 and KCNE1 (CHO-Q1/E1). In this instance, bound KCNE1 antibody (α-KCNE1) was visualized using amplified alkaline phosphatase (Bio-Rad, Hercules, CA). Three bands corresponding to differentially glycosylated forms of KCNE1 are visible. Bottom, middle, Immunoprecipitate (IP) of GC membranes by α-KCNE1, resolved by SDS-PAGE on a 4 to 20% gel, and immunoblotted (IB) with α-KCNQ1. Bottom, right, IP by α-Kv1.6 and the associated supernatant (SP) were probed with α-KCNE1 as a negative control. Arrows indicate molecular mass.

Fig. 8. Changes in expression of Kv channels during culture. Left, immunoblot (IB) of GC membrane proteins (30 μg) from fresh isolates (F) and 24 h primary cultures (C), separated by SDS-PAGE on a 4 to 20% gel, and probed with antibody to KCNQ1 (top) or KCNQ1 (bottom). Right, immunoblots of membrane proteins (30 μg) isolated from freshly harvested PGC cells (F) or PGC maintained in culture (C) for 72 h. Membrane proteins were separated by SDS-PAGE on a 4 to 20% gradient gel, transferred to nitrocellulose, and detected with anti-Kv1.2 (top), anti-Kv1.6 (middle), or anti-Kv1.16 (bottom). Arrows indicate molecular mass.

TABLE 2
Molecular basis for I
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We used coimmunoprecipitation (pull-down) to determine which, if any, of the Kv1 α-subunits contributed to heterotetramer formation in porcine GC. Table 2 summarizes the results of a series of experiments similar to those shown in Fig. 9. It should be noted that the scoring system used in Table 2 to depict the apparent relative abundance of the different molecular mass forms of the various Kv channel subunits reflects not only their actual expression but also differences between antibody efficiency and interexperimen- tation variation in the chemiluminescence reactions.

To define the nature of some of the oligomeric assemblies of Kv α-subunits in porcine GC, we used a strategy similar to that of Shamotienko et al. (1997). GC membranes were immunoprecipitated with antibody against Kv1.1, 1.2, or 1.5 then the resultant precipitate and supernatant fractions were immunoblotted with the same antibody to demonstrate complete precipitation of the targeted Kv channel protein from the membrane fraction. These supernatants were then subjected to additional rounds of immunoprecipitation and immunoblotting with the other Kv antibodies to determine and 88 kDa), Kv1.4 (88 and 110 kDa), Kv1.5 (62 and 85 kDa), and Kv1.6 (60 and 88 kDa). The lower molecular mass for Kv1.6 (60 kDa; Fig. 8) has been described in other cell types (Scott et al., 1994; Koch et al., 1997). An 88-kDa form of Kv1.6 has also been described previously, although the basis for the higher molecular mass is not known (Attali et al., 1997). Addition of N-linked oligosaccharide is unlikely because Kv1.6 lacks the N-glycosylation site present in Kv1.1, Kv1.2, Kv1.3, Kv1.4, and Kv1.5 (Shi and Trimmer, 1999). The low- and high-molecular-mass forms of Kv1.1, Kv1.2, and Kv1.4 have been shown previously to correspond, respec- tively, to incompletely glycosylated (high mannose), immature, and fully glycosylated (sialylated) mature forms of these channel subunits (Scott et al., 1994; Shi and Trimmer, 1999; Manganas and Trimmer, 2000). High- and low-molecular-mass forms of Kv1.3 and Kv1.5 have also be reported and are suggested to reflect post-translational modification of these proteins, which contain multiple phosphorylation sites in addition to the conserved N-glycosylation site (Attali et al., 1997; Bowblby et al., 1997; Chung and Schlichter, 1997; Sobko et al., 1998; Yuen et al., 1998). We confirmed the PNGase-F sensitivity of the high molecular form of Kv1.3 expressed in cultured but not freshly isolated GC, to provide a direct correlation between the high-molecular-mass form of this α-subunit and the presence of mature N-linked oligosacch- aride. PNGase-F digestion shifted the mobility of Kv1.3 from 88 to 64 kDa (data not shown).

We used coimmunoprecipitation (pull-down) to determine which, if any, of the Kv1 α-subunits contributed to heterotetramer formation in porcine GC. Table 2 summarizes the results of a series of experiments similar to those shown in Fig. 9. It should be noted that the scoring system used in Table 2 to depict the apparent relative abundance of the different molecular mass forms of the various Kv channel subunits reflects not only their actual expression but also differences between antibody efficiency and interexperimen- tation variation in the chemiluminescence reactions.

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which (if any) of the possible hetero-oligomeric subunit combinations were present. These experiments indicated that Kv1.1 is not found in complexes without Kv1.5 but can be found without Kv1.2. In contrast, Kv1.5 is found in complexes that have neither Kv1.1 nor Kv1.2.

To detect temporal differences in the expression of the various Kv α-subunits, immunoblots performed on membranes prepared from freshly isolated GC were compared with immunoblots performed using membranes prepared from 72-h monolayer cultures. Expression of Kv1.1, Kv1.2 (Fig. 8), and Kv1.4 (data not shown) was greater in freshly isolated GC than 72-h primary cultures. The opposite was true for Kv1.3, Kv1.5, and Kv1.6 (Figs. 8 and 10). Kv1.3 was undetectable in two of six membrane preparations from freshly isolated granulosa cells. The PNGase-F-sensitive form of Kv1.3 (80 kDa) was found only in cultured GC (Fig. 10). Similarly, high-molecular-mass forms of Kv1.5 were expressed to a greater extent in cultured than fresh GC (Fig. 8).

Interestingly, Kv1.3 was subject to constitutive tyrosine phosphorylation in freshly isolated and cultured GC; anti-phosphotyrosine consistently detected the same bands as anti-Kv1.3, on Western blots (Fig. 10) and immunoprecipitates (data not shown).

Heteromultimeric assembly of Kvα and Kvβ assembly is known to contribute to K⁺ current diversity in cardiac, neuronal, and other cells. To determine whether Kv β-subunits are expressed and capable of association with Kv α-subunits in GC, we used PCR to look for mRNA encoding Kvβ1, Kvβ2, and Kvβ3 and found that message for Kvβ2 is expressed in GC (Fig. 11). In addition, we used Western analysis to detect Kvβ1.3 in GC membrane fractions immunoprecipitated with antibody against Kvβ1.3 and three Kv α-subunits. As shown in Fig. 11, Kvβ1.3 is expressed in freshly isolated GC and coassociates with Kv1.2 and Kv1.5, but not Kv1.1. It is unclear why immunoblotting successfully detected Kvβ1.3 protein, when PCR with primers complementary to a C-terminal sequence shared by all Kvβ1.x subunits did not yield any product (Fig. 11). Product of the expected size was not seen using PCR conditions identical to those published for pulmonary artery smooth muscle (Yuan et al., 1998), or under conditions where buffer (Opti-Prime PCR Optimization kit; Stratogene, La Jolla, CA) and annealing temperature (40–56°C gradient, Tgradient Thermocycler; Whatman Biometra GmbH, Niedersachsen, Germany) were varied. Optimization of PCR conditions for Kvβ1.x was not pursued after Kvβ1.3 protein was demonstrated by immunoblotting.

**Fig. 9.** Heteromultimeric coassembly of Kv channel subunits in porcine granulosa cells. Immunoprecipitation (IP) from the membrane protein fraction was performed then the resulting IP and supernatant (SP) were resolved by SDS-PAGE (4 to 20% gradient gels) and analyzed by immunoblotting (IB). A, IP of 35S-labeled proteins from Chinese hamster ovary cells stably transfected with Kv1.5 (Kv1.5) and porcine GC. B, proteins from GC and Chinese hamster ovary cells stably transfected with Kv1.5 (CHO-Kv1.5) were IP with antibody to Kv1.1. The IP and SP were then IP with anti-Kv1.5, in the absence and presence (bottom) of Kv1.5 fusion protein (FP). C, membrane proteins from porcine granulosa cells were IP with antibody to Kv1.1. The IP and SP were probed with antibody to Kv1.1. D, proteins IP with antibodies (rabbit polyclonal) to Kv1.1–Kv1.6 were IB with antibody (mouse monoclonal) to Kv1.4. Arrows indicate molecular mass.

**Fig. 10.** Expression and phosphorylation of Kv1.3 in fresh and cultured porcine granulosa cells. Western blot of membrane proteins isolated from freshly harvested PGC cells (F) or PGC maintained in culture for 72 h (C). Membrane proteins were separated by SDS-PAGE on a 4 to 20% gradient gel, transferred to nitrocellulose, and detected with anti-Kv1.3 (top). The blot was then stripped and reprobed with HRP-linked anti-phosphotyrosine (bottom).

**Fig. 11.** Expression of Kv β-subunits in porcine GC. Top, left, membrane proteins from porcine GC were immunoprecipitated with antibody to Kvβ1.3, Kv1.1, Kv1.2, or Kv1.5 and resolved by SDS-PAGE (4 to 20% gradient gels). The Western blot was probed with antibody to Kvβ1.3. The wide band at 50 kDa in the lane sequentially immunoprecipitated then immunoblotted with rabbit anti-Kvβ1.3 is IgG detected by the anti-rabbit secondary antibody. This band is lacking in the other lanes, because Kv α-subunits were immunoprecipitated with mouse monoclonal antibodies. Left arrows indicate molecular mass standards. Right arrow (40 kDa) indicates Kvβ1.3 protein. Top, right, mRNA for Kvβ2, but not Kvβ1 nor Kvβ3, is detected in porcine GC. RT-PCR was performed using gene-specific primers and poly(A)⁺ mRNA. Amplified products were separated by gel electrophoresis and visualized by ethidium bromide staining. Lanes: 1) 50-bp ladder; 2) primers for β1, 3) primers for β2, and 4) primers for β3. Right arrow (~140 bp) indicates Kvβ2 product. Bottom, partial deduced amino acid sequence of pig granulosa Kvβ2 (GenBank accession number AF348084), compared with previous clones. Dashes indicate homology to Pig GC Kvβ2.
**Discussion**

Kv channels not only influence the electrical properties of excitable and nonexcitable cells but also regulate cell proliferation and differentiation (Attali et al., 1997; Sobko et al., 1998; Kotecha and Schlichter, 1999; Rane, 1999). K+-selective channel pores are formed by tetrameric complexes of integral membrane proteins with six transmembrane-spanning domains. Formation of homo- and heterotetramers of Kv α-subunits has been demonstrated not only in heterologous expression systems but also native cells (Scott et al., 1994; Attali et al., 1997; Koch et al., 1997; Shamotienko et al., 1997; Sobko et al., 1998; Yuan et al., 1998; Schmidt et al., 1999).

The subunit composition of Kv channels influences the expression, and electrophysiological and pharmacological properties of the associated currents (Hopkins, 1998). Kv channel expression and function may be modulated not only by coassociation of the various pore-forming α-subunits with accessory (β)-subunits and other regulatory proteins but also by post-translational modifications of either the Kv α- or Kv β-subunits (Martens et al., 1999; Petersen and Nerboune, 1999). Several K+-channel subunits are typically expressed in a single cell. The extensive diversity in Kv channels has made it difficult to determine definitively the molecular identity of native K+-currents in many cases (Attali et al., 1997; Shamotienko et al., 1997; Sobko et al., 1998; Yuan et al., 1998; Schmidt et al., 1999).

A variety of K+ currents with different activation and inactivation kinetics have been described in porcine GC subjected to whole-cell patch clamp. In GC cultured for 2 days in serum-free media containing FSH (10 ng/ml) then an additional 3 to 5 days in FSH-free media, Kusaka et al. (1993) described a rapidly activating transient outward (A-type) current activated from a −70-mV holding potential, and a rapidly activating nonactivating delayed rectifier K+ current activated from a holding potential of −30 mV. Both currents were sensitive to 4-AP but only the delayed rectifier was sensitive to TEA (10 mM). In freshly isolated GC and GC maintained cultured for up to 3 days in serum-containing media (10% fetal bovine serum), Mattioli et al. (1991, 1993) described two currents; a slowly activating, noninactivating, TEA- and 4-AP-insensitive K+ current that disappeared after 24 h in monolayer culture, and a rapidly activating K+ current with an inactivation time constant that increased from 10 to 300 ms over 72 h in culture. The latter could be activated from a holding potential of −70 but not −40 mV and was sensitive to 4-AP. We recorded routinely a slowly activating, nonactivating delayed rectifier K+ current and an ultrarapidly activating, slowly inactivating K+ current. A rapidly inactivating A-type current was elicited from a −80-mV holding potential in less than 5% of screened GC.

The slowly activating, nonactivating current in Fig. 1 is identical to that described previously (Mattioli et al., 1991, 1993). Its electrophysiological properties and drug sensitivity are consistent with those of IKS currents and channels formed by coassociation of KCNQ1 and KCNE1 proteins (Barhanin et al., 1996; Busch et al., 1996; Sanguinetti et al., 1996; Selnick et al., 1997). In fact, both of these channel proteins are expressed in GC. The disappearance of IKS, as GC differentiate in culture, along with the observation that the current is inhibited by luteinizing hormone, cAMP, and protein kinase C, has led to the speculation that IKS may play a specific role in granulosa cell maturation (Mattioli et al., 1991, 1993; L. C. Freeman, unpublished observations). Our identification not only of KCNQ1 and KCNE1 as the molecular correlates of GC IKS but also of 293B, L-735,821, and L-768,673 as specific antagonists of the current, will facilitate further investigation of its functional role. Additional experiments will be required to determine the basis for tissue-specific differences in IKS modulation, particularly to address the discrepant effects of cAMP on GC IKS compared with recombinant KCNQ1/KCNE1 currents (Blumenthal and Kaczmarek, 1992) and native cardiac currents in guinea pig (Walsh et al., 1989) and pig (L. C. Freeman, unpublished observations).

The ultrarapidly activating, slowly inactivating K+ current recorded from GC by using whole-cell patch clamp seems to reflect overlapping components carried by homomorphic and heteromeric Kv channels. The variability in the electrophysiological and pharmacological properties of granulosa cell delayed rectifier currents described here and elsewhere (Kusaka et al., 1993; Mattioli et al., 1993) is not surprising given the results shown in Figs. 7 to 10. GC can express at least six Kv α-subunits, and their expression varies with time in culture. The diversity of granulosa cell K+ currents is further increased by the potential for not only coassembly of nonidentical Kv α-subunits but also modulation by accessory subunits. Our data indicate that the potential for K+ channel diversity in GC is comparable with that seen in the central nervous system. Further experiments are required to determine whether GC also expresses non–Shaker-related Kv channel proteins (Kv2.x, Kv3.x, Kv4.x, etc.).

Heterotetrameric complexes of α-subunits have been demonstrated previously among the following: Kv1.1/Kv1.2, Kv1.2/Kv1.4, Kv1.2/Kv1.3, Kv1.4/Kv1.6, Kv1.5/Kv1.2, Kv1.5/Kv1.4, Kv1.1/Kv1.2/Kv1.4, Kv1.1/Kv1.2/Kv1.6, Kv1.1/Kv1.2/Kv1.3, Kv1.2/Kv1.3/Kv1.4, and Kv1.2/Kv1.4/Kv1.6 (Koch et al., 1997; Shamotienko et al., 1997; Koschak et al., 1998; Sobko et al., 1998). In addition, the potential for heteromultimer formation has been demonstrated in oligodendrocyte progenitors for Kv1.4, Kv1.5, and Kv1.6 (Attali et al., 1997; Schmidt et al., 1999). In GC that express Kv1.1 to Kv1.6 proteins, we were able to demonstrate many of these Kv α-subunit coassemblies (Table 2).

The strong coassociation observed between the glycosylated forms of Kv1.4 and Kv1.6 is noteworthy and may explain our failure to detect rapidly inactivating A-type currents in a significant number of GC. Heteromorphic expression of Kv1.4 and Kv1.6 has been shown to result in a slowly inactivating current (Roeppe et al., 1998), because the N terminus of Kv1.6 possesses an N-type inactivation prevention domain, which prevents the fast, N-type inactivation typically associated with homomorphic Kv1.4 channels. A potentially significant pattern of coassociation was also evident between the 60-kDa form of Kv1.1 and the low-molecular-mass forms of Kv1.2, Kv1.3, Kv1.4, and Kv1.5. These relationships may reflect a physiological role for Kv1.1 in limiting surface expression of the other Kv subunits. Kv1.1 has been reported to have a dominant negative effect on the surface expression of Kv1.2 and Kv1.4 (Manganas and Trimmer, 2000).

Kv β-subunits can also affect dramatically both the gating and cell surface expression of coassociated Kv α-subunits (Martens et al., 1999). Our data demonstrate that GC ex-
press at least two β-subunits encoded by distinct genes, Kvβ1 and Kvβ2 family members not only interact with α-subunits via distinct functional stoichiometries (αββ3 for Kvβ1 and αββ1β3 for Kvβ2) but also affect Kv currents in distinct manners (Xu et al., 1998; Martens et al., 1999). For example, Kvβ1.3 converts Kv1.5 from a delayed rectifier channel to one with rapid, but partial inactivation, whereas Kvβ2.1 shifts the state-state activation and inactivation of Kv1.5 without inducing rapid inactivation. Kvβ2 subunits have also been shown to enhance N-glycosylation and/or surface expression of associated Kv α-subunits, including Kv1.1, Kv1.2, Kv1.3, and Kv1.6. Clearly, the presence of Kvβ1 and Kvβ2 accessory subunits in GC could contribute substantially to Kv current diversity, not only by influencing the electrophysiological properties of expressed delayed rectifier currents but also by controlling the number of α-subunits available for tetramer assembly.

Phosphorylation has been shown also to modulate current amplitudes and kinetin by influencing interactions between not only α- and β-subunits but also Kv channel complexes and other intracellular proteins (Bolwby et al., 1997; Martens et al., 1999; Rane, 1999). Basal tyrosine phosphorylation of Kv1.3 suggests that the activity of GC Kv channels may be influenced by signaling pathways associated with intracellular growth factors (Steele and Leung, 1993). Tyrosine phosphorylation has been identified previously as an important influence on the activity of Kv1.3 (Bolwby et al., 1997; Rane, 1999), an ion channel with a well established role in modulating proliferation and differentiation of other nonexcitable cells (Kotecha and Schlichter, 1999; Rane, 1999).

As GC spontaneously luteinized in culture, expression of Kv1.3 increased. The mature, fully glycosylated form of Kv1.3 was present only in cultured GC. Whole-cell K+ currents recorded from cultured GC contained a TEA-sensitive component that exhibited cumulative inactivation, consistent with expression of Kv1.3 homotetramers. It will be interesting to determine what role, if any, Kv1.3 plays in granulosa cell maturation. Increased expression of Kv1.3 has been positively correlated with days in culture and acquisition of a proliferative phenotype in microgla (Kotecha and Schlichter, 1999).

Associating specific K+ channels with cellular processes in GC will be technically challenging as a result of the molecular diversity. However, it will be critical to define the roles of specific K+ channels in granulosa cell proliferation, differentiation, and apoptosis, and the temporal pattern of K+ channel expression during the estrous cycle, because these proteins may represent either novel targets for assisted reproduction or potential sites of toxicity for drugs designed to act on channels in other tissues, including heart, brain, and lymphocytes. We document here distinct differences in the pharmacological sensitivities and temporal expression patterns of voltage-gated K+ currents and channel proteins in fresh isolates and primary cultures of porcine GC. Our findings provide an essential background for experimental definition of granulosa K+ channel function(s).

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