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Ca^{2+}-Mobilizing Endothelin-A Receptors Inhibit Voltage-Gated Ca^{2+} Influx through $G_{i/o}$ Signaling Pathway in Pituitary Lactotrophs

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ABSTRACT

In excitable cells, receptor-induced Ca^{2+} release from intracellular stores is usually accompanied by sustained depolarization of cells and facilitated voltage-gated Ca^{2+} influx (VGCI). In quiescent pituitary lactotrophs, however, endothelin-1 (ET-1) induced rapid Ca^{2+} release without triggering Ca^{2+} influx. Furthermore, in spontaneously firing and depolarized lactotrophs, the Ca^{2+}-mobilizing action of ET-1 was followed by inhibition of spontaneous VGCI caused by prolonged cell hyperpolarization and abolition of action potential-driven Ca^{2+} influx. Agonist-induced depolarization of cells and enhancement of VGCI upon Ca^{2+} mobilization was established in both quiescent and firing lactotrophs treated overnight with pertussis toxin (PTX). Activation of adenylyl cyclase by forskolin and addition of cell-permeable 8-bromo-cAMP did not affect ET-1–induced sustained inhibition of VGCI, suggesting that the cAMP-protein kinase A signaling pathway does not mediate the inhibitory action of ET-1 on VGCI. Consistent with the role of PTX-sensitive K^+ channels in ET-1–induced hyperpolarization of control cells, but not PTX-treated cells, ET-1 decreased the cell input resistance and activated a 5 mM Cs^+-sensitive K^+ current. In the presence of Cs^+, ET-1 stimulated VGCI in a manner comparable with that observed in PTX-treated cells, whereas E-4031, a specific blocker of *ether-a-go-go*–related gene-like K^+ channels, was ineffective. Similar effects of PTX and Cs^+ were also observed in GH_3 immortalized cells transiently expressing ET_A receptors. These results indicate that signaling of ET_A receptors through the $G_{i/o}$ pathway in lactotrophs and the subsequent activation of inward rectifier K^+ channels provide an effective and adenylyl cyclase-independent mechanism for a prolonged uncoupling of Ca^{2+} mobilization and influx pathways.

In cells operated by calcium-mobilizing agonists, intracellular Ca^{2+} release is coupled to extracellular Ca^{2+} influx. The coordinate action of these two pathways provides a sustained rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) during prolonged agonist stimulation (Putney and Bird, 1993), which is critical to normal cell function. In nonexcitable cells, capacitative calcium entry accounts for agonist-induced Ca^{2+} influx (Putney and Bird, 1993). This pathway is also operative in some excitable cell types and is functionally integrated with voltage-gated Ca^{2+} influx (VGCI) (Berridge, 1998). Coupling of Ca^{2+} mobilization and influx pathways has been extensively studied in pituitary lactotrophs and GH-immortalized lactosomatotrophs (Stojilkovic and Catt, 1992). These cells exhibit spontaneous action potential firing and express multiple types of Ca^{2+}-mobilizing receptors. For example, TRH receptors in both cell types are coupled to the phospholipase C signaling pathway through G_q/G_{11}-proteins (Yu et al., 1998). Activation of this pathway leads to inositol trisphosphate-induced Ca^{2+} mobilization and sustained Ca^{2+} influx through voltage-gated and/or capacitative Ca^{2+} entry pathways. Facilitation of VGCI by TRH-induced Ca^{2+} mobilization has been attributed to membrane depolarization in response to inhibition of K^+ current(s) (Sankaranarayanan and Simasko, 1996a; Schafer et al., 1999).

Calcium-mobilizing endothelin (ET) receptors are also expressed in several pituitary cell types (Stojilkovic and Catt, 1996), and their activation in lactotrophs stimulates prolactin (PRL) release (Kanyicska et al., 1995; Lachowicz et al., 1997). In contrast to TRH action, the ET-1–induced transient increase in PRL secretion is followed by a prolonged inhibi-

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; VGCI, voltage-gated Ca^{2+} influx; GH, growth hormone; TRH, thyrotropin-releasing hormone; PRL, prolactin; ET, endothelin; $I_{K\text{-}Ca}$, Ca^{2+}-sensitive K^+ channel; K_{ir}, inward rectifier K^+ channel; PTX, pertussis toxin; V_m, membrane potential; indo-1, 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SSC, standard saline citrate; VGCC, voltage-gated Ca^{2+} channel; 8-Br-cAMP, 8-bromo-cAMP; erg, *ether-a go-go*–related gene; E-4031, 1-(2-(6-methyl-2-pyridyl)ethyl)-(4-methanesulfonamidobenzoyl)piperidine; Bay K 8644, 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylic acid methyl ester.

tion below basal levels. This sustained inhibitory action was initially observed by Samson et al. (1990) and Kanyicska et al. (1991), who also indicated the involvement of the ET_A receptor subtype in mediating this action (Samson, 1992; Kanyicska and Freeman, 1993). In parallel to the secretory profiles, activation of ET_A receptors stimulates a transient spike in $[Ca^{2+}]_i$ due to Ca^{2+} mobilization, followed by a sustained inhibition in response to the uncoupling of Ca^{2+} mobilization and entry pathways (Lachowicz et al., 1997). Such a bidirectional effect of a calcium-mobilizing agonist on Ca^{2+} signaling and secretion is unique among endocrine and neuroendocrine cells expressing $G_{q/G11}$-coupled receptors. Moreover, the cellular mechanisms mediating the sustained inhibition of PRL secretion and $[Ca^{2+}]_i$ are not known. Although it has been found that ET-1 activates Ca^{2+}-sensitive K^+ channels ($I_{K\text{-}Ca}$) (Kanyicska et al., 1997), this probably accounts only for the rapid hyperpolarization of cells during the transient rise in $[Ca^{2+}]_i$, but not the sustained hyperpolarization when the $[Ca^{2+}]_i$ is well below basal levels. To investigate the cellular mechanisms underlying the sustained inhibition in PRL secretion, we used enriched lactotrophs, as well as GH_3 cells in which ET_A receptors have been transiently expressed. Cells were stimulated with ET-1, a common agonist for ET_A and ET_B receptors (Rubanyi and Polokoff, 1994). Our results indicate that the prolonged uncoupling between Ca^{2+} mobilization and VGCI pathways is due to activation of inward rectifier K^+ (K_{ir}) channels via PTX-sensitive G-proteins.

Materials and Methods

Cell Cultures and Treatments. Experiments were performed on anterior pituitary cells from normal postpubertal female Sprague-Dawley rats obtained from Taconic Farms (Germantown, NY) and GH_3 immortalized pituitary cells. Pituitary cells were dispersed as described previously (Koshimizu et al., 2000) and cultured as mixed cells or enriched lactotrophs at a density of 10^6 cells/25-mm coverslip in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, and antibiotics. A two-stage Percoll discontinuous density gradient procedure (Koshimizu et al., 2000) was used to obtain an enriched lactotroph population. In single-cell measurements, lactotrophs were further identified by the addition of TRH.

GH_3 cells were cultured in F12K nutrient mixture containing 1.5 g/l $NaHCO_3$, 2.5% fetal bovine serum, and 15% horse serum. Procedures for transient transfection of ET_A receptors and expression constructs pME_{ETA} were performed as described previously (Koshimizu et al., 2000), with minor modifications. Briefly, cells were plated on coverslips coated with poly(L-lysine) at a density of 70,000/25-mm dish and allowed to grow for 24 h. On the following day, a total of 1.2 to 2.5 μg of expression constructs encoding rat ET_A receptors was mixed with 7 μl of LipofectAMINE in 0.5 ml of Opti-MEM (both from Invitrogen, Carlsbad, CA) for 15 to 30 min at ambient temperature. The DNA mixture was then applied to cells for 6 h and replaced by normal culture medium. Cells were subjected to experiments 24 h after transfection.

Hormone secretion was monitored using rapid cell column perfusion experiments and static cultures. For perfusion experiments, 1.2×10^7 cells were incubated with preswollen Cytodex-1 beads in 60-mm Petri dishes for 2 days. The beads were then transferred to 0.5-ml chambers and perfused with Hanks' medium 199 containing 20 mM HEPES, 0.1% bovine serum albumin, and penicillin/streptomycin for 2 h at a flow rate of 0.8 ml/min and 37°C to establish a stable basal PRL secretion. During experiments, 1-min fractions were collected, stored at −20°C, and later assayed for GH, PRL, and luteinizing hormone content using radioimmunoassay. For static culture experiments, 0.5×10^6 cells/well were plated in 24-well plates for 2 days. Cells were then washed and stimulated with ET-1 and Bay K 8644 for 3 h. All reagents and standards were provided by the National Pituitary Agency and Dr. A. F. Parlow (Harbor-University of California-Los Angeles Medical Center, Torrance, CA).

Intracellular Calcium Measurements. $[Ca^{2+}]_i$ measurements were performed by imaging of fura-2–loaded cells. Cells attached to coverslips were immersed in 2 μM fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR) in medium 199 with Hanks' salts at 37°C for 1 h. The medium was changed to Krebs-Ringer buffer, and cells were kept in this medium at room temperature throughout the experiment. The samples were excited by alternating 334- and 380-nm light beams, and the emitted fluorescence was measured at 520 nm using an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) and an Attofluor imaging system (Atto Instruments, Rockville, MD). The ratio of the two intensities (F_{340}/F_{380}), which reflects the changes in $[Ca^{2+}]_i$, was monitored in up to 75 cells simultaneously.

Electrophysiological Measurements. Current- and voltage-clamp recordings were performed at room temperature using an Axopatch 200 B patch-clamp amplifier (Axon Instruments, Union City, CA) and were low-pass-filtered at 2 kHz. Membrane potential (V_m) and current were measured using the perforated-patch recording technique (Rae et al., 1991). Briefly, amphotericin B (Sigma-Aldrich, St. Louis, MO) stock solutions (60 mg/ml) were prepared in dimethyl sulfoxide and stored for up to 1 week at −20°C. Just before use, the stock solution was diluted in pipette solution and sonicated for 30 s to yield a final amphotericin B concentration of 240 μg/ml. Patch electrodes used for perforated-patch recordings were fabricated from borosilicate glass (1.5 mm o.d.; World Precision Instruments, Sarasota, FL) using a Flaming Brown horizontal puller (P-87; Sutter Instruments, Novato, CA). Electrodes were heat polished to a final tip resistance of 3 to 6 MΩ and then coated with Sylgard (Dow Corning Corporation, Midland, MI) to reduce pipette capacitance. Pipette tips were briefly immersed in amphotericin B-free solution and then backfilled with the amphotericin B-containing solution. A series resistance of <15 MΩ was reached 10 min after the formation of a gigaohm seal (seal resistance >5 GΩ) and remained stable for up to 1 h. When necessary, series resistance compensation was optimized. Pulse generation, data acquisition, and analysis were done with a personal computer equipped with a Digidata 1200 A/D interface in conjunction with Clampex 8 (Axon Instruments). The extracellular medium contained 120 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 4.7 mM KCl, 0.7 mM $MgSO_4$, 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 with NaOH), and the pipette solution contained 50 mM KCl, 90 mM K^+-aspartate, 1 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.2 with KOH). The bath contained <500 μl of saline and was continuously perfused at a rate of 2 ml/min using a gravity-driven perfusion system.

Simultaneous Recording of $[Ca^{2+}]_i$ and V_m. Pituitary cells were incubated for 15 min at 37°C in phenol red-free medium 199 containing Hanks' salts, 20 mM sodium bicarbonate, 20 mM HEPES, and 0.5 μM indo-1 acetoxymethyl ester (Molecular Probes). The V_m was recorded as described above, and $[Ca^{2+}]_i$ was simultaneously monitored using a Nikon photon counter system as described previously (Van Goor et al., 2001b). The membrane potential and $[Ca^{2+}]_i$ were captured simultaneously at a rate of 5 kHz using a personal computer equipped with a Digidata 1200 A/D interface in conjunction with Clampex 8 (Axon Instruments). The $[Ca^{2+}]_i$ was calibrated in vivo according to the method of Kao (1994), and the values for R_{min}, R_{max}, $S_{f,480}/S_{b,480}$, and K_d were determined to be 0.75, 3.40, 2.45, and 230 nM, respectively.

Reverse transcription-PCR (RT-PCR) Analysis of K_{ir} 3.0 Isoform mRNA Expression. Total RNA from rat mixed pituitary cells, enriched lactotroph, or GH_3 cells was extracted using TRIzol reagent (Invitrogen). After DNase digestion, 2 μg of total RNA was reverse-transcribed into first-strand cDNA with oligo-dT primers

and SuperscriptII reverse transcriptase (Invitrogen). The cDNAs were then amplified with different K_{ir} 3.0 isoform-specific primer sets in the nonconserved carboxyl-terminal region. The oligonucleotide sequences of primers used for PCR amplification, with accession numbers of the GenBank database given in parentheses, are listed as K_{ir} 3.1 (NM_031610): sense primer (1051–1072 bp, 5′-GCAACCTTTGAAGTCCCCACCC-3′), antisense primer (1435–1457 bp, 5′-GTAGGTCCTCCAGCCATCTTTTG-3′); K_{ir} 3.2 (NM_013192): sense primer (1335–1354 bp, 5′-TGGCTAACCGGGCAGAGCTG-3′), antisense primer (1477–1499 bp, 5′-CAGCTGAGGTCTACACTTTGGAC-3′); K_{ir} 3.3 (L77929): sense primer (1258–1275 bp, 5′-GGGAACTGGCAGAAGCTG-3′), antisense primer (1442–465 bp, 5′-CTGGTCTGCCACAGGGGGTTGTAG-3′); K_{ir} 3.4 (NM_017297): sense primer (1198–1220 bp, 5′-GGAAAAGGGCTTCTATGAGGTGG-3′), antisense primer (1437–1461 bp, 5′-CTCTGGGTAGCACACAGGCTTCACA-3′).

The amplification was conducted in a Robocycler Thermal Cycler (Stratagene, La Jolla, CA) in a 50-μl reaction volume containing 1 μl of the first-strand cDNA as template, 1 unit of *Taq* DNA polymerase (Invitrogen), a 0.5 μM concentration of each primer, 0.2 mM dNTP, and 1× PCR buffer (2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl, pH 8.4). Amplification of DNA templates was initiated by a denaturation step at 94°C for 150 s, followed by 35 cycles of denaturing at 94°C for 40 s, annealing at 58 to 63°C for 30 s, and extension at 72°C for 1 min. The reaction was then terminated by a final extension step at 72°C for 5 min. To check for the integrity of RNA preparation, RT-PCR of GAPDH was also conducted as an internal control using

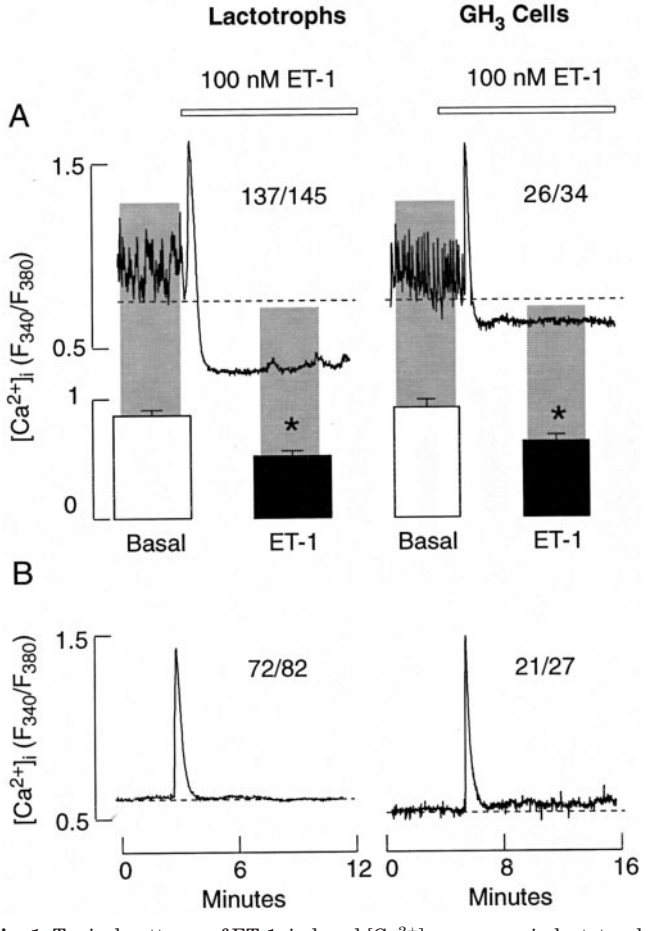

Fig. 1. Typical patterns of ET-1–induced $[Ca^{2+}]_i$ responses in lactotrophs (left) and GH_3 cells expressing rat ET_A receptors (right). A and B, representative traces in spontaneously active (A) and quiescent (B) cells. The numbers above traces indicate the fraction of spontaneously active or quiescent cells responding to ET-1with representative patterns. A, bottom, mean values ± S.E. of the $[Ca^{2+}]_i$ estimated before and during the sustained ET-1 stimulation (shown in gray areas). Asterisks indicate a significant ($P < 0.05$) difference among means. In this and the following figures, calcium recordings were done in fura-2–loaded cells, and $[Ca^{2+}]_i$ was expressed as the F_{340}/F_{380} ratio, if not specified otherwise.

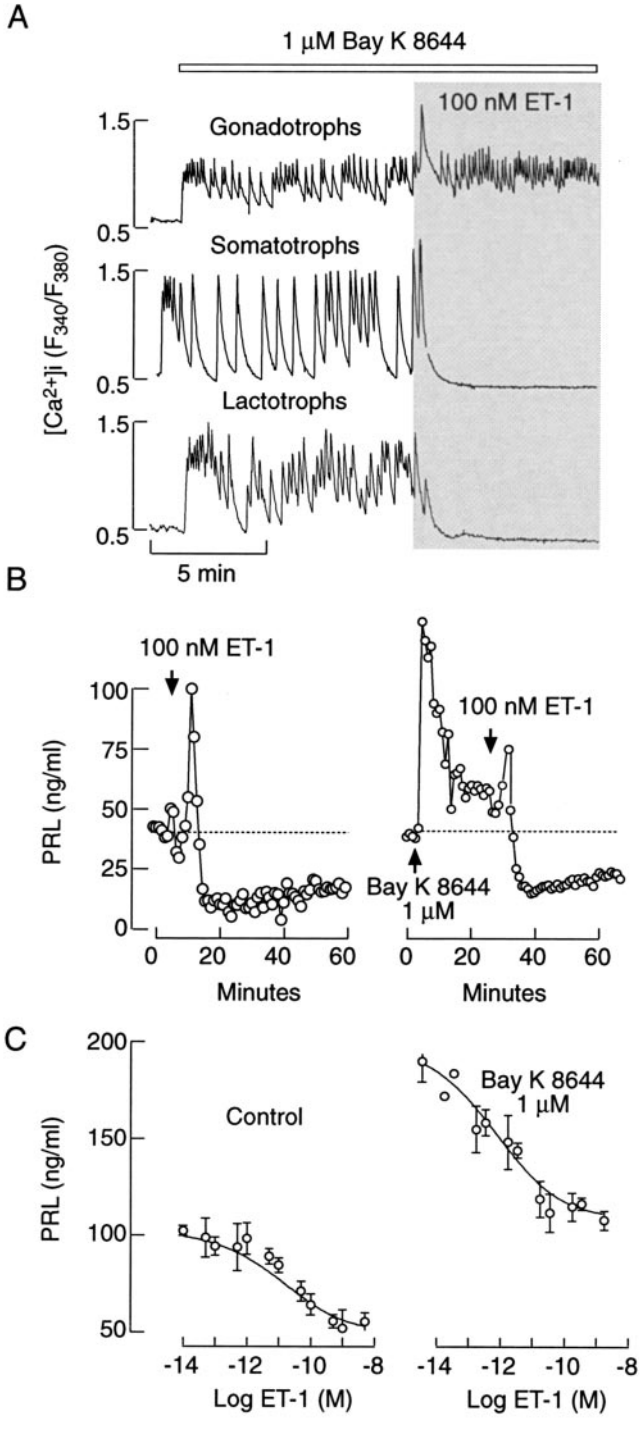

Fig. 2. Effects of ET-1 on Bay K 8644-induced calcium signaling and PRL release in pituitary cells. A, cell type-specific action of ET-1 on Bay K 8644-induced $[Ca^{2+}]_i$ transients; typical traces are shown. B, profiles of ET-1–induced PRL secretion in perfused cells; representative records from control cells (left) and Bay K 8644-treated cells (right) are shown. ET-1 was added in the presence of Bay K 8644. C, concentration-dependent effect of ET-1 on PRL release in static cultures of control cells (left) and Bay K 8644-stimulated cells (right). Data points are means ± S.E. from sextuplicate incubations.

primers GAPDH-sense (5′-GGCATCCTGGGCTACACTG-3′) and GAPDH-antisense (5′-TGAGGTCCACCACCCTGTT-3′).

After PCR, a 10-μl aliquot of PCR products was size-fractionated by electrophoresis in a 1.2% agarose gel, visualized by ethidium bromide staining, and further analyzed by Southern blotting. Oligonucleotide specific for each K_{ir} 3.0 isoform was used as a probe to identify the PCR products. The sequences of these oligonucleotides are as follows: for K_{ir} 3.1: (1129–1153 bp) 5′-GCACCAGCCATAACCAACAGCAAAG-3′; for K_{ir} 3.2: (1366–1387 bp) 5′-GTCTGTGTCCAGCAAACTGAAC-3′; for K_{ir} 3.3: (1287–1310 bp), 5′- GACGCCCATCTCTACTGGTCCATC-3′; and for K_{ir} 3.4: (1220–1243 bp) 5′-GACTACAACACTTTCCACGACACC-3′. The probes were 3′-end-labeled with digoxigenin-11-ddUTP using terminal transferase and hybridized to the blots at 52 to 62°C for overnight in DIG Easy Hyb Solution (Roche Diagnostics, Indianapolis, IN). The blots were washed at room temperature two times for 5 min each in 2× SSC-0.1% SDS and at hybridization temperature two times for 15 min each in 0.5× SSC-0.1% SDS. The hybridization signals were generated through a chemiluminescent reaction using CSPD as substrate (Roche Diagnostics) and exposed to X-ray film (Amersham Biosciences, Piscataway, NJ).

Results

Uncoupling of Ca^{2+} Mobilization and Influx Pathways. About 60% of identified lactotrophs exhibited spontaneous fluctuations in $[Ca^{2+}]_i$ (Fig. 1A, left trace), whereas the residual cells were quiescent (Fig. 1B, left trace). The actions of ET-1 on Ca^{2+} signaling were studied in both spontaneously active and quiescent cells. In spontaneously active lactotrophs, addition of 100 nM ET-1 evoked a spike in $[Ca^{2+}]_i$, which usually had a higher amplitude and longer duration than the spontaneously generated $[Ca^{2+}]_i$ transients. The spike phase was followed by the abolition of the $[Ca^{2+}]_i$ transients and a decrease in $[Ca^{2+}]_i$ below the initial level (Fig. 1A, left trace). The averaged basal $[Ca^{2+}]_i$ measured before and during the sustained stimulation with 100 nM ET-1 was significantly different (Fig. 1A, left bottom). In quiescent cells, 100 nM ET-1 induced a monophasic and extracellular Ca^{2+}-independent rise in $[Ca^{2+}]_i$ (Fig. 1B, left trace). The amplitude and duration of the ET-1–induced spikes were comparable in quiescent and spontaneously active cells. Furthermore, depletion of extracellular Ca^{2+} abolished spontaneous $[Ca^{2+}]_i$ transients but did not affect ET-1–induced spike response (not shown).

A majority of GH_3 lactosomatotrophs also exhibited spontaneous and extracellular Ca^{2+}-sensitive fluctuations in $[Ca^{2+}]_i$ comparable with those observed in native lactotrophs (Fig. 1A, right trace). Although transcripts for ET_A receptors have been identified in GH_3 cells (Tomic et al., 1999), only a few cells responded to 100 nM ET-1, indicating a low level of protein expression. To increase the number of ET-responsive cells, GH_3 cells were transfected with rat ET_A receptors. In such cells, ET-1 induced a rise in $[Ca^{2+}]_i$ in about 80% of cells examined. The $[Ca^{2+}]_i$ signaling pattern was similar to that observed in native lactotrophs (Fig. 1A, right trace). As in lactotrophs, the average basal $[Ca^{2+}]_i$ levels measured before and during the sustained stimulation with 100 nM ET-1 were significantly different (Fig. 1A, bottom right). Also, in quiescent GH_3 cells, ET-1 induced a monophasic $[Ca^{2+}]_i$ response (Fig. 1B, right). These results indicate that ET-1 induces rapid Ca^{2+} mobilization from intracellular stores in both quiescent and spontaneously active lactotrophs, and inhibits influx-dependent $[Ca^{2+}]_i$ transients in spontaneously active cells. The results further indicate that the ET_A receptor subtype can mediate the action of ET-1 on uncoupling of Ca^{2+} mobilization and VGCI pathways when expressed in immortalized lactosomatotrophs.

Role of VGCCs in ET-1–Induced Uncoupling. To study the dependence of ET-1–induced $[Ca^{2+}]_i$ responses in quiescent lactotrophs on the status of VGCI, we depolarized cells using Bay K 8644, an L-type Ca^{2+} channel agonist, and high concentrations of K^+. Bay K 8644 (1 μM) initiated $[Ca^{2+}]_i$ transients in quiescent lactotrophs, as well as in somatotrophs and gonadotrophs (Fig. 2A) and several other unidentified cell types (not shown). These $[Ca^{2+}]_i$ transients in lactotrophs were inhibited by ET-1 in the same manner as in spontaneously active lactotrophs. Inhibition was also observed in somatotrophs, but not in gonadotrophs (Fig. 2A),

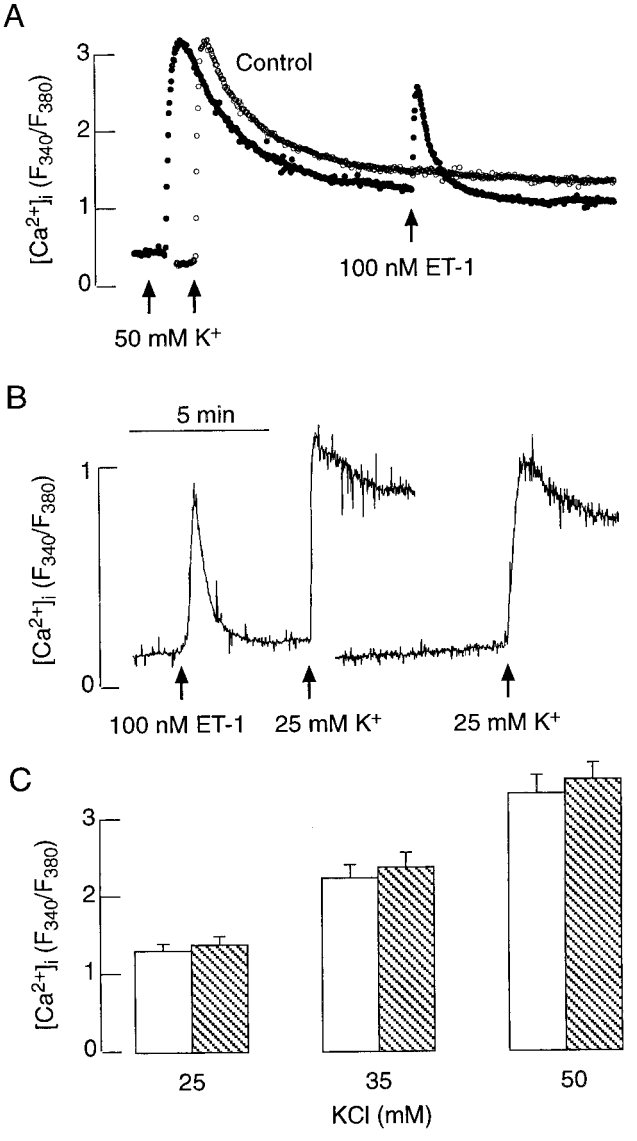

Fig. 3. Depolarization-induced $[Ca^{2+}]_i$ response in ET-1–stimulated and control lactotrophs. A, $[Ca^{2+}]_i$ signaling in cells depolarized with 50 mM K^+ and subsequently stimulated with ET-1. Data points are means derived from 32 control and 25 ET-1–stimulated cell records. B, representative traces of 25 mM K^+-induced $[Ca^{2+}]_i$ response in 100 nM ET-1–treated (left trace) and control cells (right trace). C, averaged data (means ± S.E.) of peak K^+-induced $[Ca^{2+}]_i$ responses from at least 25 cells per dose.

indicating the cell type-specific action of ET-1 on inhibition of VGCI. In parallel with $[Ca^{2+}]_i$ signaling, in perfused pituitary cells ET-1 induced a transient stimulation of PRL release, followed by sustained inhibition below the basal level (Fig. 2B, left). An elevation in PRL secretion induced by 1 μM Bay K 8644 was also inhibited by ET-1 in the same manner (Fig. 2B, right). During 3-h incubation of pituitary cells in static culture, ET-1 inhibited basal and Bay K 8644 (1 μM)-stimulated PRL release with comparable IC_{50} values in a picomolar concentration range (Fig. 2C).

In contrast to Bay K 8644 treatment, depolarization of lactotrophs with 50 mM K^+ was followed by a nonoscillatory elevation in $[Ca^{2+}]_i$ composed of an early spike phase and a sustained plateau phase. Earlier studies have revealed that the kinetics of activation and inactivation of VGCCs underlines such a profile of $[Ca^{2+}]_i$ (Stojilkovic et al., 1990a). The addition of ET-1 during the sustained depolarization of cells induced a spike $[Ca^{2+}]_i$ response, but not inhibition of VGCI (Fig. 3A), typically observed in control cells (Fig. 1A). To further test the gating status of VGCCs during ET-1 stimulation, we initially added ET-1 or medium and, subsequently, high K^+ concentrations. Figure 3B shows representative traces from such experiments; the peak amplitudes of depolarization-induced $[Ca^{2+}]_i$ responses were comparable in both cells. Figure 3C shows the mean values of peak $[Ca^{2+}]_i$ responses in cells treated with three K^+ concentrations, 25 mM, 35 mM, and 50 mM. Thus, although ET-1 inhibits spontaneous and Bay K 8644-induced voltage-gated $[Ca^{2+}]_i$ transients, it is unlikely that this agonist inhibits VGCCs directly.

Signaling Pathway Mediating ET-1–Induced Uncoupling. Simultaneous measurements of V_m and $[Ca^{2+}]_i$ in lactotrophs revealed the existence of bursting V_m oscillations that gave rise to $[Ca^{2+}]_i$ transients similar to those observed in unclamped cells (Figs. 4A). Addition of 100 nM ET-1 evoked a rapid and prolonged hyperpolarization that terminated action potential firing and decreased the $[Ca^{2+}]_i$ (Fig. 4A). To test whether the ET-1–induced membrane hyperpolarization was caused by inhibition of a depolarizing current or activation of a hyperpolarizing current, the input resistance of the membrane was monitored by the application of hyperpolarizing current injections. In all lactotrophs examined (n = 5), ET-1 decreased the cell input resistance (Fig. 4B), indicating the activation of a hyperpolarizing current. Consistent with this, ET-1 increased the amplitude of a current evoked by hyperpolarizing step from −70 mV to −150 mV (Fig. 4C). The ET-1–induced current was observed in −90 to −170 mV voltage range and reversed its direction at about −80 mV (Fig. 4D), the reversal potential for K^+ in these experimental conditions. The current was masked in potentials positive to −70 mV due to activation of other channels (not shown). These results suggest that ET-1 activates a K^+ current, leading to hyperpolarization of cells and abolition of action potential firing.

To examine the dependence of ET-1–induced calcium signaling on the $G_{i/o}$ signaling pathway, we treated cells with PTX overnight. As in control cells, the ET-1–induced spike $[Ca^{2+}]_i$ response was observed in PTX-treated cells; however, the spike $[Ca^{2+}]_i$ response was followed by a sustained elevation in $[Ca^{2+}]_i$. This was observed in both spontaneously

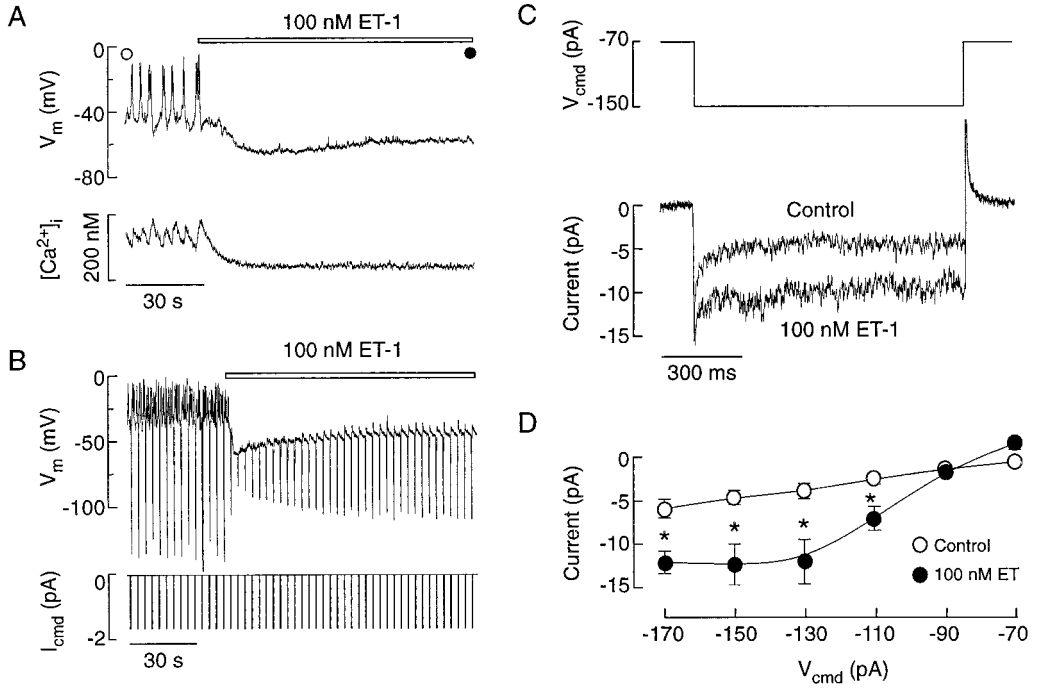

Fig. 4. ET-1–induced membrane hyperpolarization and activation of a K^+ current. A, simultaneous measurement of V_m and $[Ca^{2+}]_i$ in response to 100 nM ET-1. B, ET-1–induced reduction in membrane input resistance (upper trace), monitored by injecting regular hyperpolarizing current pulses (lower trace). C, activation of an inward current by a hyperpolarizing voltage step from −70 mV to −150 mV before and during application of 100 nM ET-1. D, current-voltage relation of ET-1–activated current before (○) and during (●) application of 100 nM ET-1. The traces shown in A and C are from the same cell. ○ and ● in A indicate the time when currents shown in C and D were measured. Data points in D are mean ± S.E. from five experiments. In this figure and Figs. 6 and 8, the current activated by hyperpolarizing pulses was recorded in perforated patch-clamp conditions with 4.7 mM extracellular K^+. V_{cmd}, the command voltage. Calcium recordings were done in indo-1–loaded cells, and $[Ca^{2+}]_i$ was calculated as described under *Materials and Methods*.

active cells (Fig. 5A, left) and quiescent cells (Fig. 5B, left trace). In PTX-treated, spontaneously active and quiescent GH_3 cells expressing ET_A receptors, the spike phase was also accompanied by a significant increase in $[Ca^{2+}]_i$ compared with prestimulated levels (Fig. 5, A and B, right). The sustained rise in $[Ca^{2+}]_i$ was abolished by 1 μM nifedipine (Fig. 5C), indicating a role of VGCCs in sustained Ca^{2+} influx in PTX-treated cells.

In current-clamped PTX-treated cells, ET-1–induced Ca^{2+} mobilization was associated with a sustained V_m depolarization and an increase in firing frequency (Fig. 6A, upper trace), resulting in the elevation of $[Ca^{2+}]_i$ (Fig. 6A, bottom trace). In addition, the ET-1–induced reduction in input resistance observed in control cells (Fig. 4B) was abolished by PTX treatment (Fig. 6B), as well as the agonist-induced enhancement of K^+ current (Fig. 6, C and D). These results indicate that a PTX-sensitive pathway mediates the increase in input resistance and modulation of K^+ current. Moreover, these results demonstrate that the cross-coupling of ET_A receptors to the $G_{i/o}$ signaling pathway accounts for the disconnection of Ca^{2+} influx, whereas Ca^{2+} mobilization is independent of the $G_{i/o}$ signaling pathway.

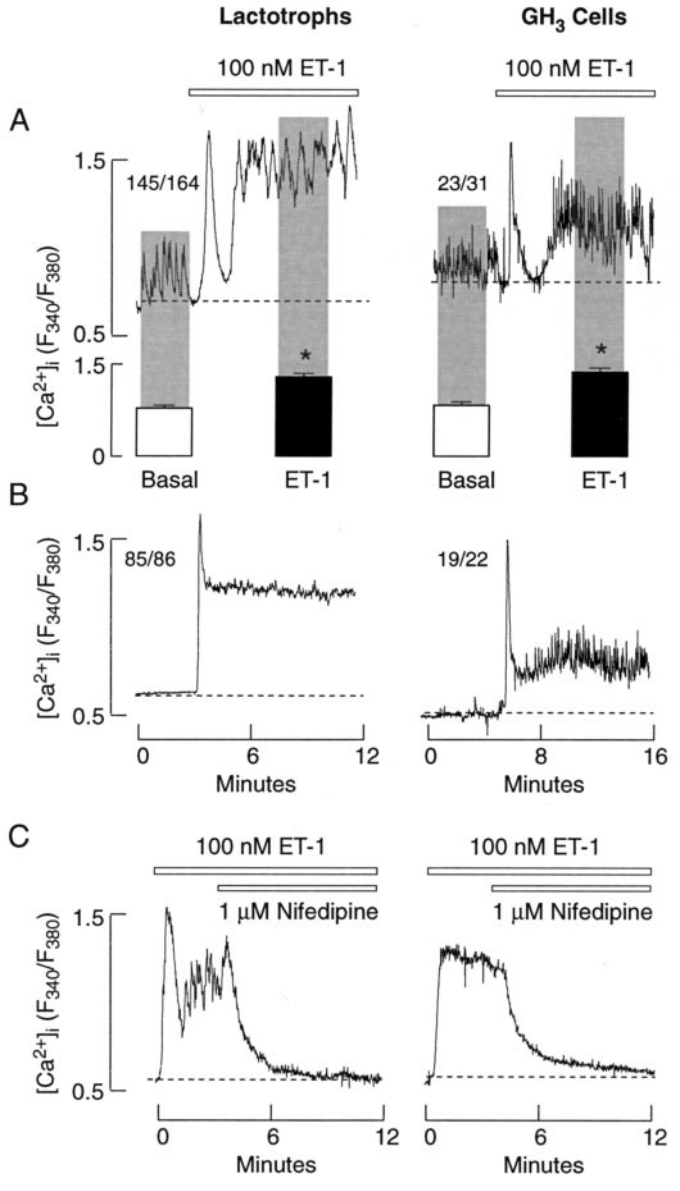

Fig. 5. Effects of PTX (250 ng/ml overnight) on ET-1–induced calcium signaling in lactotrophs and GH_3 cells transfected with rat ET_A receptors. ET-1–induced $[Ca^{2+}]_i$ response in spontaneously active (A) and quiescent (B) cells. The tracings shown are representative, with numbers above traces indicating the fraction of spontaneously active or quiescent cells responding to ET-1 with representative patterns. A, bottom, mean values ± S.E. of $[Ca^{2+}]_i$ estimated before and during the sustained ET-1 stimulation (shown by gray areas). Asterisks indicate significant ($P < 0.05$) differences among means. C, effects of nifedipine on sustained ET-1–induced VGCI; representative traces are shown.

To test the relevance of ET-1–induced inhibition of adenylyl cyclase activity on the pattern of Ca^{2+} signaling, lactotrophs were treated with 1 μM forskolin, an activator of adenylyl cyclase, and 1 mM 8-Br-cAMP, a membrane-permeable cAMP analog, for variable times (between 15 min and 3 h). In these cells, ET-1 induced a typical long-lasting inhibition of VGCI. Figure 7, A and B, shows the effect of ET-1 on Ca^{2+} signaling in spontaneously active (left and right) and quiescent lactotrophs (middle) treated with forskolin or 8-Br-cAMP for 60 min. In quiescent lactotrophs, TRH in the presence of ET-1 induced the typical biphasic $[Ca^{2+}]_i$ response (center traces). Thus, the PTX-sensitive inhibition of adenylyl cyclase activity by ET_A receptors does not account for uncoupling of Ca^{2+} mobilization and influx pathways, suggesting that β/γ dimer mediates the action of these receptors on K^+ channels.

Characterization of K^+ Channels Involved in ET-1–Induced Uncoupling. In accordance with literature data in other pituitary cell types (Kuryshev et al., 1997), 5 mM Cs^+ reduced the K^+ current in lactotrophs (Fig. 8, A and B). Single-cell $[Ca^{2+}]_i$ measurements also revealed that the addition of 5 mM Cs^+ initiated $[Ca^{2+}]_i$ transients in a fraction of quiescent lactotrophs (33%) and increased the frequency of transients in spontaneously active cells (80%; Fig. 8C). In the presence of Cs^+, the ET-1–induced spike $[Ca^{2+}]_i$ response was followed by a sustained plateau response in a large fraction of lactotrophs. The sustained plateau response was frequently observed in lactotrophs in which Cs^+ initiated $[Ca^{2+}]_i$ transients (Fig. 9A, left trace), as well as in cells in which Cs^+ per se was ineffective (Fig. 9A, right trace). These results indicate that a Cs^+-sensitive current is constitutively active in lactotrophs and contributes to the control of spontaneous action potential firing, as well as to uncoupling of Ca^{2+} mobilization and influx pathways in ET-1–stimulated lactotrophs.

Two channels expressed in pituitary lactotrophs could mediate the ET-1–induced and Cs^+-sensitive hyperpolarizing current: K_{ir} channels (Einhorn et al., 1991; Kuryshev et al., 1997) and/or *ether-a go-go*–related gene (erg)-like K^+ channels (Schafer et al., 1999; Schledermann et al., 2001). Both channels are activated by hyperpolarizing pulses, exhibit inward rectification, and can be blocked by Cs^+. To dissociate between these two currents, E-4031, a specific erg-like channel blocker, was used. As in Cs^+-treated cells, a fraction of lactotrophs responded to E-4031 with elevation in $[Ca^{2+}]_i$ (Fig. 9, B and C). In contrast to Cs^+-treated lactotrophs, the pattern of ET-1–induced $[Ca^{2+}]_i$ signaling was not affected by E-4031 (Fig. 9B). The averaged sustained $[Ca^{2+}]_i$ responses in ET-1–treated cells in the presence and absence of E-4031 were comparable and significantly lower than they were before ET-1 application. In contrast, in ET-1–stimulated cells in the presence of Cs^+, the $[Ca^{2+}]_i$ was signifi-

cantly higher (Fig. 9C). These results indicate that erg channels may participate in control of spontaneous $[Ca^{2+}]_i$ transients in a fraction of lactotrophs, but do not mediate ET-1–induced inhibition of VGCI.

In PTX- and Cs^+-treated cells, the initial Ca^{2+}-mobilizing phase was frequently separated from the sustained spiking (Figs. 5A and 9A), suggesting a role of $I_{K\text{-}Ca}$ channels in early ET-1 action. To test this hypothesis more directly, ET-1–induced responses were monitored in cells pretreated with apamin, a blocker of the small-conductance type of $I_{K\text{-}Ca}$ channels, and charybdotoxin, a blocker of large-conductance type $I_{K\text{-}Ca}$ channels. As shown in Fig. 9D, ET-1–induced $[Ca^{2+}]_i$ responses in spontaneously active (Fig. 9D, left trace) and quiescent lactotrophs (Fig. 9D, right trace) were unaffected by blocking of $I_{K\text{-}Ca}$ channels, arguing against the role of these channels in sustained inhibition of VGCI.

The current-voltage characteristics, Cs^+ sensitivity, and E-4031, forskolin, and 8-Br-cAMP insensitivity of ET-1–induced current are consistent with the role of $G_{i/o}$-controlled K_{ir} 3.0 channels in ET-1–induced sustained hyperpolarization of cells. In accord with these findings and literature data in pituitary tissue and immortalized pituitary cells (Wulfsen et al., 2000; Gregerson et al., 2001), the robust PCR products of predicted sizes for K_{ir} 3.1, 3.2, and 3.4 were also observed

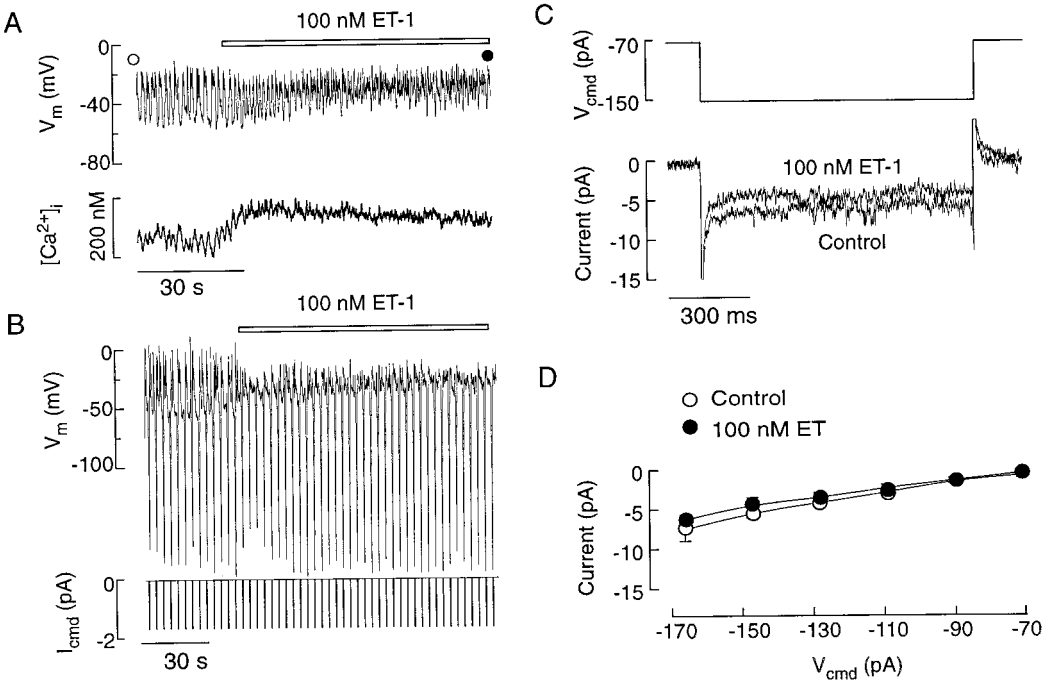

Fig. 6. ET-1–induced membrane depolarization in PTX-treated (250 ng/ml overnight) lactotrophs. A, simultaneous measurement of V_m and $[Ca^{2+}]_i$ in response to 100 nM ET-1 (bar). B, abolition of the ET-1–induced reduction in input resistance by PTX treatment. C, the lack of ET-1–induced activation of K^+ current in PTX-treated cells. D, current-voltage relation of K^+ current before (○) and during (●) application of 100 nM ET-1. The traces shown in A and C are from the same cell. ○ and ● in A indicate the time when currents shown in C and D were measured. Data points in D are mean ± S.E.M. from five experiments. Calcium recordings were done in indo-1–loaded cells.

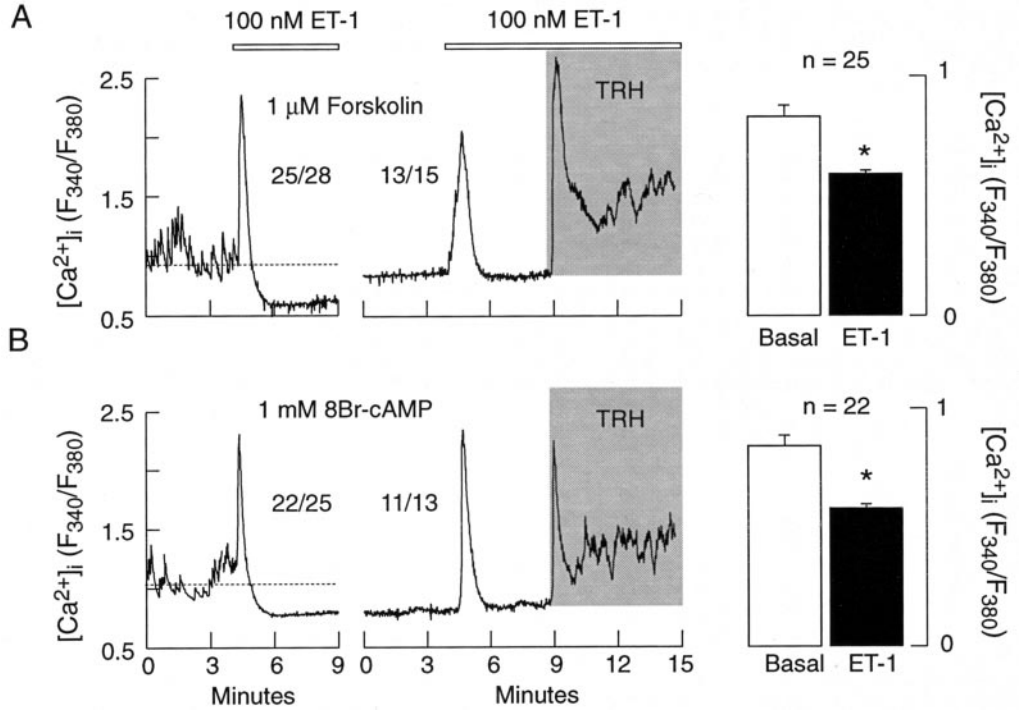

Fig. 7. The lack of effects of forskolin (A) and 8-Br-cAMP (B) on Ca^{2+} signaling in ET-1–stimulated lactotrophs. Left, representative patterns of Ca^{2+} signaling in spontaneously active lactotrophs. Center, comparison of ET-1– and 100 nM TRH-induced $[Ca^{2+}]_i$ responses in quiescent lactotrophs. Right, mean values ± S.E. of $[Ca^{2+}]_i$ in spontaneously active lactotrophs estimated during the first 3 min (basal) and between 6 and 9 min of recording (ET-1–treated). Asterisks indicate significant ($P < 0.05$) differences among means.

in a mixed population of anterior pituitary cells cultured for 24 h after dispersion, whereas a PCR product of predicted size for K_{ir} 3.3 was less robust. K_{ir} 3.1, 3.2, 3.3, and 3.4 were also amplified from purified lactotrophs. Figure 10 illustrates the products for each K_{ir} 3.0 family subunit, as well as GAPDH product, obtained from mixed anterior pituitary cells (Fig. 10, lane 4) and purified lactotrophs (Fig. 10, lane 6) after 35 amplification cycles. Southern blot analysis of the same gels further confirmed these results. No PCR products were detected from control cells containing all the components except for the RT, ruling out the possibility of genomic DNA contamination. In GH_3 cells, the specific PCR products for K_{ir} 3.1, 3.2, and 3.3, but not 3.4, were also found (not shown).

Discussion

Activation of Ca^{2+}-mobilizing receptors in excitable cells usually generates biphasic $[Ca^{2+}]_i$ and secretory profiles composed of an early spike response mediated by inositol trisphosphate-induced Ca^{2+} release and a sustained plateau response that is dependent on Ca^{2+} influx through voltage-gated and -insensitive calcium channels (Stojilkovic and Catt, 1992). In lactotrophs, stimulation of Ca^{2+} mobilization and PRL secretion by ET-1 and their PTX insensitivity are consistent with the coupling of ET_A receptors to the $G_{q/11}$ signaling pathway and activation of the phospholipase C signaling pathway (Stojilkovic et al., 1990b, 1992). In contrast to other Ca^{2+}-mobilizing receptors, the ET-1–induced Ca^{2+} mobilization in lactotrophs is not followed by sustained increase in Ca^{2+} influx due to the prolonged membrane hyperpolarization, which inhibits VGCI (Lachowicz et al., 1997). Here we show that in PTX- and Cs^+-treated lactotrophs, ET-1 induced biphasic $[Ca^{2+}]_i$ and secretory responses comparable with those observed upon stimulation with other Ca^{2+}-mobilizing agonists (Hinkle et al., 1996; Lachowicz et al., 1997). Thus, ET_A receptors in these cells also trigger a common mechanism for the activation of Ca^{2+} influx with Ca^{2+} mobilization, but the coupling of these receptors to the PTX-sensitive $G_{i/o}$ signaling pathway under physiological conditions prevents the development of the second phase in Ca^{2+} signaling.

In other cell types, intracellular signaling through the $G_{i/o}$ pathway is well characterized. During receptor activation, the α subunit of these heteromeric proteins couples to adenylyl cyclase, leading to inhibition of cAMP production, whereas β/γ dimer activates several other effector molecules in a cell-specific manner, including phospholipase C, VGCCs, and K_{ir} channels (family 3.0) (Wickman and Clapham, 1995). As discussed above, the ET_A receptor signals for activation of phospholipase C through PTX-insensitive G proteins. Furthermore, the $[Ca^{2+}]_i$ response to the addition of extracellular K^+ was comparable in control and ET-treated cells, suggesting that VGCCs are not directly inhibited. In accordance with this finding, activation of ET_A receptors in other pituitary cell types was found not to affect voltage-gated Ca^{2+} current (Tomic et al., 1999). Although in pituitary cells basal adenylyl cyclase activity is controlled by spontaneous VGCI (Kostic et al., 2001) and ET-1 inhibits it in a dose-dependent and PTX-sensitive manner (Tomic et al., 1999), it is unlikely that the adenylyl cyclase-signaling pathway mediates the action of ET_A receptors on inhibition of VGCI. First, activation of adenylyl cyclase by forskolin did not affect the pattern of ET-1–induced $[Ca^{2+}]_i$ signaling. Second, the addition of 8-Br-cAMP, a permeable cAMP analog, was also ineffective.

On the other hand, several lines of evidence support the role of K^+ channels in preventing VGCI after intracellular Ca^{2+} mobilization. The sustained membrane hyperpolarization and decrease in input resistance observed in ET-1–stimulated cells indicate that ET_A receptors activate or augment a hyperpolarizing current rather than inhibit a depolarizing

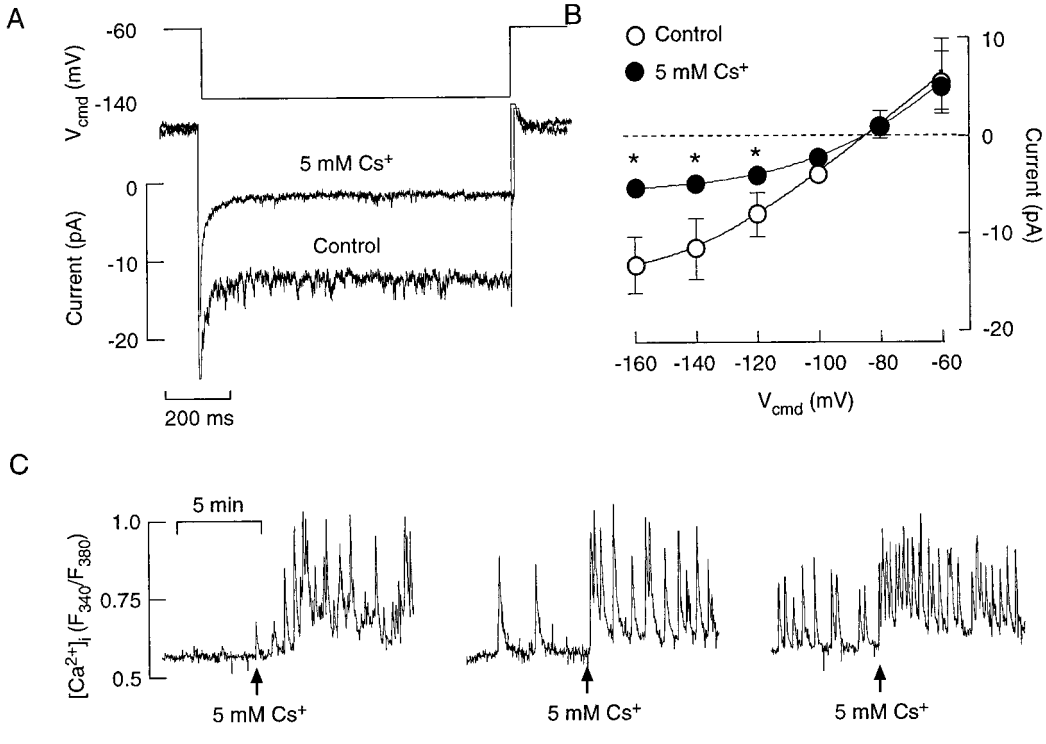

Fig. 8. Effects of extracellular Cs^+ on K^+ current and $[Ca^{2+}]_i$ signaling in lactotrophs. A and B, activation of K^+ current by a hyperpolarizing voltage step from −60 mV to −140 mV before and during application of 5 mM Cs^+. A, representative traces. B, current-voltage relation. C, effects of 5 mM Cs^+ on $[Ca^{2+}]_i$ in quiescent (left trace) and spontaneously active cells (center and right trace).

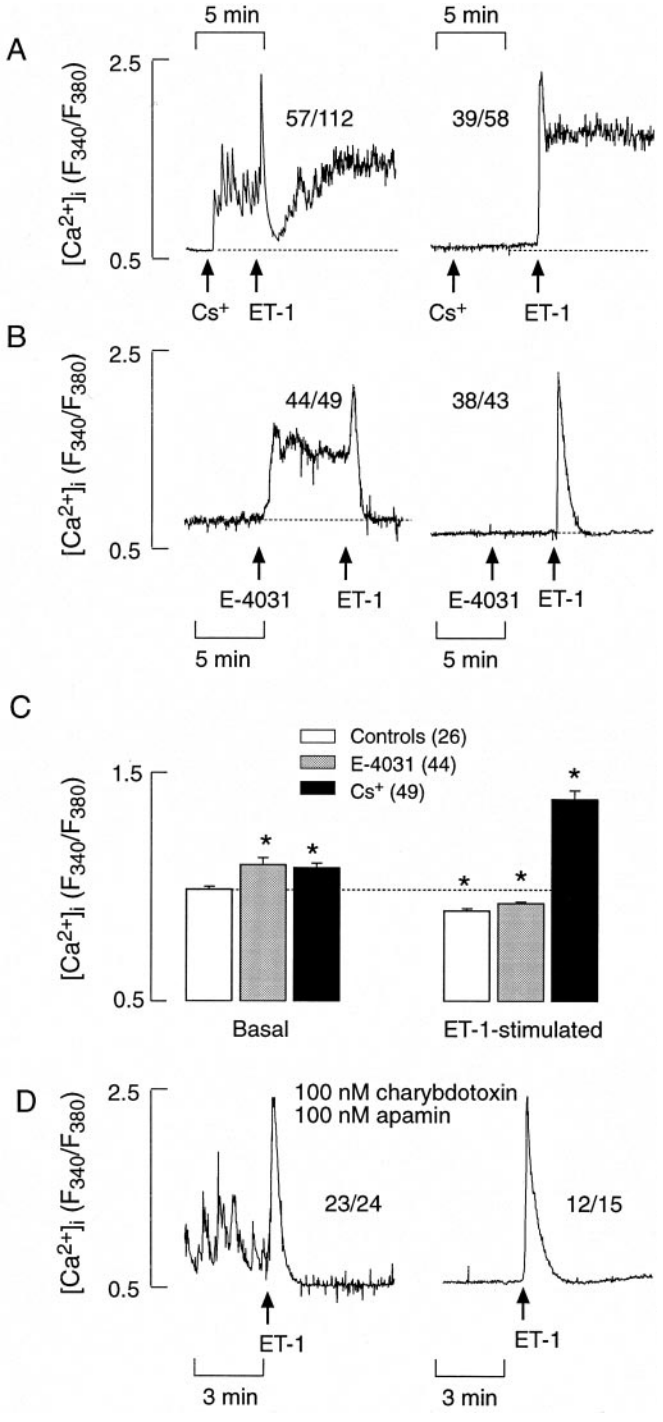

Fig. 9. Effects of K^+ channel blockers on $[Ca^{2+}]_i$ signaling in 100 nM ET-1–stimulated lactotrophs. A, pattern of ET-1–induced $[Ca^{2+}]_i$ signaling in lactotrophs bathed in medium containing Cs^+; representative traces in lactotrophs responding to the addition of 5 mM Cs^+ with (left trace) and without (right trace) elevation in $[Ca^+]_i$ are shown. B, pattern of ET-1–induced $[Ca^{2+}]_i$ signaling in lactotrophs bathed in medium containing 1 μM E-4031, a specific erg-like channel blocker; representative traces are shown. The numbers above traces in A and B indicate the fraction of cells responding with a particular pattern to Cs^+ + ET-1 and E-4031 + ET-1 stimulation. C, mean values ± S.E. of basal and sustained ET-1–induced $[Ca^{2+}]_i$ responses in control cells, and 5 mM Cs^+- and 1 μM E-4031-treated cells, with *N* indicated in parentheses. Asterisks indicate significant ($P < 0.05$) difference among means. D, the lack of effect of 100 nM apamine and 100 nM charybdotoxin cocktail on the pattern of ET-1–induced Ca^{2+} signaling in spontaneously active (left trace) and quiescent lactotrophs (right trace). Numbers above traces indicate the fraction of cells responding with a particular pattern.

current. In voltage-clamped cells stimulated with ET-1, application of hyperpolarizing pulses revealed stimulation of a K^+ current. Agonist-induced hyperpolarization of cells, reduction in input resistance, and activation of K^+ current were abolished in PTX-treated cells. Addition of extracellular Cs^+ inhibited hyperpolarization-induced K^+ current. This treatment also led to the coupling of Ca^{2+} influx to Ca^{2+} mobilization in a manner comparable with that observed in PTX-treated cells, suggesting a role of Cs^+-sensitive K^+ channels in sustained hyperpolarization of cells.

Pituitary lactotrophs express two channel subtypes that are sensitive to Cs^+ at the concentrations used in our experiments, K_{ir} and erg-like channels, but only the latter is also sensitive to E-4031 (Einhorn et al., 1991; Schafer et al., 1999; Schledermann et al., 2001). Initiation of $[Ca^{2+}]_i$ transients in quiescent lactotrophs and the increase in the frequency of $[Ca^{2+}]_i$ transients in spontaneously active cells after the addition of 5 mM Cs^+ and 1 μM E-4031 are consistent with the role of erg-like channels in controlling the firing frequency in unstimulated cells. However, in cells treated with

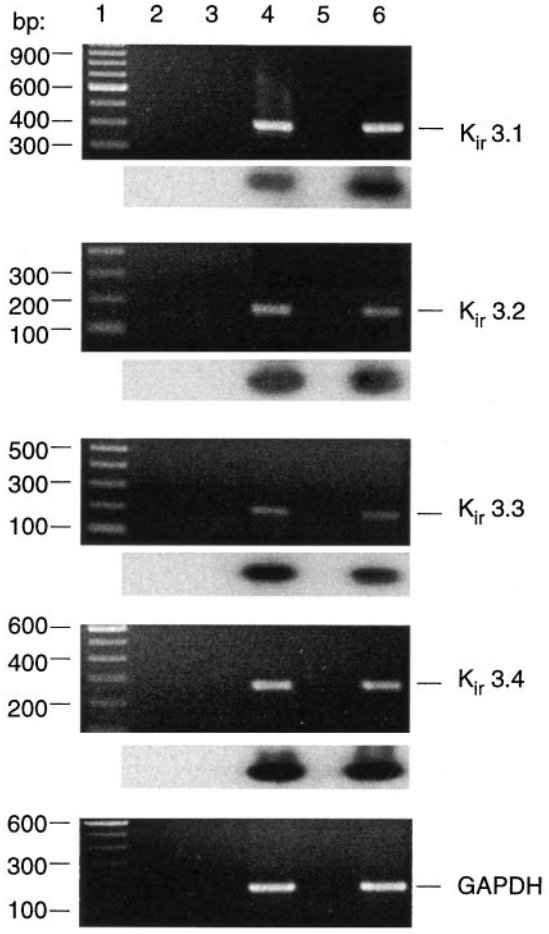

Fig. 10. Expression of K_{ir} 3.0 channels in anterior pituitary cells and enriched lactotrophs. Black, detection of K_{ir} 3.0 isoforms in mixed anterior pituitary cells (lanes 3 and 4) and purified lactotrophs (lanes 5 and 6). For negative control cells, PCR was conducted using first-strand cDNA samples without RT (lanes 3 and 5). In the case of "no template" control (lane 2), water was substituted for the first-strand cDNA sample in the PCR reaction. DNA markers are shown in lane 1. GAPDH primers were used as an internal control to monitor the quality of RNA preparation. Gray, Southern blot analysis of the same gels shown above. Blots were probed with 3′-end-labeled oligonucleotide specific to a corresponding type of K_{ir} channel.

E-4031, ET-1–induced inhibition of sustained VGCI did not differ from that observed in control cells, supporting a role for K_{ir} rather than erg-like channels in mediating the ET-induced sustained V_m hyperpolarization. Consistent with these findings, our results indicate that lactotrophs express the K_{ir} 3.0 subfamily of channels. Others have also observed the expression of K_{ir} 3.0 in pituitary tissue and GH cells (Wulfsen et al., 2000; Gregerson et al., 2001). Further single-channel recordings are needed to fully characterize these channels and their regulation by ET_A receptors.

The cross-coupling of ET_A receptors to the $G_{i/o}$ signaling pathway is not unique to pituitary lactotrophs, as it is also observed in other cell types (James et al., 1994; Ono et al., 1994). Other Ca^{2+}-mobilizing receptors also cross-couple to the $G_{i/o}$ signaling pathway (Krsmanovic et al., 1998). In lactotrophs, the pattern of ET-1 action depends on the firing status of individual cells. In quiescent cells, the ET-1–induced monophasic $[Ca^{2+}]_i$ response was solely dependent on Ca^{2+} mobilization, whereas in spontaneously active cells, the Ca^{2+}-mobilizing action was accompanied by a prolonged inhibition of spontaneous pacemaking and VGCI. Because spontaneous action potential firing and the associated Ca^{2+} influx are sufficient to trigger PRL secretion (Sankaranarayanan and Simasko, 1996b; Van Goor et al., 2001a), the ET-induced hyperpolarization and the ensuing inhibition of VGCI are an effective mechanism for the sustained inhibition of PRL secretion. The similar actions of ET-1 in lactotrophs and GH cells, the latter not expressing K_{ir} 3.4, further indicate that this particular isoform of channels is not essential for ET-1–induced inhibition of VGCI.

The coupling of ET_A receptors to K_{ir} in spontaneously active cells and their ability to induce sustained membrane hyperpolarization and inhibition of hormone secretion are comparable with the action of dopamine and somatostatin receptor activation in pituitary lactotrophs and other cell types. Like ET_A receptors (Tomic et al., 1999), these receptors are negatively coupled to adenylyl cyclase; i.e., their activation leads to inhibition of cAMP production in a PTX-sensitive manner (Freeman et al., 2000). Dopamine and somatostatin also directly activate K_{ir} in a PTX-sensitive manner (Einhorn et al., 1991; Sims et al., 1991; Lledo et al., 1992). However, in contrast to ET_A receptors, dopamine and somatostatin receptors do not stimulate the phospholipase C in pituitary cells (Tallent et al., 1996; Freeman et al., 2000). Furthermore, dopamine and somatostatin inhibit L-type calcium channels (Lewis et al., 1986; Kleuss, 1995; Tallent et al., 1996), whereas ET_A receptors do not. At the present time, the biochemical reason for the difference in the coupling of these receptors to the L-type channels is not clear.

Despite the importance of K_{ir} channels in mediating the sustained membrane hyperpolarization, they are not exclusively responsible for ET-1–induced termination of action potential firing in lactotrophs. Both lactotrophs and GH_3 cells express $I_{K\text{-}Ca}$ channels (Ritchie, 1987b; Van Goor et al., 2001b), and their activation by Ca^{2+} mobilization in response to ET-1 and TRH has been shown previously (Ritchie, 1987a; Kanyicska et al., 1997). Consistent with this, in PTX- and Cs^+-treated cells, the sustained facilitation of VGCI often occurs with a delay, effectively dissociating the spike and plateau $[Ca^{2+}]_i$ responses. Under normal conditions, however, depletion of the intracellular Ca^{2+} pool(s) within 1 to 2 min and the marked reduction in $[Ca^{2+}]_i$ caused by inhibition of VGCI will prevent the sustained activation of $I_{K\text{-}Ca}$ channels and, thus, their participation in facilitating membrane hyperpolarization.

In conclusion, our results indicate that ET-1 prevents VGCI after intracellular Ca^{2+} mobilization in lactotrophs by activating the K_{ir} 3.0 family of channels. The cross-coupling of ET_A receptors to the G_i/G_o signaling pathway leads to activation of these channels, hyperpolarization of cells, and abolition of spontaneous firing of action potentials and action potential-driven Ca^{2+} influx. Abolition of this cross-coupling by PTX unmasks the depolarizing action of ET-1, typically observed in response to other Ca^{2+}-mobilizing receptors. The uncoupling of Ca^{2+} mobilization and influx pathways and the inhibition of spontaneous firing of action potentials are effective mechanisms for the prolonged inhibition of PRL secretion.

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