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The In Vitro Regulation of Growth Hormone Secretion by Orexins

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Orexins, orexigenic neuropeptides, have recently been discovered in lateral hypothalamus and play an important role in the regulation of pituitary hormone secretion. Two subtypes of orexin receptors (orexin-1 and orexin-2) have been demonstrated in pituitaries. In this experiment, the effects of orexins on voltage-gated Ca^{2+} currents and the GH release in primary cultured ovine somatotropes were examined. Voltage-gated Ca^{2+} currents were isolated in ovine somatotropes as L, T, and N currents using whole-cell patch-clamp techniques and specific Ca^{2+} channel blocker and toxin. Application of orexin-A or orexin-B (100 n*M*) significantly, dose-depen-dently, and reversibly increased only nifedipine-sensitive L-type Ca^{2+} current. Inhibitors of PKC (calphostin C, PKC inhibitory peptide) but not inhibitors of PKA (H89, PKA inhibitory peptide) cancelled the increase in the L current by orexins. Co-administration of orexin-A and GHRH (10 n*M*) showed an additive effect on the L current. Specific intracellular Ca^{2+}-store-depleting reagent, thapsigargin (1 μ*M*), did not affect the orexin-induced increase in the L current. Orexin-B alone slightly increased GH release and co-administration of orexin-A and GHRH synergistically stimulated GH secretion in vitro. It is therefore suggested that orexins may play an important role in regulating GHRH-stimulated GH secretion through an increase in the L-type Ca^{2+} current and the PKC-mediated signaling pathways in ovine somatotropes.

Key Words: Orexin; GH; Ca^{2+} currents; GHRH; PKC.

Introduction

Orexin-A and orexin-B (also known as hypocretin-1 and hypocretin-2) are two hypothalamic peptides identified a few years ago acting through two types of receptors (orexin 1-R and orexin 2-R) belonging to the G-protein–coupled seven-transmembrane-domain receptor superfamily *(1,2)*. Orexin-A and orexin-B are 33- and 28-amino-acid peptides with 46% sequence homology between the two. Orexin 1-R is highly specific for orexin-A, whereas orexin 2-R has equal affinity for both orexin-A and orexin-B *(2)*. It has been suggested that orexins play a significant role in the control of food intake and sleep–wake cycle *(3,4)*. Recent studies have demonstrated that orexins, orexin receptors, and orexin-containing projections spread not only throughout the brain and spinal cord but also in several peripheral systems *(4–6)*. Orexins and orexin receptors have been shown in median eminence and pituitary gland, which suggests a regulatory role of orexins on pituitary hormones *(7–11)*. Laboratory data have linked orexins to both hypothalamus–pituitary–adrenal (H-P-A) and hypothalamus–pituitary–gonadal (H-P-D) axes influencing the secretion of a number of hormones including adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), corticosterone, and prolactin both in vivo and in vitro *(3,12–16)*. In addition, the stimulatory effects of orexins on the secretion of insulin and TSH have been reported *(17,18)*. The role of orexins in the regulation of growth hormone (GH), an anabolic hormone in the pituitary, is not clear with limited and controversial results *(3, 19)*. Orexins have been shown to change the electrophysiological activities in a number of neuronal and nonneuronal cells *(20,21)*. Modification of ion channels in somatotropes is one of the most important mechanisms by which GH secretion is regulated by hypothalamic peptides including GH-releasing hormone (GHRH), GH-releasing peptides (GHRP), and somatostatin *(22,23)*.

In this experiment, the effects of orexin-A and orexin-B on voltage-gated Ca^{2+} currents and GH secretion were studied in primary cultured ovine somatotropes. Types of Ca^{2+} channels were isolated by specifically designed voltage-clamp protocols and selective blocker and toxin. The involvement of second messenger systems in the orexins-induced effect on Ca^{2+} channels was determined.

Results

Voltage-Gated Ca^{2+} Currents

Voltage-gated calcium channels have been reported as L, T, N, P/Q, and R subtypes in different cells *(24,25)*. In ovine somatotropes, L and T currents were previously isolated, with the L type predominant *(26)*. In this study, we extended our

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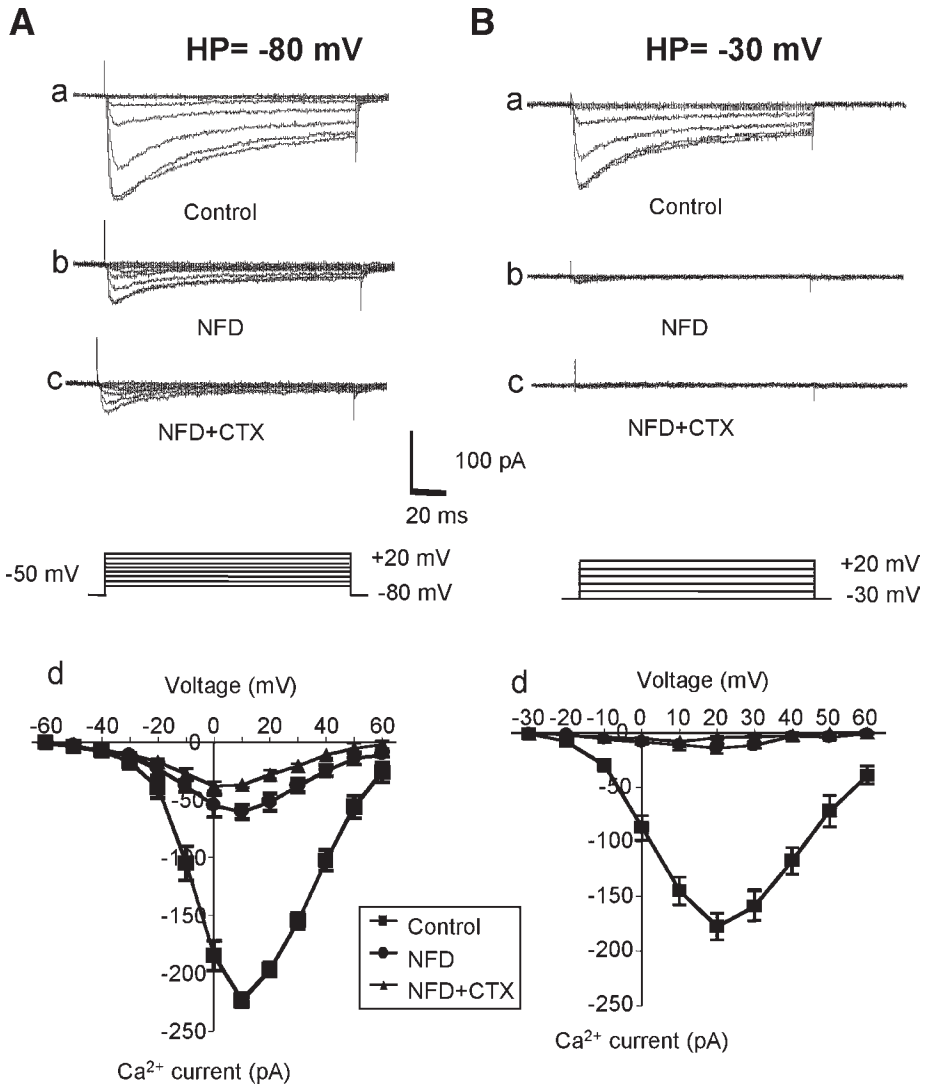

Fig. 1. Characterization of voltage-gated Ca^{2+} currents in ovine somatotropes. (**A**) Voltage-gated Ca^{2+} currents were evoked by depolarizing test pulses from a holding potential of –80 mV with a 10 mV interval for 250 ms, as indicated in the lower panel. (**Aa**) Total Ca^{2+} currents were evoked by depolarizing test pulses up to +20 mV. (**Ab**), Combined N and T Ca^{2+} currents were evoked in the presence of the L-type Ca^{2+} current blocker, nifedipine (NFD, 10 μ*M*). (**Ac**) T-type Ca^{2+} current was evoked in the presence of L-type and N-type Ca^{2+} current blockers, NFD, and conotoxin (CTX, 1 μ*M*), respectively. (**Ad**) Current–voltage (*I-V*) relationships for three subtypes of recorded Ca^{2+} currents measured at the highest points of the peak traces. The currents were obtained with depolarizing pulses from –50 mV to +60 mV, with a holding potential of –80 mV (mean ± SEM, $n = 3$, in each case). Note that the total Ca^{2+} current (indicated as ■), the T plus N types (indicated as ●, in the presence of NFD), and T-type currents (indicated as ▲, in the presence of NFD and CTX) were isolated. (**B**) Voltage-gated Ca^{2+} currents were evoked by depolarising pulses with a 10-mV interval from a holding potential of –30 mV for 200 ms, as indicated in the lower panel. (**Ba**) Ca^{2+} currents were evoked by depolarizing pulses up to +20 mV. (**Bb**) Ca^{2+} currents were evoked in the presence of NFD. (**Bc**) Ca^{2+} currents were evoked in the presence of NFD and CTX. (**Bd**) Current–voltage (*I-V*) relationships for three subtypes of recorded Ca^{2+} currents measured at the highest points of the peak traces. The currents were obtained with the depolarizing pulses from –30 mV (**B**) to +60 mV with a holding potential of –80 mV (mean ± SEM, $n = 3$, in each case). Note that majority of the Ca^{2+} current was the L-type (indicated as ■, where NFD blocked most of the current). (Reproduced with permission from ref. *63*.)

research using L and N current blockers, nifedipine (NFD) and ω-conotoxin (CTX), respectively, to systemically examine the components of voltage-gated Ca^{2+} currents in the somatotropes.

Figure 1 shows a group of recordings for such a characterization in a representative cell. Total Ca^{2+} currents (Fig. 1A) were evoked by depolarizing test pulses from –50 mV up to +20 mV for 250 ms with a holding potential of –80 mV. Incubation with NFD (10 μ*M*) in the bath solution reduced approx 75% of the total Ca^{2+} current (Fig. 1A, part b), suggesting the presence of a large proportion of the L current. The rest of Ca^{2+} current was further suppressed by

the addition of the N current blocker, CTX (1 μ*M*) (comparing Fig. 1A, parts b and c). The remaining current (Fig. 1A, part c) was predominantly the T current, distinguishable by its quick inactivation (within 100 ms, unlike currents of the P, Q, or R types). While using a holding potential of –30 mV to exclude the T and N currents, the total current recorded was predominantly of the L type. Addition of NFD almost totally suppressed the current (comparing Fig. 1B, parts a and b). Only a minor effect was evident with further addition of CTX (comparing Fig. 1B, parts b and c), indicating probably an incomplete inactivation of the N current. In comparison, the currents blocked by NFD (L type) under the two holding potentials were almost identical (167 pA in Fig. 1A vs 170 pA in Fig. 1B). The *I-V* curves for three subtypes of currents under different holding potentials obtained from a group of three cells are shown in Fig. 1A; part d and B; part d, respectively.

Effect of Orexins on Total Ca^{2+} Currents

In order to obtain Ca^{2+} currents stable for at least 15 min, an ATP regenerative system comprising ATP (2 m*M*), Na_2-phosphocreatine (5 m*M*), creatine phosphokinase (20 unit/mL) plus 0.1 m*M* GTP was included in the pipet solution. It was previously shown that this inclusion did not alter the voltage-gated Ca^{2+} current *(27)*. Figure 2A shows the effect of orexin-A (100 n*M*) on the current–voltage relationship curve of total Ca^{2+} currents. Figure 2B demonstrates the data of peak Ca^{2+} current from a group of eight cells with addition of orexin-A and orexin-B (100 n*M*). Orexins significantly and reversibly increased the total Ca^{2+} current.

Effect of Orexins on L, T, and N Currents

As ovine somatotropes possess three subtypes of Ca^{2+} currents, we further examined the effect of orexin-B on the individual currents. The protocols for the isolation of these currents were similar to those described in Fig. 1. Application of orexins significantly increased the L-type Ca^{2+} current with a holding potential of –30 mV (Fig. 3A). When the L current was blocked by NFD with a holding potential of –80 mV, orexin-A and orexin-B did not influence the other Ca^{2+} currents, including the T and N currents (Fig. 3B and C). Because only the L current was increased by orexins, the total Ca^{2+} current was used to investigate the involvement of intracellular signaling systems in the following experiments.

PKA System and Intracellular Ca^{2+} Store

Specific blockers have previously been used in the electrophysiological assessment of the involvement of PKA in GHRH-induced changes in both Ca^{2+} and K^+ currents in somatotropes *(28–30)*. In this study, cell permeable blocker H89 (1 μ*M*, final concentration) was added directly into the bath solution. For an even distribution of the reagent in the bath solution to completely quench the PKA activities, cells were incubated with H89 for about 5 min. No change was

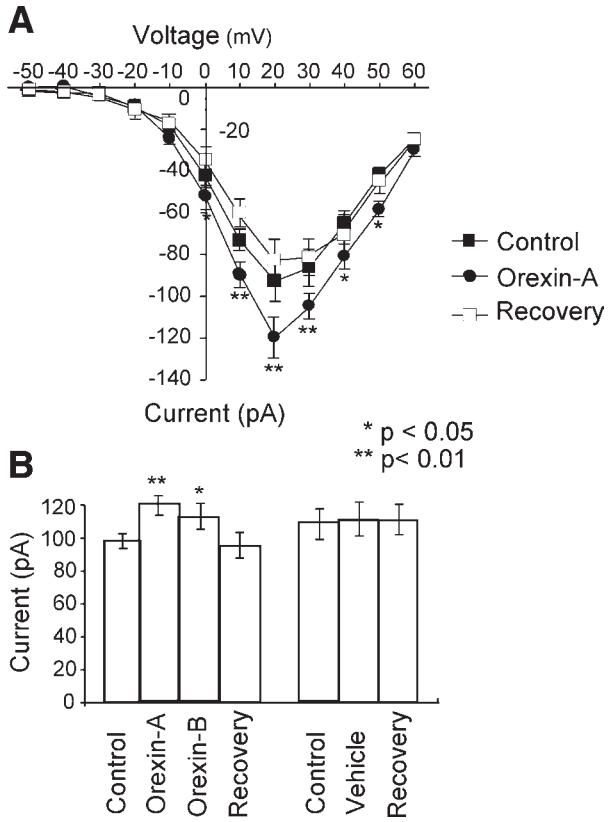

Fig. 2. Effect of orexins on Ca^{2+} currents in ovine somatotropes. (**A**) Statistical data of the current–voltage relationship curves obtained from a group of four recordings. Orexin-A significantly and reversibly increased the amplitude of the voltage-gated Ca^{2+} current (means ± SEM, $n = 4$). **(B)** Statistical data demonstrate that orexin-A and orexin-B significantly increases voltage-gated calcium currents while there is no change in the current amplitude when vehicle is used. (Adapted with permission from refs. *9,63*.)

observed in the basal amplitude of the Ca^{2+} currents when the cells were incubated with H89. Moreover, the significant increase in the Ca^{2+} current by orexin-A and orexin-B was not influenced by the presence of H89 (Fig. 4A). PKI is a synthetic PKA inhibitory peptide that cannot pass through the cell membrane, so it was included in the pipet solution to be dialyzed into the cells. The increase in the Ca^{2+} current by orexin-A and orexin-B was maintained during the intracellular dialysis with PKI (10 μ*M*, Fig. 4B). It has been reported that similar introduction of PKI blocked PKA activities on the voltage-gated Ca^{2+} and Na^+ channels *(31)*. Current results hence indicate that the PKA system is not involved in the orexin-B–induced augmentation in the Ca^{2+} current in ovine somatotropes.

In order to rule out the possible involvement of intracellular Ca^{2+} store in an orexin-mediated effect, we depleted the Ca^{2+} store by pretreatment of somatotropes with thapsigargin (10 μ*M*) for 30 min. After such a pretreatment, orexin-A and orexin-B still elevated the Ca^{2+} current significantly

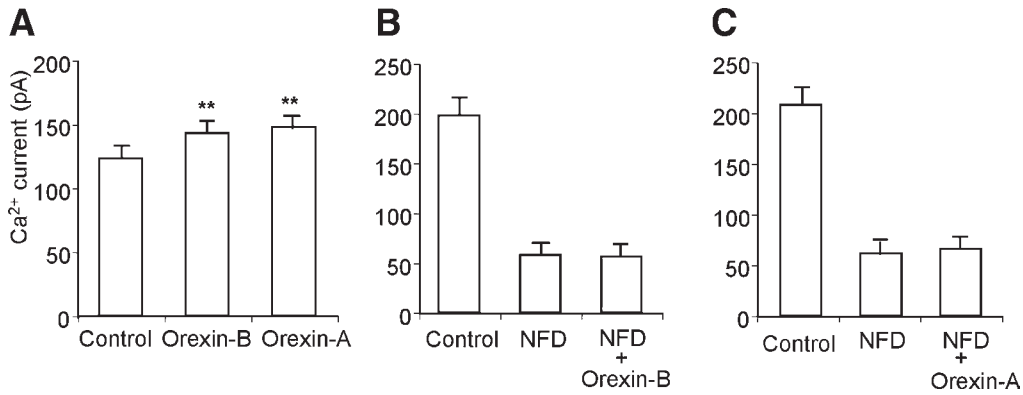

Fig. 3. Effect of orexins on the L-, T-, and N-type Ca^{2+} currents. Three subtypes of Ca^{2+} currents were isolated as depicted in Fig. 1. (**A**) The effect of orexin-B and orexin-A (100 n*M*) on the L-type Ca^{2+} current recorded at +20 mV test voltage from a holding potential of –30 mV (mean ± SEM, $n = 4$, in each case). No effect was observed in the currents containing the T and N subtypes (**B** and **C**) recorded at +10 mV test voltage from a holding potential of –80 mV. $^{**}p < 0.01$. (Adapted with permission from refs. *9,63*.)

(Fig. 4C), suggesting that the intracellular Ca^{2+} store does not contribute to the orexin-induced increase in Ca^{2+} currents.

PKC System

To test the possible involvement of PKC in the action of orexins on Ca^{2+} channels, we examined the inhibitory effects of Cal-C, $PKC_{19–36}$, and PDBu, each of which effectively and selectively inhibits PKC activities in ovine somatotropes *(30,32)*. Incubation of the cells with Cal-C (100 n*M*) for 5 min did not change the basal amplitude of Ca^{2+} currents. However, the increase in Ca^{2+} currents by orexin-A and orexin-B was abolished in the presence of Cal-C (Fig. 5A). When we microdialyzed $PKC_{19–36}$, a specific PKC inhibitory peptide, into the cells at a concentration of 1 μ*M*, the increase in Ca^{2+} currents by orexin-A and orexin-B was inhibited (Fig. 5B). PDBu itself (0.5 μ*M*) for 5 min significantly increased Ca^{2+} currents, mimicking the effect of orexins (Fig. 5C). Furthermore, when the cells were pretreated with PDBu (0.5 μ*M*) for 16 h to down-regulate PKC, the orexin-induced increase in Ca^{2+} currents was completely suppressed (Fig. 5D). These results strongly suggested that PKC is required in the orexin-mediated increase in Ca^{2+} currents in ovine somatotropes.

Additive Effect of Orexin-A and GHRH on Ca^{2+} Currents

As Ca^{2+} currents have previously been demonstrated to be increased by GHRH in ovine somatotropes, we tested the interactions between orexin-A and GHRH (100 n*M*) in the modification of the Ca^{2+} current. In order to exclude the possible cross-effect between the receptors of orexin-A and GHRH, we administered orexin-A (100 n*M*) and GHRH in different orders. In the first series of experiments, GHRH was delivered 8 min before orexin-A. GHRH increased the Ca^{2+} current significantly and subsequent addition of orexin-A further elevated the level of the Ca^{2+} currents (Fig. 6A). When orexin-A was added 8 min before GHRH, it was found that orexin-A alone augmented the Ca^{2+} currents significantly and addition of GHRH further enhanced the Ca^{2+} currents (Fig. 6B). A complete recovery was achievable in both experimental groups. The total increase in the Ca^{2+} currents by orexin-A and GHRH was similar between two sets of experiments.

Effect of Orexins on Ovine GH Release

Finally, we examined the direct effect of orexins on the GH secretion in ovine somatotropes in vitro. Cells were incubated with orexin-B (1–100 n*M*) or GHRH (10 n*M*) for 30 min. Orexin-B dose dependently increased the GH release, but the potency of orexin-B is much less than the releasing effect of GHRH (Fig. 7A). When orexin-A (100 n*M*) or GHRH (10 or 100 n*M*) or the combination of orexin-A and GHRH were included in incubation medium for 30 min, GHRH dose dependently increased the GH release and orexin-A alone did not increase the GH secretion (Fig. 7B). Co-administration of GHRH and orexin-A synergistically augmented the GH release (Fig. 7B).

Discussion

In this set of experiments, we characterized the Ca^{2+} currents and studied the effect of orexins on the individual subtypes of Ca^{2+} current in primary cultured ovine somatotropes. Three subtypes of Ca^{2+} currents, L, T, and N currents, were identified. Orexins dose dependently and reversibly augmented only the L current. Furthermore, the PKC signaling pathway is responsible for mediating the orexin-induced increase in the L-type Ca^{2+} current in ovine somatotropes. Orexins also induced a slight dose-dependent increase in GH secretion and a synergistic effect on GH release with GHRH.

Numerous studies have shown that Ca^{2+} influx through the transmembrane voltage-gated Ca^{2+} channels is important for $[Ca^{2+}]_i$ levels, which are critical events associated with the release of GH in somatotropes *(33–36)*. In order to investigate the effect of orexins on the membrane Ca^{2+} channels, it is essential to isolate the subtypes of Ca^{2+} currents. Three Ca^{2+} currents, the L, T, and N currents, were found to

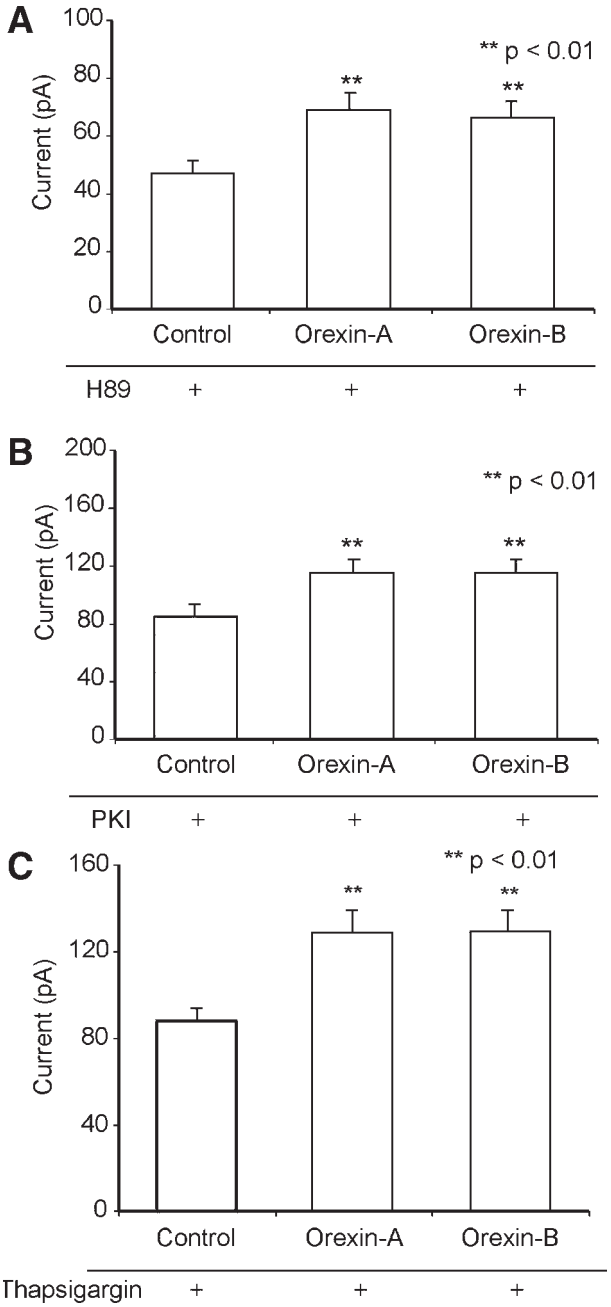

Fig. 4. Involvement of PKA-cAMP system and intracellular Ca^{2+} stores. (**A**) In the presence of a specific PKA blocker H89 (1 μ*M*), orexin-A and orexin-B increased Ca^{2+} currents in ovine somatotropes. (**B**) In the intracellular presence of a specific PKA blocker PKI (10 μ*M*), orexin-A and orexin-B increased Ca^{2+} currents in ovine somatotropes. (**C**) After incubation in presence of a specific intracellular Ca^{2+} stores depleting reagent thapsigargin (1 μ*M*), orexin-A and orexin-B increased Ca^{2+} currents in ovine somatotropes. (Adapted with permission from refs. *9,63*.)

operate in ovine somatotropes, with the L current carrying the largest proportion of Ca^{2+} influx. This confirmed previous reports that the L-type current is the predominant Ca^{2+} current subtype in somatotropes *(25,26)*. Although the N current is ubiquitous in the brain *(37)*, an N-type current has not always been observed in pituitary GH3 cells and ovine somatotropes *(25,26,38)*. In the present study, we examined the N current using conventional whole-cell patch-clamp configuration. We demonstrated that a CTX-sensitive N current exists in ovine somatotropes, but this current accounts for less than 10% of the total Ca^{2+} current, a much smaller proportion than it accounts for in neurons *(39)*. The T current has been demonstrated in ovine somatotropes and gonadotropes, but is negligible in the GH3/B6 cell line *(23,40–42)*. In the current study, the T current was recorded under the holding potential of –80 mV when the L and N currents were excluded from the total currents by NFD and CTX. This T current is fast-activated and inactivated within 100 ms, a kinetic characteristic allowing it to be easily distinguished from P/Q currents, inactivation of which is much slower *(25)*. The T current only contributed about 15% of the total Ca^{2+} current in ovine somatotropes. The differing presentation of the Ca^{2+} current subtypes reflects the difference in tissues and animal species. Furthermore, different recording techniques and cell culture conditions may also influence the characterization of Ca^{2+} currents, in particular, when the current is very small.

The present study demonstrates that both orexin-A and orexin-B increase the L-type, but not the T- or N-type, Ca^{2+} current in ovine somatotropes. This is not surprising considering that the L current is the predominant voltage-gated Ca^{2+} current in somatotropes and plays a key role in regulating the $[Ca^{2+}]_i$ levels *(43,44)*. When the effects of orexins were studied using Ca^{2+} imaging and other electrophysiological techniques in neurons, it was initially reported that orexins increase postsynaptic firing in hypothalamic neurons *(1)*. Further evidence showed that the frequency of firing of action potential was significantly increased by orexins in locus ceruleus cells *(3)*. However, the effects of orexins on the ion channels in somatotropes have not previously been reported. We demonstrate that orexins directly enhance only the L current, which suggests a functional role for this peptide in somatotropes. We also isolated the N and T currents, but failed to detect any response to orexins in either. The reasons for the different responses to orexins in these subtypes of Ca^{2+} currents are still unclear. However, our results strongly suggest that the enhancement of the L current by orexins may influence $[Ca^{2+}]_i$, the latter subsequently determining GH secretion.

Using specific PKA and PKC pathway blockers, we further examined the involvement of second messenger systems in this orexin-induced increase in the Ca^{2+} currents. In somatotropes, we and others have previously showed that the K^+, Na^+, and Ca^{2+} channels were modified by GHRH, GHRP, and somatostatin *(22,29,45)*, and both PKA-cAMP and PKC systems were involved in the intracellular signal transduction used by these peptides *(29,46)*. We therefore tried to elucidate the possible intracellular signaling pathways by which orexins induced the increase in the L current in ovine somatotropes. Using cAMP/PKA specific blockers, we demonstrated that PKA-cAMP pathway was not impli-

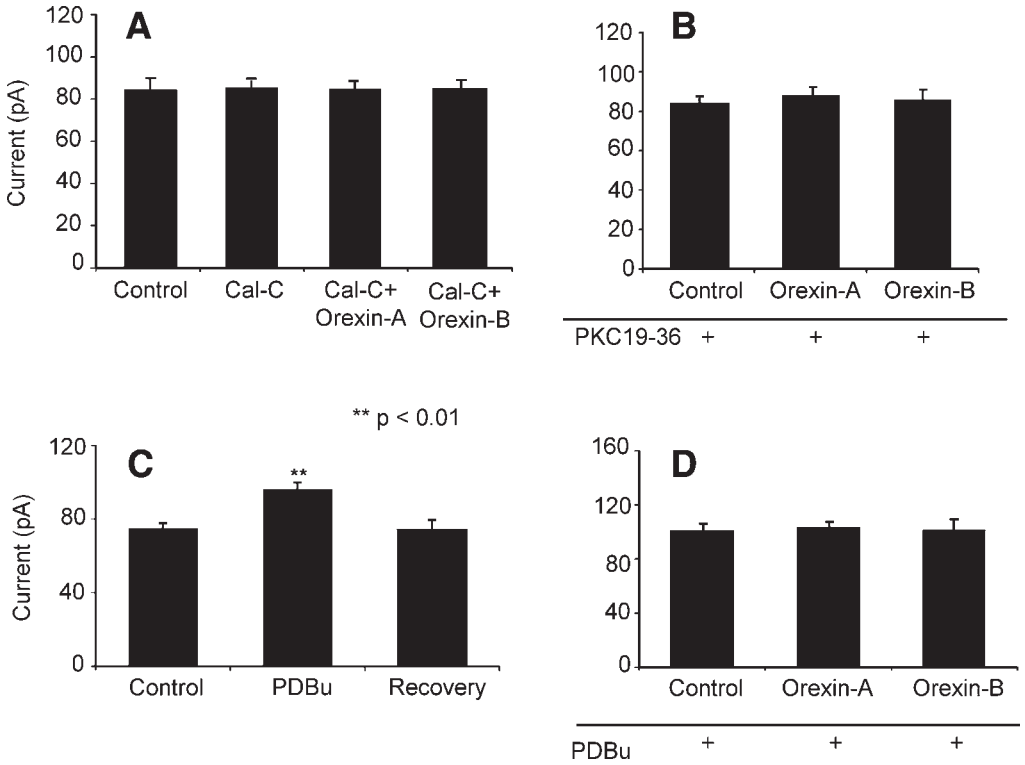

Fig. 5. Involvement of PKC system in the Ca^{2+} current response to orexins. (**A**) Statistical data show that calphostin C (Cal-C, 100 n*M*) totally abolished the orexin-induced increase in Ca^{2+} current (means ± SEM, $n = 4$). (**B**) Statistical data show that intracellular PKC inhibitory peptide $PKC_{19–36}$ completely abolished the orexin-induced increase in Ca^{2+} current. As $PKC_{19–36}$ is not permeable through cell membrane, it has been included in pipet solution to be introduced into the recorded cells (means ± SEM, $n = 4$). (**C**) Statistical data (means ± SEM, $n = 5$) show the acute application of PDBu (0.5 μ*M* for 5 min) on the Ca^{2+} current. (**D**) Statistical data (means ± SEM, $n = 5$) show the effect of long-term pretreatment (16 h) on the orexin-induced increase in the Ca^{2+} current. (Adapted with permission from refs. *9,63*.)

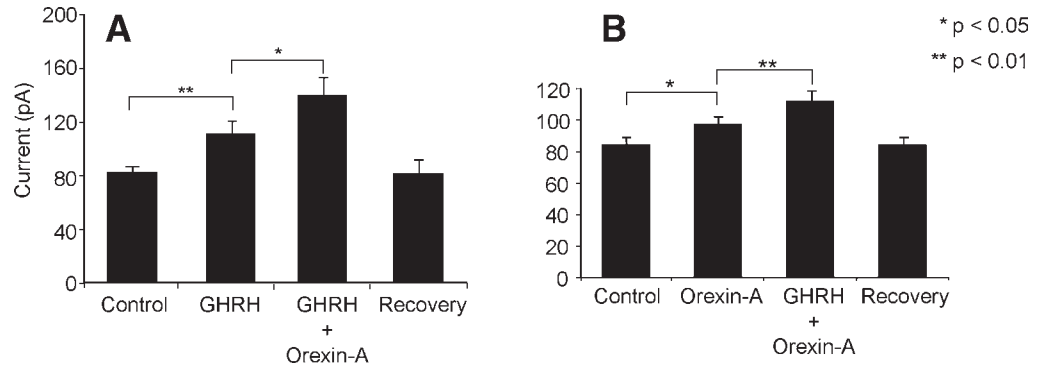

Fig. 6. Orexin-A and GHRH additively increase the voltage-gated Ca^{2+} currents. (**A**) Statistical analysis (means ± SEM, $n = 6$) of the effects of GHRH followed by the co-administration of GHRH and orexin-A on the voltage-gated Ca^{2+} currents. (**B**) Statistical analysis (means ± SEM, $n = 5$) of the effects of orexin-A followed by the co-administration of orexin-A and GHRH on the voltage-gated Ca^{2+} currents. (Reproduced with permission from ref. *9*.)

cated in orexin-induced Ca^{2+} increase. PKC and calcium have long been recognized as the important messengers mediating the effects of orexins in neurons and pheochromocytoma cells *(1,16,47)*, but no studies has been undertaken in somatotropes. We used several approaches to stimulate or block the intracellular PKC activities. Acute stimulation of the intracellular PKC activities by PDBu mimicked the effect of orexins on the Ca^{2+} current, suggesting the involvement of PKC signaling pathway. Meanwhile, we showed that the affect of orexins on the Ca^{2+} currents was completely abolished by pretreatment of the cells with PDBu for 16 h (down-regulation of the PKC system), or a PKC blocker Cal-C, or the specific PKC inhibitory peptide $PKC_{19–36}$. These results strongly indicate that orexins augment the L-type Ca^{2+} current through a PKC-dependent pathway. Our observations were in agreement with previous studies that orexins increased $[Ca^{2+}]_i$ via the PKC pathway, leading to the elevated activities of the neurons in arcuate nuclei and

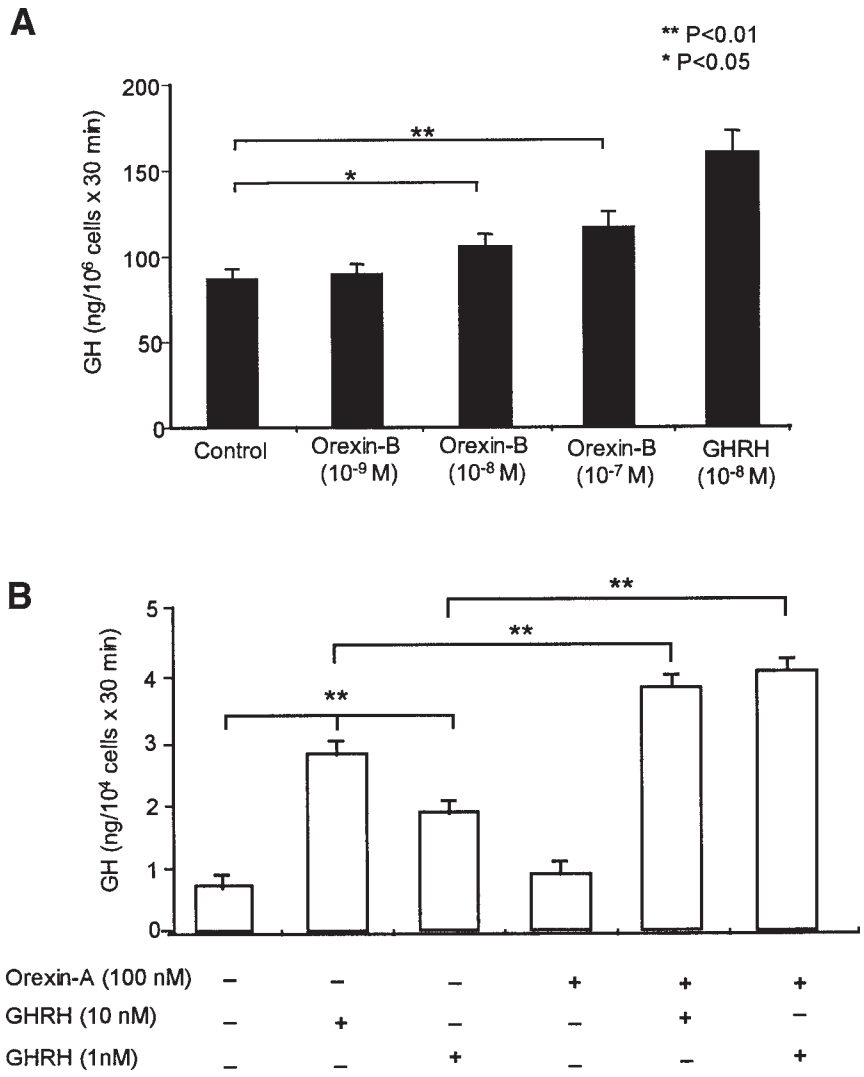

Fig. 7. Effect of orexins and GHRH on the GH secretion. (**A**) Effects of different doses of orexin-B (10^{-9}–10^{-7} *M*) and GHRH (10^{-8} *M*) on GH release in cultured ovine somatotropes were observed. The columns show (mean ± SEM, $n = 4$) the values of GH release in response to the individual peptides indicated at the bottom of the figure. Orexin-B induced a dose-dependent increase in GH release with statistic significance in doses of 10^{-8} and 10^{-7} *M* although the response is much smaller than that induced by 10^{-8} *M* GHRH. (Reproduced with permission from ref. *63*.) (**B**) Effects of orexin-A, different concentrations of GHRH, and the co-administration of orexin-A and GHRH on GH release in cultured ovine somatotropes were observed. The columns show (mean ± SEM, $n = 4$) the values of GH release in response to the individual peptides or the combination of both peptides indicated at the bottom of the figure (Reproduced with permission from ref. *9*.) Note that the units for *Y* axis in **A** and **B** are different.

the ventral tegmental area *(47,48)*. These findings are also in line with the report that PKC signaling cascade was implicated in orexin-stimulated catecholamine secretion in human adrenal cells *(16)*. Because PKA-cAMP and PKC signal transduction pathways are involved in the modification of the K^+ and Ca^{2+} currents by GHRH in somatotropes *(29,49–51)*, orexins may interact with GHRH through these intracellular second messenger systems to increase the Ca^{2+} current.

PLC pathway may play a role in the functional regulation in somatotropes *(52)*. GHS receptor was coupled to IP_3 in triggering the release of Ca^{2+} from the intracellular store and activating the PKC system in rat somatotropes *(53)*. It has recently been demonstrated that the effect of orexins is mediated by both Ca^{2+} influx and the IP_3 production in Chinese hamster ovary cells expressing the orexin receptors *(54,55)*. It is, however, unclear whether IP_3 was implicated in the effect of orexins on the membrane Ca^{2+} current in ovine somatotropes. In our experimental conditions, thapsigargin was used to deplete the intracellular Ca^{2+} store before orexins were applied, and we found that the increase in the L-type current by orexins was not affected. These results suggest that the elevation in the Ca^{2+} current by orexins was not dependent on the calcium release from the intracellular calcium store. This is not surprising given that receptor-operated Ca^{2+} entry may not be the consequence of intracellular Ca^{2+} release in several cell types *(56,57)*.

In comparison to the effects of GHRH and GHRP-2 on voltage-gated Ca^{2+} currents in ovine somatotropes *(26)*, the increase in Ca^{2+} currents by orexins was rather small *(29)*. The increase in L-type Ca^{2+} current brought about by orexins in somatotropes was also smaller than that in neurons *(58)*. Interaction between orexin-A and GHRH on Ca^{2+} currents was investigated. Orexin-A or GHRH individually induces a significant increase in the L-type current, and an additive effect on the current was observed when they were co-administered. This can be explained by the fact that orexin-A and GHRH modify the L-type current through different signalling pathways, e.g., orexin-A mainly activates the PKC system whereas GHRH predominantly uses PKA-cAMP to increase the L-type current. These data indeed prompted us to study further the direct stimulatory effects of orexins on the GH secretion in vitro. Orexin-B slightly increased GH release in a dose-dependent manner. This stimulation on GH secretion is much weaker than the stimulation by GHRH. Using orexin-A, a synergistic effect was evident on GH release when orexin-A and GHRH were co-administered, although orexin-A by itself did not significantly increase GH release. This synergistic effect corresponded well with the previous electrophysiological study that an additive effect on the L-type current was generated by the co-administration of orexin-A and GHRH. Therefore, the major function of orexins in the regulation of GH secretion may be that orexins prime the somatotropes for further action of GHRH. This process by orexins significantly increases the susceptibility and excitability of the somatotropes, so that GHRH subsequently generates a much stronger effect on GH release in, specifically, a normal pulsatile pattern. Meanwhile, the L-type Ca^{2+} current may exert a pivotal role on the enhanced GH secretion as both GHRH and orexins activate this current.

It should be emphasized, however, that the possible role of orexins in the control of GH secretion is still controversial. It has been reported that GH secretion may or may not be affected by orexins in different experimental conditions *(3,19)*. In this study, the orexin-induced increase in the L-type Ca^{2+} current is a modest modification rather than a marked enlargement. The release and the regulatory effect of orexins in vivo may reflect the actions of a number of other factors from the hypothalamus, such as leptin or neuropeptide Y (NPY). GH release may be significantly influenced by hypothalamic peptides and their complex inter action *(59–61)*. Hence, orexins may play a complementary role in the control of GH release. In the light of our current results, further studies on interactions between orexins and other GH secretagogues in GH control are warranted.

In conclusion, we have presented data demonstrating that the L-type current was significantly increased by orexins via the PKC signaling pathway in ovine somatotropes. GHRH and orexins exhibit an additive and a synergistic effect in stimulating the L current and GH release, respectively. The functions of orexins, therefore, may include the regulation of GH at the pituitary level by priming the somatotropes for further stimulation by GHRH.

Materials and Methods

Chemicals

Orexin-A and orexin-B were obtained as a generous gift from Dr. Yoichi Ueta (Department of Physiology, University of Occupational and Environmental Health, Kitakyushu, Japan) and Dr. Thomas Kilduff (SRI International, Menlo Park, CA, USA). Dulbecco's modified Eagle's medium (DMEM), *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES), and carbohydrate solutions were purchased from Trace Biosciences Pty Ltd (Clayton, Victoria, Australia). Medium 199 (M199) was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA) and penicillin–streptomycin fungizone solution from CSL Limited (Parkville, Victoria, Australia). Tissue culture reagents (DNAse, hyaluronidase, trypsin inhibitor, pancreatin), sera, adenosine-5'-triphosphate (ATP), creatine phosphokinase, phosphocreatine, guanosine 5'-triphosphates (GTP), nifedipine (NFD), ω-conotoxin (CTX), and all salts for recording solutions were purchased from Sigma (St. Louis, MO, USA). Tetrodotoxin (TTX) was from Alomone Labs (Jerusalem, Israel). L-Glutamine was purchased from GIBCO (Gaithersburg, MD, USA). Protein kinase A (PKA) and protein kinase C (PKC) inhibitory peptides (PKI and $PKC_{19–36}$) and phorbol 12,13-dibutyrate (PDBu) were obtained from Research Chemicals International (Natick, MA, USA). H89 and calphostin C (Cal-C) were from Cal-Biochem (San Diego, CA, USA).

Cell Preparation

Adult sheep pituitary glands were obtained at a local abattoir and then subjected to collagenase / pancreatin treatments to dissociate the cells, as described previously *(62)*. Briefly, whole pituitaries had their encapsulating tissue, the neurohypophysis, and pituitary stalk tissue removed. The anterior pituitaries were then minced and placed into calcium-free phosphate-buffered solution (PBS) + bovine serum albumin (BSA). The tissue fragments were gently washed and incubated with DNAse, hyaluronidase, trypsin inhibitor, pancreatin, and collagenase (approx 2.5 mg/mL) for 30–40 min at 37°C in a shaking water bath. After centrifugation at 2000*g*, the cells were suspended in Medium 199 and counted by hemocytometer. Cell yield was usually $2 \infty 10^7$ per pituitary gland, with more than 95% viability (trypan blue exclusion test). The cell suspension (3–5 mL) was placed above a column of layers of increasing density of Percoll solutions. In our culture condition, seven Percoll dilutions were prepared as follows for the discontinuous density gradient: 1.10, 1.074, 1.071, 1.068, 1.063, 1.058, 1.040, 1.029 g/mL (calibrated by density mark beads from Pharmacia Biotech, Uppsala, Sweden). Tubes were then centrifuged (J6-HC Centrifuge, Beckman Instruments, CA, USA)

at 2500 rpm for 30 min at 4ºC without brake. About 80% of cells in fractions ranging in density from 1.063 to 1.071 g/mL were somatotropes as determined by immunocytochemical staining *(62)*. Cells recovered from these fractions were subsequently seeded into 35 mm culture dishes for electrophysiology study and 48-well plates (10^4 or 10^5 cells/well) for the incubation experiments (GH radioimmunoassay) containing DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) L-glutamine (200 m*M* stock), and penicillin–streptomycin fungizone solution, and were maintained in a humidified incubator (37ºC, 5% CO_2). The culture medium was replenished every 2–3 d, and electrophysiological recordings were performed on cells maintained between 3 and 7 d in culture.

Patch-Clamp Recording

On the day of recording, culture medium was replaced by patch-clamp bath solution through a gravity pressure perfusion system 10 min before recording. Transmembrane Ca^{2+} currents were recorded using "gigaseal" patch-clamp techniques in classic whole-cell recording (WCR) configuration. Electrodes were pulled by a Sutter P-87 microelectrode puller from borosilicate micropipets with inner filament (Clark Electromedical Instruments, Pangbourne Reading, England) and had an initial input resistance of 3–5 MΩ. All recordings were made using the Axopatch 200A amplifier (Axon Instruments, CA, USA). The bath solution was composed of the following: 40 m*M* tetraethylammonium chloride (TEA-Cl), 90 m*M* NaCl, 5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES at pH 7.4 (adjusted with NaOH) and osmolarity of 300 mosmol/L (adjusted with sucrose). To exclude contamination by the Na^+ current, TTX was added into the bath solution at a final concentration of 1 μ*M* just before commencing experimentation. The pipet solution was composed of the following: 120 m*M* Cs-Aspartate, 20 m*M* TEA-Cl, 10 m*M* EGTA, 10 m*M* glucose, 10 m*M* HEPES. Just before recording, an ATP regenerative system (2 m*M* ATP, 5 m*M* Na_2-phosphocreatine, 20 units/mL creatine phosphokinase) plus 0.1 m*M* GTP was added into the pipet solution with pH adjusted to 7.4 and osmolarity to 300 mosmol/L (except in the recordings using PKI or $PKC_{19–36}$).

After obtaining a high-resistance seal, the voltage in the pipet was held at –80 mV, and voltage pulses (10 mV, 200 ms duration) were delivered periodically to monitor the access resistance. Access to the cell interior was judged by the appearance of a membrane capacitance transient current after a gentle suction was applied through the recording pipet. Whole-cell capacitance (7.7 ± 0.8 pF, n = 53) and series resistance were compensated (approx 80%) before experimentation, and leak current was routinely subtracted using the option offered by the Clampex 7 program (Axon Instruments). We also monitored the change in series resistance over the course of each experiment, and recordings with significant change in series resistance were finally excluded from the data analysis. The electrical signal was filtered at 2 kHz with a low-pass filter built on the amplifier, and the sweeps were sampled at 1 ms intervals between data points in our recording protocols.

Cell culture dishes were fixed on the stage of an Olympus inverted microscope, and a gravity pressure system was used to perfuse the cells at a rate of approx 1 mL/min. cAMP/PKA and PKC blockers, including H89 and Cal-C, were added into the culture dishes containing the cells to be recorded by free hand when the perfusion system was temporarily in pause. Cells were incubated with these blockers for at least 5 min in the bath solution in order to achieve an even distribution (the concentrations cited in the *Results* section are the final concentrations when diluted in the bath solution). Orexins or vehicle was applied through the perfusion system. Application of vehicle from the same system did not change Ca^{2+} currents in any of the various recording conditions. All experiments were performed at room temperature (20–22ºC).

Incubation and Radioimmunoassay (RIA)

Before each incubation experiment, the cells were washed three times with incubation medium (M199 containing 0.1% BSA) and then preincubated for 1 h with incubation medium as equilibration period. The medium after 1 h incubation was subsequently discarded and 0.5 mL/well of fresh incubation medium with or without the test substances was added for 30 min. Parallel incubations containing vehicle alone were included in the incubation as control. At the end of the incubation, the medium was collected and stored at –20ºC before the GH radioimmunoassay. The concentration of GH in the incubation medium was measured in a double-antibody RIA system using kits provided by the National Hormone and Pituitary Program of the USA (ovine GH and ovine GH antisera). All samples were assayed in duplicate. The sensitivity of the assay was 0.3 ng/mL. The inter- and intraassay coefficients of variation were <15% and <8%, respectively (n = 4). All samples from one experiment were measured in the same assay and GH values were expressed as nanogram equivalents ovine GH standard.

Data Analysis

The pCLAMP 7.0 software (Axon Instrument) was used to record and analyse the transmembrane current data. Student's paired t-test was used, where appropriate, to evaluate the statistical significance of differences between two group means, and the effect was considered to be significant at the value of $p < 5\%$. Group data are expressed as mean ± SEM in the *Results* section. The traces in figures were in each case representative of at least four recordings under the same experimental condition unless indicated otherwise in the text. GH data in incubation studies are also presented as means ± SEM. Statistical comparisons were made using Student's t-test and significance was taken as $p < 0.05$.

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References

1. de Lecea, L., Kilduff, T. S., Peyron, C., et al. (1998). *Proc. Natl. Acad. Sci. USA* **95,** 322–327.
2. Sakurai, T., Amemiya, A., Ishii, M., et al. (1998). *Cell* **92,** 573–585.
3. Hagan, J. J., Leslie, R. A., Patel, S., et al. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 10911–10916.
4. Mondal, M. S., Nakazato, M., and Matsukura, S. (2000). *Biochem. Cell Biol.* **78,** 299–305.
5. de Lecea, L. and Sutcliffe, J. G. (1999). *Cell Mol. Life Sci.* **56,** 473–480.
6. Mazzocchi, G., Malendowicz, L. K., Aragona, F., Rebuffat, P., Gottardo, L., and Nussdorfer, G. G. (2001). *J. Clin. Endocrinol. Metab.* **86,** 4818–4821.
7. Date, Y., Ueta, Y., Yamashita, H., et al. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 748–753.
8. Date, Y., Mondal, M. S., Matsukura, S., et al. (2000). *Brain Res. Mol. Brain Res.* **76,** 1–6.
9. Xu, R., Wang, Q., Yan, M., et al. (2002). *Endocrinology* **143,** 4609–4619.
10. Blanco, M., Lopez, M., GarcIa-Caballero, T., et al. (2001). *J. Clin. Endocrinol. Metab.* **86,** 1616–1619.
11. Johren, O., Neidert, S. J., Kummer, M., Dendorfer, A., and Dominiak, P. (2001). *Endocrinology* **142,** 3324–3331.
12. Pu, S., Jain, M. R., Kalra, P. S., and Kalra, S. P. (1998). *Regul. Pept.* **78,** 133–136.
13. Russell, S. H., Kim, M. S., Small, C. J., et al. (2000). *J. Neuroendocrinol.* **12,** 1213–1218.
14. Jaszberenyi, M., Bujdoso, E., Pataki, I., and Telegdy, G. (2000). *J. Neuroendocrinol.* **12,** 1174–1178.
15. Irahara, M., Tamura, T., Matuzaki, T., et al. (2001). *Biochem. Biophys. Res. Commun.* **281,** 232–236.
16. Mazzocchi, G., Malendowicz, L. K., Gottardo, L., Aragona, F., and Nussdorfer, G. G. (2001). *J. Clin. Endocrinol. Metab.* **86,** 778–782.
17. Mitsuma, T., Hirooka, Y., Mori, Y., et al. (1999). *Horm. Metab. Res.* **31,** 606–609.
18. Nowak, K. W., Mackowiak, P., Switonska, M. M., Fabis, M., and Malendowicz, L. K. (2000). *Life Sci.* **66,** 449–454.
19. Samson, W. K. and Taylor, M. M. (2001). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281,** R1140–R1145.
20. van den Pol, A. N., Gao, X. B., Obrietan, K., Kilduff, T. S., and Belousov, A. B. (1998). *J. Neurosci.* **18,** 7962–7971.
21. Grudt, T. J., van den Pol, A. N., and Perl, E. R. (2002). *J. Physiol.* **538,** 517–525.
22. Chen, C., Vincent, J. D., and Clarke, I. J. (1994). *Trend Endocrinol. Metab.* **5,** 227–233.
23. Chen, C. and Clarke, I. J. (1995). *Am. J. Physiol.* **268,** E204–E212.
24. Tsien, R. W., Fox, A. P., Hess, P., et al. (1987). *Soc. Gen. Physiol. Ser.* **41,** 167–187.
25. Glassmeier, G., Hauber, M., Wulfsen, I., Weinsberg, F., Bauer, C. K., and Schwarz, J. R. (2001). *Pflugers Arch.* **442,** 577–587.
26. Chen, C. and Clarke, I. J. (1995). *J. Neuroendocrinol.* **7,** 179–186.
27. Chen, C., Zhang, J., Dayanithi, G., Vincent, J. D., and Israel, J. M. (1989). *Neurochem. Int.* **15,** 265–275.
28. Kato, M. and Sakuma, Y. (1997). *Endocrinology* **138,** 5096–5100.
29. Takei, T., Takano, K., Yasufuku-Takano, J., Fujita, T., and Yamashita, N. (1996). *Am. J. Physiol.* **271,** E801–E807.
30. Xu, R., Clarke, I. J., Chen, S., and Chen, C. (2000). *J. Neuroendocrinol.* **12,** 147–157.
31. Wetzel, C. H., Spehr, M., and Hatt, H. (2001). *Eur. J. Neurosci.* **14,** 1056–1064.
32. Wu, D., Chen, C., Zhang, J., Bowers, C. Y., and Clarke, I. J. (1996). *J. Endocrinol.* **148,** 197–205.
33. Stojilkovic, S. S., Izumi, S., and Catt, K. J. (1988). *J. Biol. Chem.* **263,** 13054–13061.
34. Ramirez, J. L., Torronteras, R., Malagon, M. M., et al. (1998). *Cell Calcium* **23,** 207–217.
35. Kwiecien, R. and Hammond, C. (1998). *Neuroendocrinology* **68,** 135–151.
36. Rodgers, R. J., Halford, J. C., Nunes de Souza, R. L., et al. (2000). *Regul. Pept.* **96,** 71–84.
37. Stea, A., Soong, T. W., and Snutch, T. P. (1995). *Neuron* **15,** 929–940.
38. Zong, S., Yassin, M., and Tanabe, T. (1995). *Biochem. Biophys. Res. Commun.* **215,** 302–308.
39. Washburn, D. L. and Ferguson, A. V. (2001). *J. Physiol.* **536,** 667–675.
40. Cataldi, M., Taglialatela, M., Guerriero, S., et al. (1996). *J. Biol. Chem.* **271,** 9441–9446.
41. Tse, A. and Hille, B. (1993). *Endocrinology* **132,** 1475–1481.
42. Heyward, P. M., Chen, C., and Clarke, I. J. (1995). *Neuroendocrinology* **61,** 609–621.
43. Charles, A. C., Piros, E. T., Evans, C. J., and Hales, T. G. (1999). *J. Biol. Chem.* **274,** 7508–7515.
44. Shiraishi, T., Oomura, Y., Sasaki, K., and Wayner, M. J. (2000). *Physiol. Behav.* **71,** 251–261.
45. Takei, T., Yasufuku-Takano, J., Takano, K., Fujita, T., and Yamashita, N. (1998). *Am. J. Physiol.* **275,** E649–E654.
46. Adams, E. F., Lei, T., Buchfelder, M., Bowers, C. Y., and Fahlbusch, R. (1996). *Mol. Endocrinol.* **10,** 432–438.
47. Uramura, K., Funahashi, H., Muroya, S., Shioda, S., Takigawa, M., and Yada, T. (2001). *Neuroreport* **12,** 1885–1889.
48. van den Pol, A. N. (1999). *J. Neurosci.* **19,** 3171–3182.
49. Lussier, B. T., French, M. B., Moor, B. C., and Kraicer, J. (1991). *Endocrinology* **128,** 592–603.
50. Chen, C., Wu, D., and Clarke, I. J. (1996). *J. Endocrinol.* **148,** 381–386.
51. Xu, R., Roh, S. G., Loneragan, K., Pullar, M., and Chen, C. (1999). *J. Physiol.* **520(Pt 3),** 697–707.
52. Lei, T., Adams, E. F., Buchfelder, M., and Fahlbusch, R. (1996). *Neurosurgery* **39,** 569–575.
53. Cheng, K., Chan, W. W., Butler, B., Barreto, A. Jr., and Smith, R. G. (1991). *Endocrinology* **129,** 3337–3342.
54. Lund, P. E., Shariatmadari, R., Uustare, A., et al. (2000). *J. Biol. Chem.* **275,** 30806–30812.
55. Kukkonen, J. P. and Akerman, K. E. (2001). *Neuroreport* **12,** 2017–2020.
56. Felder, C. C., Poulter, M. O., and Wess, J. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 509–513.
57. Zerbes, M., Bunn, S. J., and Powis, D. A. (1998). *Cell Calcium* **23,** 379–386.
58. Hwang, L. L., Chen, C. T., and Dun, N. J. (2001). *J. Physiol.* **537,** 511–520.
59. Rauch, M., Riediger, T., Schmid, H. A., and Simon, E. (2000). *Pflugers Arch.* **440,** 699–703.
60. Ida, T., Nakahara, K., Kuroiwa, T., et al. (2000). *Neurosci. Lett.* **293,** 119–122.
61. Komaki, G., Matsumoto, Y., Nishikata, H., et al. (2001). *Eur. J. Endocrinol.* **144,** 645–651.
62. Chen, C., Heyward, P., Zhang, J., Wu, D., and Clarke, I. J. (1994). *Neuroendocrinology* **59,** 1–9.
63. Xu, R., Zhao, Y., and Chen, C. (2003). *Neuroendocrinology* **77,** 141–152.