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Dependence of Prolactin Release on Coupling Between Ca^{2+} Mobilization and Voltage-Gated Ca^{2+} Influx Pathways in Rat Lactotrophs

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Two Ca^{2+}-mobilizing receptors expressed in lactotrophs, endothelin-A (ET_A) and thyrotropin-releasing hormone (TRH), induce a rapid Ca^{2+} release from intracellular stores and prolactin (PRL) secretion but differ in their actions during the sustained stimulation; TRH facilitates and ET-1 inhibits voltage-gated calcium influx (VGCI) and PRL secretion. In pertussis toxin (PTX)–treated cells, ET-1-induced inhibition of VGCI was abolished and the pattern of Ca^{2+} signaling was highly comparable with that observed in TRH-stimulated cells. The addition of Cs^+, a relatively specific blocker of inward rectifier K^+ channels, mimicked the effect of PTX on the pattern of ET-1-induced sustained Ca^{2+} signaling, but only in about 50% of cells, and did not affect agonist-induced inhibition of PRL secretion. Extracellular Cs^+ was also ineffective in altering the TRH-induced facilitation of VGCI and PRL secretion. Furthermore, apamin and paxilline, specific blockers of Ca^{2+}-activated SK- and BK-type K^+ channels, respectively; E-4031, a blocker of *ether a-go-go* K^+ channel; and linopirdine, a blocker of M-type K^+ channel, did not affect the agonist-specific patterns of calcium signaling and PRL secretion. These results suggest that ET-1 inhibits VGCI through activation of Cs^+-sensitive channels, presumably the $G_{i/o}$-controlled inward rectifier K^+ channels, and that this agonist also inhibits PRL release, but downstream of Ca^{2+} influx. Further studies are required to identify the mechanism of sustained TRH-induced facilitation of VGCI and PRL secretion.

Key Words: Endothelin-A; thyrotropin-releasing hormone; calcium; prolactin; voltage-gated calcium channels; inward rectifier potassium channels.

Introduction

Several G protein–coupled receptors expressed in lactotrophs stimulate or inhibit spontaneous voltage-gated calcium influx (VGCI) and prolactin (PRL) secretion *(1)*. Here, we focus on two Ca^{2+}-mobilizing receptors, thyrotropin-releasing hormone (TRH) and endothelin-A (ET_A). TRH receptor is expressed in lactotrophs and growth hormone (GH)–immortalized cells and is coupled to the phospholipase C (PLC) signaling pathway through $G_{q/11}$ proteins *(2)*. Activation of these receptors leads to the rapid increase in Ca^{2+} mobilization from intracellular stores and PRL release, accompanied by sustained facilitation of VGCI and secretion *(3)*. Whereas the Ca^{2+}-mobilizing action of TRH receptors is well defined, the mechanism of facilitation of sustained Ca^{2+} influx and PRL secretion has not been clarified. Different plasma membrane channels have been suggested to account for TRH-induced increase in calcium influx, including capacitative Ca^{2+} entry channels *(4,5)*, M-type K^+ channels *(6)*, *ether a-go-go* (erg) K^+ channels *(7,8)*, and inward rectifier K^+ (K_{ir}) channels *(9)*. The impact of blockade of these channels on TRH-induced Ca^{2+} signaling and secretion has not been systematically addressed.

ET_A receptors are also expressed in lactotrophs and are coupled to the PLC signaling pathway through the $G_{q/11}$ signaling pathway, which accounts for rapid increases in $[Ca^{2+}]_i$ and PRL release *(10,11)*. In contrast to TRH receptor, ET_A receptor–induced elevation in intracellular Ca^{2+} and PRL release is followed by a prolonged inhibition of spontaneous VGCI and secretion *(10–16)* in a manner that resembles the action of two other G protein–coupled receptors that are expressed in lactotrophs: dopamine and somatostatin *(17–21)*. Like these receptors, ET_A receptor is negatively coupled to adenylyl cyclase through pertussis toxin (PTX)–sensitive signaling pathway *(22)*. In PTX-treated cells, the ET_A receptor agonist ET-1 induces Ca^{2+} signaling profiles comparable with those observed in TRH-stimulated cells *(11)*. Dopamine and somatostatin induce inhibition of spontaneous firing of action potentials and the accompanied VGCI in pituitary cells by activating K_{ir} channels *(17,18, 20,23)*, as well as by inhibiting voltage-gated Ca^{2+} channels *(20,21)*. Activation of calcium-controlled potassium

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($I_{K\text{-}Ca}$) channels *(24)* and K_{ir} channels *(11)* has been suggested to account for ET-1-induced inhibition of VGCI and PRL release. As with TRH receptors, no systematic evaluation of the impact of blockade of these and other K^+ channels on ET_A receptor–induced sustained VGCI and PRL secretion has been done.

To study the role of K^+ channels in diverse actions of these two receptors on VGCI and secretion, we used Cs^+, a relatively specific blocker of K_{ir} channels; apamin and paxilline, specific blockers of Ca^{2+}-activated SK- and BK-type $I_{K\text{-}Ca}$ channels, respectively; [1-2-(6-methyl-2-pyridyl)ethyl)-(4-methanesulfonamidobenzoyl)piperidine (E-4031), a blocker of erg K^+ channel; and 1,3-dihydro-1-phenyl-3,3-*bis*(4-pyridinylmethyl)-2H-indol-2-one (linopirdine), a blocker of M-type K^+ channel. Calcium measurements were done in single identified lactotrophs. The rate of PRL secretion was analyzed in perifused pituitary cells. Our results suggest that activation of K_{ir} channels, at least in part, accounts for ET-1 inhibition of VGCI, but that ET_A receptors inhibit PRL secretion downstream of VGCI. None of the examined blockers inhibited TRH-induced stimulation of VGCI and PRL release.

Results

Patterns of ET-1 and TRH-Induced Calcium Signaling and Secretion

In pituitary cells perifused with Ca^{2+}-deficient medium, PRL secretion was 3–5 ng/(mL·min). ET-1 and TRH (both from Peninsula) induced a rapid rise in PRL secretion that lasted for several minutes and returned to prestimulated levels (monophasic response). As shown in Fig. 1A, the profiles of monophasic responses induced by two agonists were comparable. Consistent with the Ca^{2+}-mobilizing action of ET_A and TRH receptors, both agonists also induced monophasic $[Ca^{2+}]_i$ responses in cells bathed in Ca^{2+}-deficient medium (Fig. 1B). The qualitative similarities in $[Ca^{2+}]_i$ and secretory profiles in ET-1- and TRH-stimulated cells indicate that a transient rise in $[Ca^{2+}]_i$ is sufficient to activate exocytosis. The temporal correlation between Ca^{2+} signaling and secretion is more complex, as indicated by a sixfold difference in the time scales in both cases.

Pituitary cells perifused with Ca^{2+}-containing medium secreted between 30 and 50 ng/(mL·min) of PRL. In these cells, ET-1-induced spike PRL response was followed by sustained inhibition of secretion below the initial basal levels (bidirectional response), whereas TRH-induced spike response was accompanied by sustained plateau secretion above the initial levels (biphasic response; Fig. 2A). ET-1 also induced bidirectional changes in $[Ca^{2+}]_i$, whereas TRH induced a biphasic response in $[Ca^{2+}]_i$, typically observed in cells stimulated with Ca^{2+}-mobilizing receptors. In some cells, the sustained response was nonoscillatory, and in the others, such as that shown in Fig. 2B, it was oscillatory.

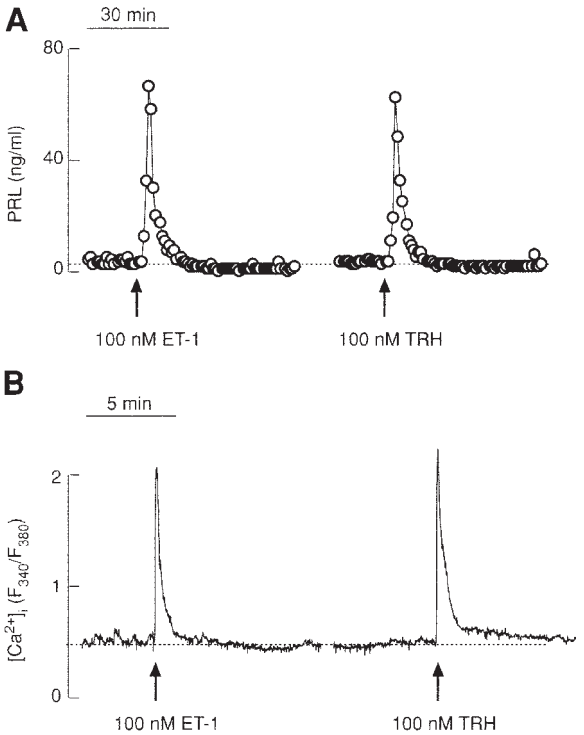

Fig. 1. Dependence of PRL secretion on Ca^{2+} mobilization from intracellular stores. Typical patterns of ET-1- and TRH-induced PRL (**A**) and $[Ca^{2+}]_i$ responses (**B**) in cells bathed in Ca^{2+}-deficient medium (about 100 n*M* free $[Ca^{2+}]_e$). Such profiles are called *monophasic*. Note the difference in time scales for calcium and secretory responses in this and following figures.

These results are in accordance with literature data *(2,3,10–16)*, indicating that ET-1 and TRH have opposite effects on VGCI and Ca^{2+}-influx-controlled PRL secretion.

Effects of PTX and Cs^+ on Agonist-Induced Calcium Signaling and Secretion

Two receptors differed in their coupling to the PTX-sensitive $G_{i/o}$ signaling pathway. ET-1-induced inhibition of VGCI was abolished in cells treated with PTX overnight (Fig. 3, left). On the other hand, TRH-induced $[Ca^{2+}]_i$ response was not obviously affected by PTX (Fig. 3, right). The patterns of $[Ca^{2+}]_i$ signals triggered by ET-1 and TRH in PTX-treated cells were highly comparable, indicating that the removal of coupling between ET_A receptor and $G_{i/o}$ signaling pathway makes this receptor capable of operating in a manner typical of Ca^{2+}-mobilizing receptors. The results further indicate that TRH receptor–induced calcium signaling is insensitive to PTX treatment.

In a fraction of cells (32 of 65 cells) bathed in 5 m*M* extracellular Cs^+-containing medium, ET-1-induced Ca^{2+} mobilization was accompanied by sustained VGCI (Fig. 4A, traces a and b). In the remaining cells, however, ET-1 inhibited VGCI in the presence of Cs^+ (Fig. 4A, traces c and d). In none of the cells studied (n = 73), did Cs^+ affect TRH-induced facilitation of VGCI (Fig. 4A, right). Note that Cs^+

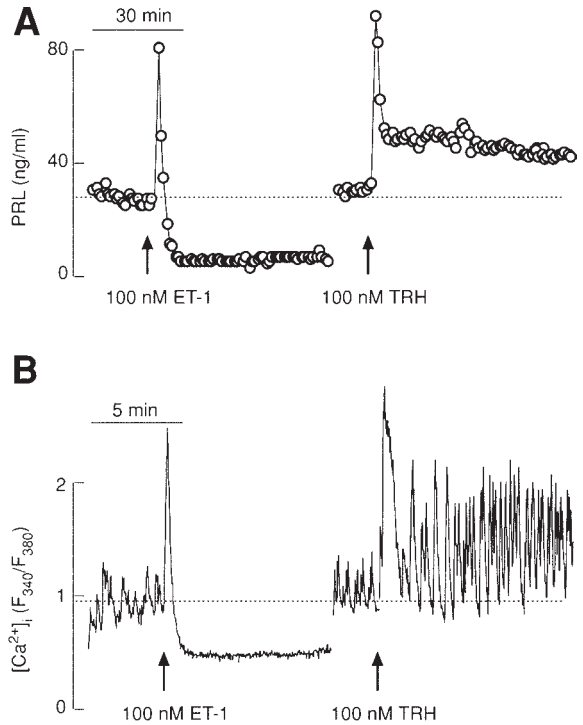

Fig. 2. Opposite effects of ET-1 and TRH on Ca^{2+} influx and sustained PRL secretion. Typical patterns of ET-1- and TRH-induced PRL (**A**) and $[Ca^{2+}]_i$ responses (**B**) in cells bathed in Ca^{2+}-containing medium ($[Ca^{2+}]_e$ is 2 m*M*). (**Left**) The profiles are called *bidirectional*; (**right**) the profiles are called *biphasic*. Note the difference in basal PRL secretion in Ca^{2+}-containing (Fig. 2) and Ca^{2+}-deficient medium (Fig. 1).

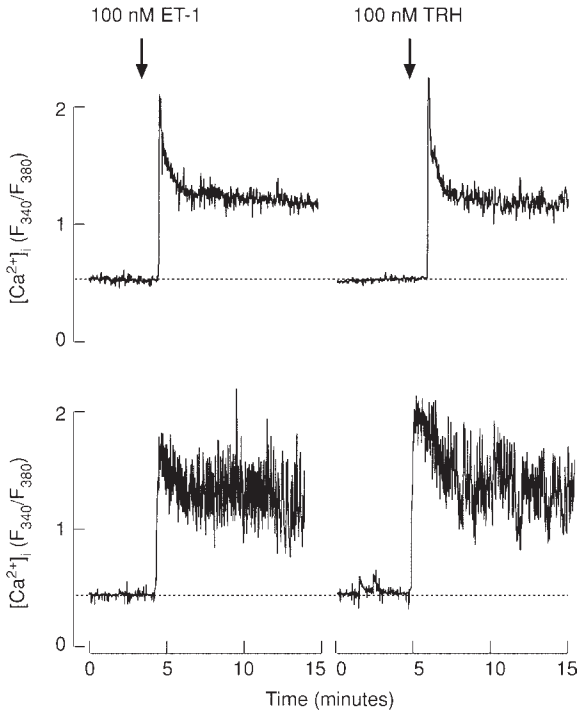

Fig. 3. Coupling of ET_A and TRH receptors to $G_{i/o}$ signaling pathway. The effects of PTX (250 ng/mL overnight) on ET-1-induced $[Ca^{2+}]_i$ response (left traces) and the lack of effects of PTX on TRH-induced $[Ca^{2+}]_i$ response (right traces) are shown. Sustained response was either a plateau (upper traces) or oscillatory (lower traces). In about 10% of lactotrophs, PTX did not alter the bidirectional effects of ET-1 on $[Ca^{2+}]_i$ response (not shown).

affects basal $[Ca^{2+}]_i$ in some cells (Fig. 4A, traces a, c, d, and g). Finally, the secretory profiles in ET-1- and TRH-stimulated cells were not significantly affected by Cs^+ (Fig. 4B). These results indicate that Cs^+-sensitive channels are accountable for ET-1-induced inhibition of VGCI. The partial action of Cs^+ on ET-1-induced calcium signaling may further suggest that there is incomplete blockade of target channels, or that the $G_{i/o}$-sensitive signaling pathway also alters the conductivity of channels other than Cs^+ sensitive. Finally, the lack of effect of Cs^+ on PRL secretion indicates that ET-1 also inhibits exocytosis downstream of VGCI.

Effects of K^+ Channel Blockers on Agonist-Induced Signaling and Secretion

In the concentrations used, Cs^+ inhibited K_{ir} channels, but also erg channels. E-4031 (Sigma-RBI) is a specific blocker of erg type K^+ channels *(7)*. Like Cs^+, this compound elevated the baseline $[Ca^{2+}]_i$ in a fraction of cells (Fig. 5A, traces b and d). In contrast to Cs^+, the bidirectional calcium and secretory responses to ET-1 were not affected by E-4031 (Fig. 5, left). TRH-induced biphasic $[Ca^{2+}]_i$ and secretory responses were also not affected by this compound (Fig. 5, right). A fraction of TRH-stimulated lactotrophs exhibited high-amplitude sustained $[Ca^{2+}]_i$ transients in the presence of E-4031 (Fig. 5A, trace f).

In further studies, we examined the potential roles of $I_{K\text{-}Ca}$ and M channels in agonist-induced signaling and secretion. To inhibit $I_{K\text{-}Ca}$ channels, we used apamin, a specific blocker of one subtype of SK $I_{K\text{-}Ca}$ channels, and paxilline, a specific blocker of BK-type $I_{K\text{-}Ca}$ channels (both from Sigma-RBI). When added together, these blockers did not change the bidirectional pattern of ET-1-induced $[Ca^{2+}]_i$ response (Fig. 6, traces a–c). TRH-induced biphasic $[Ca^{2+}]_i$ response was also preserved, but the sustained VGCI was frequently organized in the form of relatively regular $[Ca^{2+}]_i$ transients (Fig. 6, traces e and f). In some cells, the addition of $I_{K\text{-}Ca}$ blockers also affected the baseline $[Ca^{2+}]_i$ transients (trace a). These results indicate the relevance of apamine + paxilline–sensitive channels in control of the pattern of calcium signaling in TRH-stimulated cells, but not in the sustained inhibition of VGCI in ET-1-stimulated cells. The addition of linopirdine (Sigma - RBI), a relatively selective blocker of M current, was ineffective on ET-1- and TRH-induced $[Ca^{2+}]_i$ signaling and PRL secretion (Fig. 7).

Discussion

We studied the mechanism of diverse actions of two calcium-mobilizing receptors, ET_A and TRH, on VGCI and calcium influx–dependent PRL secretion in rat pituitary lactotrophs. Calcium-mobilizing agonists usually generate

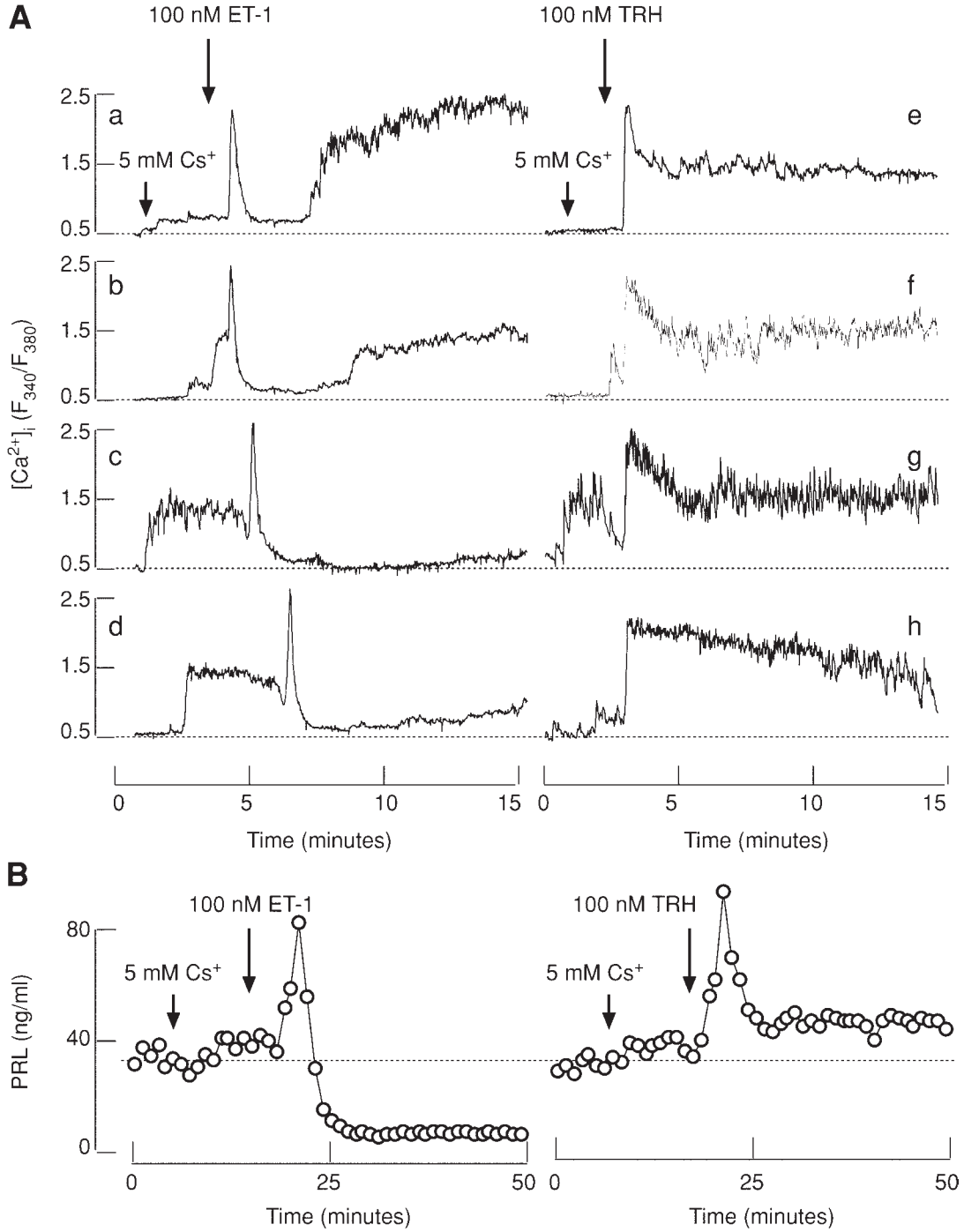

Fig. 4. Dependence of agonist-induced calcium signaling and PRL secretion on Cs^+-sensitive K^+ channels in lactotrophs. The effects of extracellular Cs^+ (5 m*M*), a blocker of K_{ir} channels, on (**A**) ET-1- (left traces) and TRH-induced $[Ca^{2+}]_i$ response (right traces) and (**B**) the lack of effects of Cs^+ on ET-1- and TRH-induced PRL secretion are shown. Note the stimulatory effects of Cs^+ on basal $[Ca^{2+}]_i$ in some cells (traces a, c, d, and g).

biphasic $[Ca^{2+}]_i$ and secretory profiles composed of an early spike response mediated by $InsP_3$-induced Ca^{2+} release and a sustained plateau response that is dependent on Ca^{2+} influx through voltage-gated and/or capacitative calcium influx channels *(25)*. In lactotrophs, TRH induced such a pattern of signaling and secretion, while ET-1 mimicked the Ca^{2+}-mobilizing action of TRH, but not the Ca^{2+} influx–dependent sustained response. Mobilization of Ca^{2+} from intracellular stores by ET-1 was followed by a profound and long-lasting inhibition of VGCI and PRL secretion in a manner resembling the actions of two other receptors expressed in lactotrophs: dopamine and somatostatin (reviewed in ref. *1*). However, in PTX-treated cells, ET-1 generated Ca^{2+} signals similar to those induced by TRH, indicating that ET_A

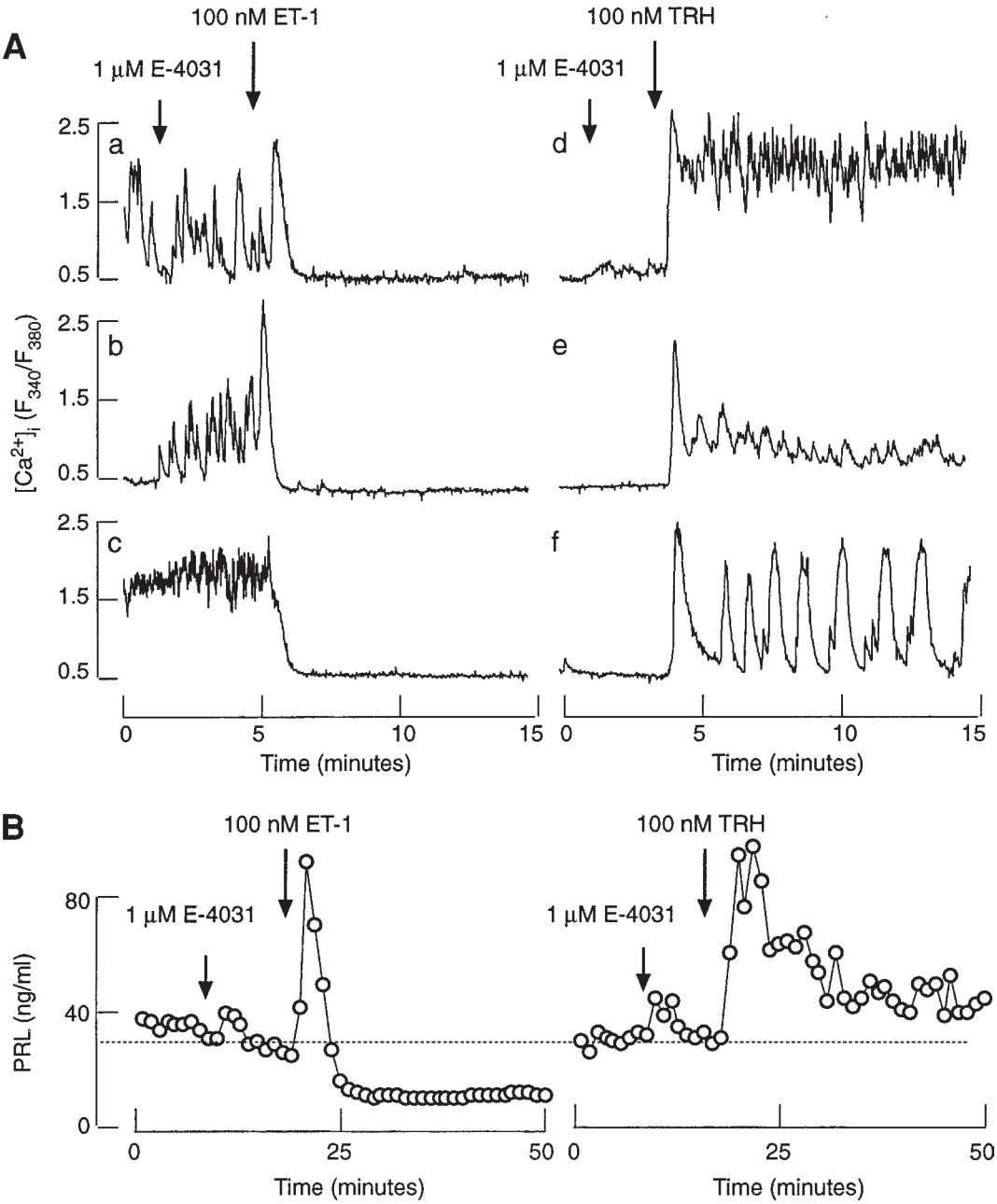

Fig. 5. Pattern of agonist-induced $[Ca^{2+}]_i$ (**A**) and secretory responses (**B**) in lactotrophs with blocked erg channels. To block these channels, cells were exposed to 1 μ*M* E-4031 for several minutes prior to the addition of ET-1 and TRH. Note the stimulatory effects of E-4031 on basal $[Ca^{2+}]_i$ and relatively regular $[Ca^{2+}]_i$ transients during the sustained TRH stimulation in some cells (traces b and d).

receptors in these cells could trigger a common mechanism for the activation of Ca^{2+} influx with Ca^{2+} mobilization, but that the cross-coupling of these receptors to $G_{i/o}$ in physiologic conditions prevents the development of the second phase in Ca^{2+} signaling. These findings raised questions about the mechanism by which TRH triggers Ca^{2+} influx in excitable lactotrophs, and about ET-1 uncoupling Ca^{2+} influx from Ca^{2+}-mobilizing pathways.

Several plasma membrane potassium channels have been shown to be affected by TRH and ET-1 in lactotrophs and GH cells, including $I_{K\text{-}Ca}$, K_{ir}, erg, and M potassium channels, and store-operated calcium channels *(5–9,11,24)*. Pituitary lactotrophs and GH cells express apamin-sensitive SK channels and charybdotoxin/paxilline-sensitive BK channels *(26–28)*. As in other cell types, the early spike $[Ca^{2+}]_i$ response is probably responsible for their activation induced by ET-1 in lactotrophs, whereas during the sustained stimulation with ET-1 at the level of $[Ca^{2+}]_i$ below the basal, it is unlikely that these channels are activated. During the sustained VGCI in TRH-stimulated cells, however, these channels may play

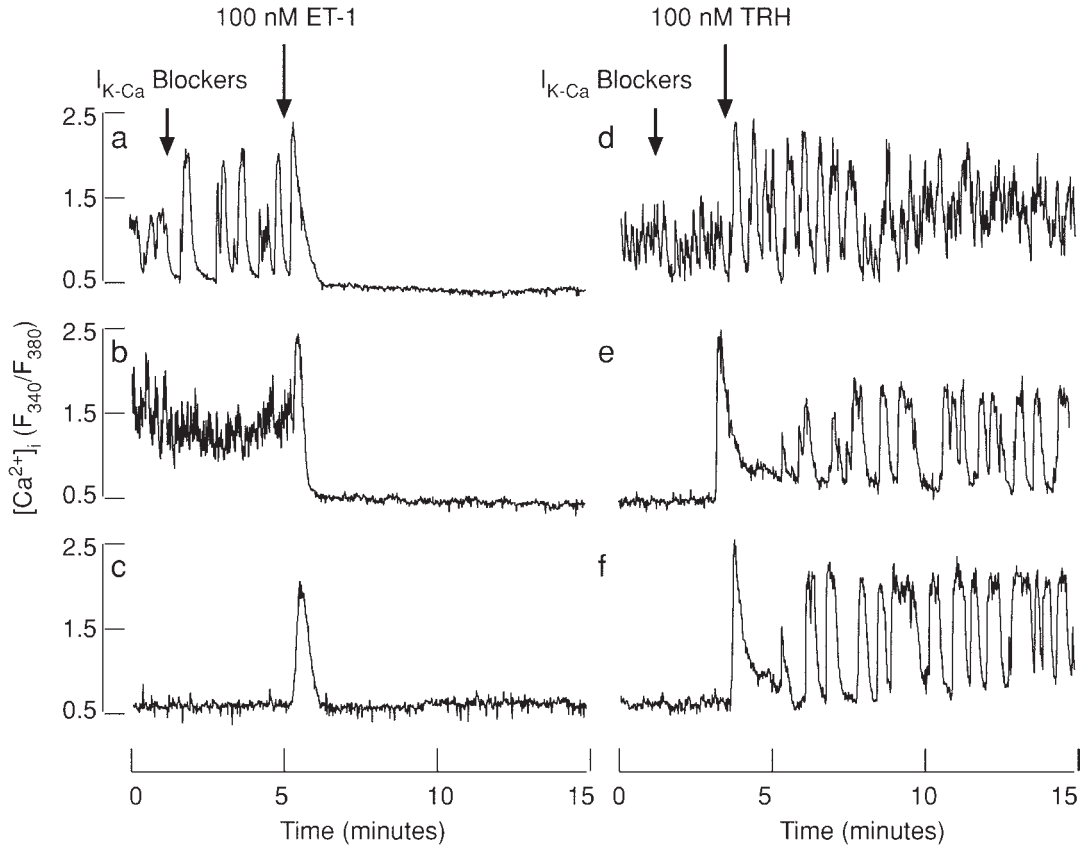

Fig. 6. Characterization of agonist-induced calcium signaling and PRL secretion in lactotrophs with blocked I_{K-Ca} channels. The lack of effects of apamin (100 n*M*) + paxilline (1 μ*M*), the blockers of I_{K-Ca} channels, on bidirectional and biphasic pattern of agonist-induced $[Ca^{2+}]_i$ response is shown. Note the presence of relatively regular baseline (trace a) and sustained TRH-induced $[Ca^{2+}]_i$ transients in some cells (traces e and f).

a role in controlling VGCI, as indicated by the generation of high-amplitude calcium transients in cells treated with apamin + paxilline. In the absence of specific blockers for apamin-insensitive SK channels, we were unable to evaluate their role in agonist-induced Ca^{2+} signaling and secretion.

In the concentrations used in our experiments, Cs^+ inhibits its K_{ir} channels, as well as the hyperpolarization-activated I_h channels and erg channels. The I_h channel is expressed in lactotrophs *(29)*, but it is an unlikely candidate to be activated by ET-1. First, this is a cyclic adenosine monophosphate–gated channel *(30)*, and ET-1 inhibits adenylyl cyclase in a PTX-sensitive manner. Second, I_h is a depolarizing channel *(30)*, whereas ET-1 hyperpolizes cells *(11)*. The ability of Cs^+ to mimic the action of PTX is more consistent with the action of ET_A receptors on K_{ir} channels. The incomplete action of Cs^+ (in about 50% of cells) also supports this hypothesis, because Cs^+ frequently acts as a partial blocker of these channels when they conduct the outward potassium current *(30)*. The PTX sensitivity of VGCI further strengthened the conclusion that the K_{ir} 3.0 subfamily of these channels accounts for ET-1-induced calcium signaling. The expression of these channels in lactotrophs has been shown previously *(11,18)*. Because lactotrophs fire action potentials spontaneously *(31)* and the action potential–driven Ca^{2+} influx is sufficient to trigger PRL secretion *(32)*, the sustained hyperpolarization of cells by activated K_{ir} should provide an effective mechanism for inhibition of VGCI and PRL secretion. Two other receptors coupled to $G_{i/o}$ signaling, dopamine and somatostatin, also activate K_{ir} and inhibit PRL secretion in a PTX-sensitive manner *(17–20)*.

Consistent with the hypothesis that spontaneously active K_{ir} and/or erg channels participate in the control of pacemaking in lactotrophs, we observed the stimulatory effects of Cs^+ on basal $[Ca^{2+}]_i$ in a fraction of cells *(11)*. Others have shown the expression of mRNA for Cs^+-sensitive K_{ir} 1.0 and 2.0 subfamilies *(33)* and Cs^+-sensitive erg channels *(7,8)* in rat pituitary and immortalized GH_3 cells. Furthermore, the stimulatory action of Ca^{2+}-mobilizing TRH agonist on VGCI in lactotrophs was suggested to occur through partial inhibition of K_{ir} *(9)* and/or erg *(7)* channels. In our experiments, however, we did not observe the effects of blockers of these channels on TRH-induced sustained Ca^{2+} signaling and PRL release. A blocker of M channels was also ineffective in inhibiting TRH-induced biphasic Ca^{2+} response. The lack of effects of these blockers on ET-1- and TRH-induced calcium signaling and secretion does not argue

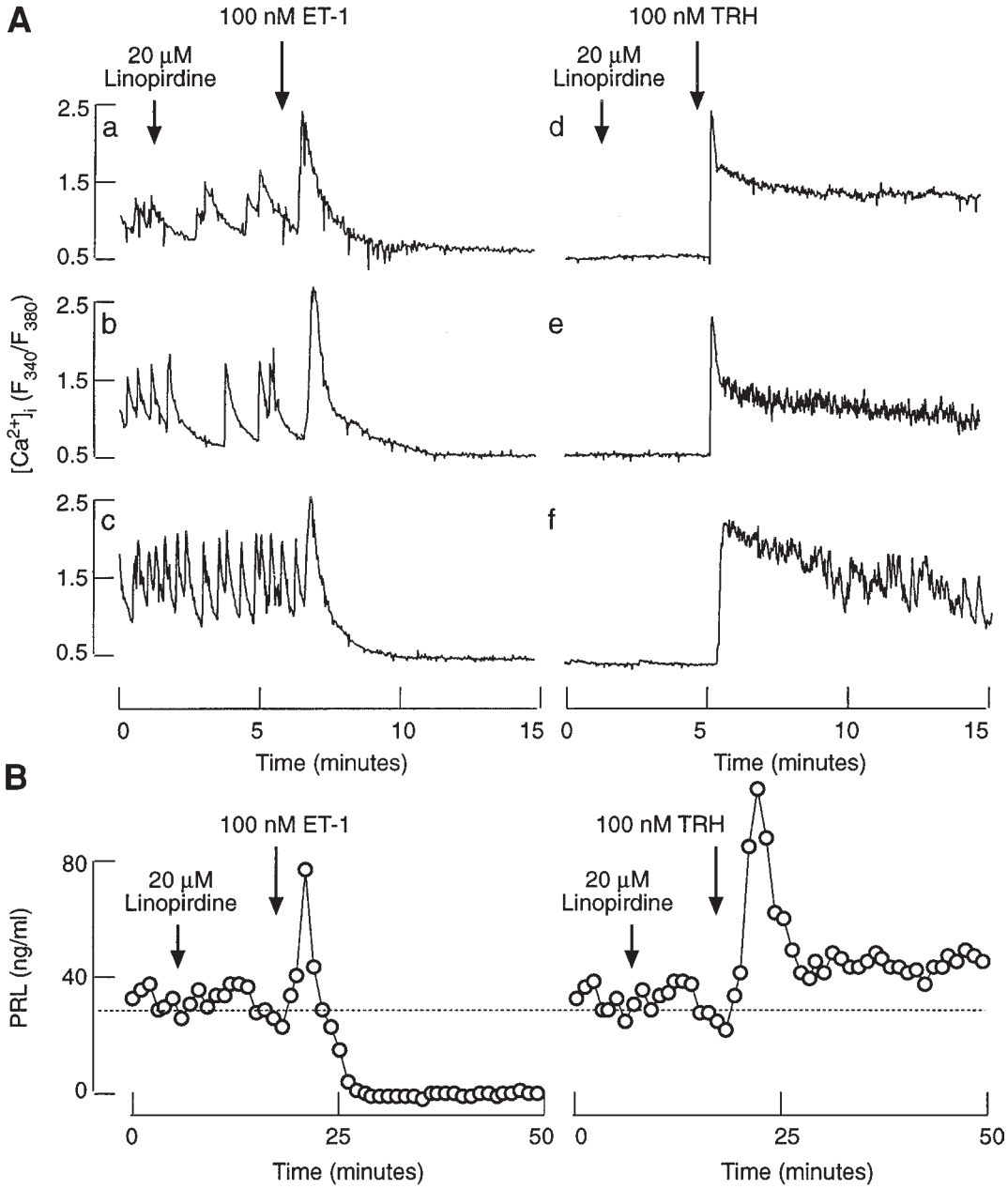

Fig. 7. Independence of ET-1- and TRH-induced Ca^{2+} signaling and PRL secretion on M current. The lack of effects of linopirdine, an M-type K^+ channel blocker, on (**A**) Ca^{2+} signaling pattern and (**B**) PRL secretion in ET-1- (**left**) and TRH-stimulated cells (**right**) is shown.

against the agonist action on currents observed by others but, rather, indicates that these currents are not critical for generating the agonist-specific bidirectional and biphasic patterns of signaling and secretion.

Two additional observations deserve attention. There is a qualitative parallelism in the actions of agonists on calcium signaling and secretion, but there is no temporal and amplitude correlation between them. The rise and fall in $[Ca^{2+}]_i$ concentrations occurred more rapidly than PRL secretion and the fold increase in PRL secretion was higher compared to calcium increase. Several factors could participate in this dissociation, including difference in the rate of recordings (minutes vs seconds), the absence of calibration for $[Ca^{2+}]_i$, the speed of washing of secreted PRL by perifusion medium, and the strength of calcium-secretion coupling that is cell specific and determined by many factors. Whereas the lack of quantitative correlation between $[Ca^{2+}]_i$ and secretion was logical and expected, the lack of effects of Cs^+ on PRL secretion in ET-1-stimulated cells was more surprising and indicates that ET_A receptors inhibit PRL secretion downstream of VGCI. Further studies are required to clarify the mechanism by which ET_A receptors inhibit PRL secretion.

Materials and Methods

Cell Cultures, Treatments, and Radioimmunoassay

Experiments were performed on anterior pituitary cells from normal postpubertal female Sprague Dawley rats obtained from Taconic Farm (Germantown, NY). Pituitary cells were dispersed as described previously *(34)* and cultured as mixed cells or enriched lactotrophs at a density of 10^6 cells/25-mm cover slip in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, and antibiotics. A two-stage Percoll discontinuous density gradient procedure *(34)* was used to obtain an enriched lactotroph population. In single-cell Ca^{2+} measurements, lactotrophs were further identified by the addition of TRH.

Hormone secretion was monitored using rapid cell column perfusion experiments. Cells (1.2×10^7) were incubated with preswollen cytodex-1 beads in 60-mm Petri dishes for 2 d. The beads were then transferred to 0.5-mL chambers and perifused with Hanks' M199 containing 20 m*M* HEPES, 0.1% bovine serum albumin, and penicillin/streptomycin for 2 h at a flow rate of 0.8 mL/min and 37ºC to establish a stable basal PRL secretion. During the experiment, 1-min fractions were collected, stored at –20ºC, and later assayed for PRL content using radioimmunoassay (RIA). All reagents and standards for RIA 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

For $[Ca^{2+}]_i$ measurement, cells attached to cover slips were immersed in 2 μ*M* fura-2 AM (Molecular Probes, Eugene, OR) in Medium 199 with Hanks' salts at 37ºC for 1 h. The medium was changed to Krebs-Ringer buffer, and the cells were further 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 Attofluor Imaging System (Atto, 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.

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