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Stimulation of catecholamine synthesis by orexin-A in bovine adrenal medullary cells through orexin receptor 1

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Abstract

Orexin-A has recently been identified as a new hypothalamic peptide working as a mediator in the regulation of feeding behavior and sleep control. To determine the role of orexin-A in peripheral metabolic processes, we examined direct effects of orexin-A on catecholamine synthesis and secretion in cultured bovine adrenal medullary cells. Incubation of cells with orexin-A (100 pM) for 20 min caused a small but significant increase in ^{14}C-catecholamine synthesis from [^{14}C]tyrosine, but not from L-3,4-dihydroxyphenyl[3-^{14}C]alanine. Orexin-A (100 pM) potentiated the stimulatory effects of acetylcholine (0.3 mM) on ^{14}C-catecholamine synthesis. Orexin-A significantly increased tyrosine hydroxylase activity, which was evident at 1 pM and maximal at 100 pM. 4β-Phorbol-12β-myristate-13α-acetate, an activator of protein kinase C, did not enhance the stimulatory effects of orexin-A on tyrosine hydroxylase activity, while H-7 and staurosporine, inhibitors of protein kinase C, nullified the effects of orexin-A. Orexin-A had little effect on catecholamine secretion from the cells. Orexin receptor 1 (OX_1R) but not orexin receptor 2 (OX_2R) mRNA was detected in bovine adrenal medullary cells by reverse transcriptase-polymerase chain reaction. These findings suggest that orexin-A activates tyrosine hydroxylase and then stimulates catecholamine synthesis, probably via activation of the OX_1R-protein kinase C pathway in adrenal medullary cells.

Keywords: Adrenal medullary cells; Catecholamine synthesis; Orexin-A; Orexin receptor 1; Protein kinase C; Tyrosine hydroxylase

1. Introduction

Orexin-A and orexin-B, also known as hypocretins 1 and 2, have recently been identified as hypothalamic peptides derived from a precursor, prepro-orexin, in neurons located within and around the lateral and posterior hypothalamus in the rat brain [1–3]. These peptides mediate feeding behaviors and energy homeostasis through two subtypes of G protein-coupled receptors, namely orexin receptor 1 (OX_1R) and orexin receptor 2 (OX_2R) [1], i.e. OX_1R is linked to Gq proteins and OX_2R to Gq or Gi proteins [1,4]. Orexin-A also regulates the sleep-wake cycle and other behaviors such as grooming [5] through a dopaminergic pathway in the central nervous system. Lin *et al.* [6] have found that canine narcolepsy is caused by a mutation in the OX_2R gene, and that orexin knockout mice exhibit a phenotype similar to human narcolepsy [7,8]. Furthermore, orexins have been shown to stimulate the cardiovascular system via activation of central sympathetic outflow [9,10]. In addition to these central functions, several lines of evidence have shown that orexin-A acts directly on some extrahypothalamic and peripheral tissues. Orexin-A, when administered intra-peritoneally to the rat, stimulates insulin secretion by direct action on the pancreatic islets [11]. Kirchgessner and Liu have reported that orexin and orexin receptors are present in the enteric nervous system and that orexin receptors participate in the initiation and/or propagation of the peristaltic reflex [12]. In dispersed zona fasciculate/reticularis cells of the rat adrenal cortex, orexin-A increases basal secretion of

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Abbreviations: KRP, Krebs–Ringer phosphate; OXR, orexin receptor; RT-PCR, reverse transcriptase-polymerase chain reaction.

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corticosterone [13]. Because orexin-A is present in the blood at a substantial level [14], there may be a possible role of orexin-A in peripheral tissues.

Adrenal medullary cells derived from the embryonic neural crest share many physiological and pharmacological properties with postganglionic sympathetic neurons. Stimulation of adrenal medullary cells by acetylcholine released from splanchnic nerves causes secretion of catecholamines into the systemic circulation [15] and also increases catecholamine synthesis, which is associated with tyrosine hydroxylase activation [16,17]. Tyrosine hydroxylase, the rate-limiting step in the biosynthesis of catecholamines, catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and its activity is acutely regulated by various factors [18], including enzyme phosphorylation [19].

Orexin receptors have been detected in the rat adrenal medulla by RT-PCR and immunohistochemistry [20]. There are, however, still conflicting reports regarding the effects of orexins on catecholamine secretion [21,22] and the type of OXR expressed in the adrenal medulla [4,20]. In the present study, we investigated the effects of orexin-A on catecholamine synthesis, and assessed the type of OXR mRNA by RT-PCR in cultured bovine adrenal medullary cells.

2. Materials and methods

2.1. Materials

Oxygenated KRP buffer was used throughout. Its composition is as follows (in mM): 154 NaCl, 5.6 KCl, 1.1 $MgSO_4$, 2.2 $CaCl_2$, 0.85 NaH_2PO_4, 2.15 Na_2HPO_4 and 10 glucose, adjusted to pH 7.4. Materials were obtained from the following sources: Eagle's minimum essential medium (MEM) was from Nissui Pharmaceutical; collagenase was from Nitta Zerachin; calf serum and acetylcholine were from Nacalai Tesque; ionomycin, and 4β-phorbol-12β-myristate-13α-acetate (PMA) were from Calbiochem-Behring; L-[1-^{14}C]tyrosine (54.45 mCi/mmol) was from Perkin Elmer Life Sciences; L-[U-^{14}C]tyrosine (460 mCi/mmol) and L-[3-^{14}C]DOPA (6.8 mCi/mmol) were from Amersham Biosciences and staurosporine from Alomone Labs. Protein kinase inhibitors (H-7 and KN-62) were from Seikagaku Corp. Human orexin-A was from Peptide Institute.

2.2. Primary culture of bovine adrenal medullary cells

From bovine adrenal glands, the medullary cells were isolated by collagenase digestion according to the previously described method [23]. The cells were plated at a density of 4×10^6 cells/dish (Falcon 35 mm, Becton Dickinson Labware) and maintained in monolayer culture in Eagle's MEM containing 10% calf serum and antibiotics at 37° under 5% CO_2/95% air. The cells were used for experiments after being cultured for between 2 and 7 days.

2.3. ^{14}C-Catecholamine synthesis from [^{14}C]tyrosine or [^{14}C]DOPA

The cells were incubated with 20 μM L-[U-^{14}C]tyrosine or L-[3-^{14}C]DOPA in 1.0 mL of KRP buffer in the presence or absence of 100 pM of orexin-A and 0.3 mM acetylcholine at 37° for 20 min for [^{14}C]tyrosine and 10 min for [^{14}C]DOPA. After aspiration of the reaction medium, the cells were harvested in 2.5 mL of 0.4 M perchloric acid and left standing for more than 30 min on ice to extract the radioactive catecholamines. The precipitated protein was removed by centrifugation at 1600 *g* for 10 min, and ^{14}C-labeled catechol compounds in the supernatant were separated further into the fractions containing ^{14}C-dopamine, and ^{14}C-epinephrine plus ^{14}C-norepinephrine by ion exchange chromatography on Duolite C-25 columns (H^+ type, 0.4 cm × 7.0 cm) [24]. The ^{14}C-labeled catecholamines separated were counted in a toluene base scintillator using a liquid scintillation counter (Aloka LSC-3500E, Aloka Co., Ltd.). ^{14}C-Catecholamine synthesis was expressed as the sum of the ^{14}C-catecholamines (epinephrine, norepinephrine and dopamine), because the ratio of ^{14}C-epinephrine plus ^{14}C-norepinephrine/^{14}C-dopamine was not significantly changed by stimulants.

2.4. Catecholamine secretion

The cells were incubated with or without up to 1 nM of orexin-A and/or 0.3 mM of acetylcholine for 20 min at 37°. Catecholamines (norepinephrine plus epinephrine) secreted into the medium were adsorbed to aluminum hydroxide and measured as previously reported [23].

2.5. Tyrosine hydroxylase activity

The cells (10^6 cells/well; 24-well, Falcon) were exposed to 250 μL of the KRP buffer with or without various agents, including orexin-A (up to 100 pM), 4β-phorbol-12β-myristate-13α-acetate (PMA) (1 μM) or ionomycin (10 μM), supplemented with 18 μM L-[1-^{14}C]tyrosine (0.2 μCi) for 20 min at 37°. Upon addition of L-[1-^{14}C]tyrosine, each well was immediately sealed with an acrylic tube capped with a rubber stopper and fitted with a small plastic cup containing 200 μL of NCS-II tissue solubilizer (Amersham Biosciences) to absorb $^{14}CO_2$ released by the cells [25]. To terminate the reaction, 250 μL of perchloric acid was injected into each well through the rubber stopper using a syringe. The protein kinase inhibitors examined were as follows; H-7 (10 μM), staurosporine (1 μM) and KN-62 (1 μM) in the presence or absence of 100 pM orexin-A.

2.6. RT-PCR of OXR mRNAs

mRNA was isolated from bovine adrenal medullary cells by guanidine hydrochloride extraction and oligo(dT) cellulose column separation. The primers of 5′-AACCGCA-

CACGGCTCTTCTCA-3′ and 5′-TAGACAGCTTCGCG-GTCACTG-3′ for OX_1R and 5′-TGGGAACGTCCTGGT-TTGTGT-3′ and 5′-CACACCGTAAAGAGGGTGGTT-3′ for OX_2R were designed on the basis of the sequence of human OXRs (1). RT-PCR was performed with a PC-701 thermocycler (Astec), using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) and a Takara Ex Taq kit (Takara), as described previously [24]. The thermocycling conditions were as follows: 94° (1 min), 67° (1 min), and 72° (2 min) for 33 cycles. The resultant PCR product was subjected to electrophoresis in a 5% polyacrylamide gel, stained with GelStar Nucleic Acid Gel Stain (Takara), and analyzed using FLA-2000 fluoroimage analyzer (Fujifilm).

2.7. Statistics

Data are presented as the means ± standard deviation (SD). The statistical evaluation of the data was performed by ANOVA. Values were considered statistically significant when P was less than 0.05. Statistical analyses were performed using StatView for Macintosh version 4.11J software (Abacus Concept Inc.).

3. Results

3.1. Time course of ^{14}C-catecholamine synthesis induced by orexin-A in cultured bovine adrenal medullary cells

The cells were incubated with or without 100 pM orexin-A at 37° for the indicated times (Fig. 1). The basal synthesis of ^{14}C-catecholamines (the sum of ^{14}C-epinephrine, ^{14}C-norepinephrine and ^{14}C-dopamine production) from [^{14}C]tyrosine was linear for up to 30 min, as previously reported [24]. Orexin-A caused a small (about 20–30% over the control) but significant ($P < 0.05$) increase in ^{14}C-catecholamine synthesis during incubation for 10–30 min. From this result, subsequent measurements of ^{14}C-catecholamine synthesis were performed with 20 min incubation.

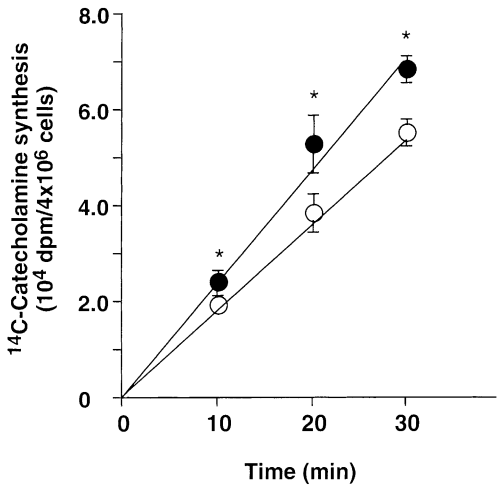

Fig. 1. Time course of ^{14}C-catecholamine synthesis stimulated by orexin-A in bovine adrenal medullary cells. Cultured cells (4×10^6 cells/dish) were incubated with (●) or without (○) orexin-A (100 pM) at 37° for the indicated times in 1.0 mL KRP buffer containing L-[U-^{14}C]tyrosine. The ^{14}C-labeled catecholamines formed are shown as the total ^{14}C-catecholamines. Data are expressed as the mean ± SD of five experiments carried out in duplicate. $^{*}P < 0.05$, compared with control.

3.2. Effects of orexin-A and acetylcholine on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine or [^{14}C]DOPA

As shown in Fig. 2A, orexin-A (100 pM) and acetylcholine (0.3 mM) increased the synthesis of ^{14}C-catecholamines from [^{14}C]tyrosine by 21 and 301% over the control, respectively. Combined treatment with orexin-A significantly potentiated the stimulatory effect of acet-

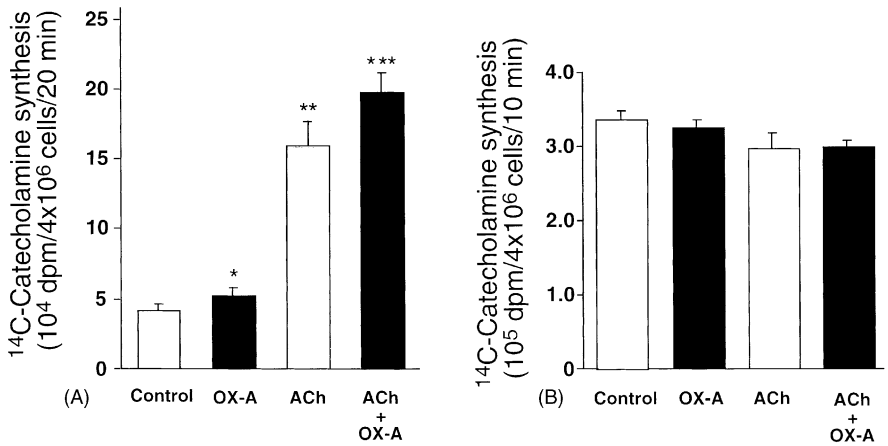

Fig. 2. Effects of orexin-A and/or acetylcholine on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine or [^{14}C]DOPA. The cells were incubated with or without orexin-A (100 pM) and acetylcholine (ACh) (0.3 mM) at 37° for 20 min and 10 min in the presence of L-[U-^{14}C]tyrosine (A) or [^{14}C]DOPA (B), respectively. Data are expressed as the mean ± SD of four experiments carried out in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with control; $^{***}P < 0.05$, compared with acetylcholine alone.

ylcholine (by 370% over the control). To know which step of catecholamine synthesis was stimulated by orexin-A, [^{14}C]DOPA was used as a substrate instead of [^{14}C]tyrosine. The basal synthesis of ^{14}C-catecholamines from [^{14}C]DOPA was much greater than that from [^{14}C]tyrosine (Fig. 2B). Neither orexin-A nor acetylcholine increased the synthesis of ^{14}C-catecholamines from [^{14}C]DOPA, indicating that stimulation of ^{14}C-catecholamine synthesis caused by orexin-A and acetylcholine occurs predominantly upstream of the DOPA decarboxylase step.

3.3. Effects of orexin-A and acetylcholine on tyrosine hydroxylase activity in the cells

Figure 3 shows the concentration–response curves for tyrosine hydroxylase activity stimulated by various concentrations of orexin-A in the presence or absence of acetylcholine (0.3 mM). In the absence of acetylcholine, orexin-A significantly enhanced tyrosine hydroxylase activity at 1 pM by 9% over the control. A maximal stimulation of tyrosine hydroxylase activity was observed at 100 pM, and the increment was 18% over the control. Acetylcholine at the concentration of 0.3 mM that induced the maximal response [17] enhanced tyrosine hydroxylase activity by 59% over the control. In the presence of acetylcholine (0.3 mM), the maximal stimulation was exhibited at 100 pM orexin-A that was increased by 42% over acetylcholine alone and by 125% over the control.

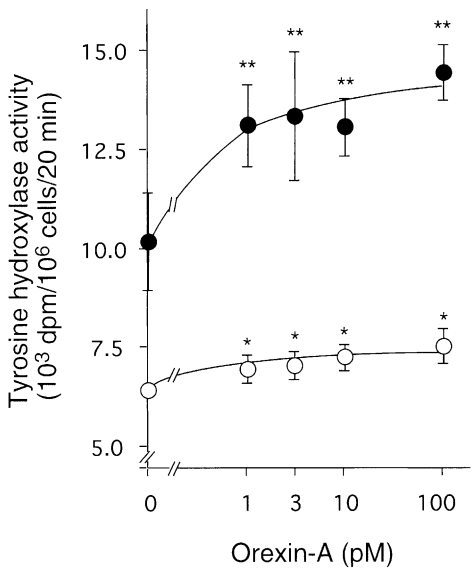

Fig. 3. Concentration–response curves for tyrosine hydroxylase activity induced by orexin-A. The cells were incubated with 18 μM L-[1-^{14}C]tyrosine (0.2 μCi) and various concentrations of orexin-A for 20 min at 37° in the presence (●) or absence (○) of acetylcholine (0.3 mM). Data are expressed as mean ± SD of five experiments carried out in triplicate. $^{*}P < 0.05$, compared with orexin-A (−); $^{**}P < 0.05$, compared with acetylcholine alone.

3.4. Effects of protein kinase C activator and a Ca^{2+} ionophore on tyrosine hydroxylase activity in cells stimulated by orexin-A

To examine the mechanism of tyrosine hydroxylase activation by orexin-A, cells were incubated with orexin-A in the presence of protein kinase activators and then the tyrosine hydroxylase activity was measured (Fig. 4). PMA and ionomycin were used to activate protein kinase C and Ca^{2+}-dependent protein kinases, respectively, all of which produce an activation of tyrosine hydroxylase [26]. At the concentration (1 μM) producing the maximal response, PMA increased tyrosine hydroxylase activity by 47% over the control as did orexin-A, but orexin-A did not produce any additive effect on PMA stimulation. On the other hand, ionomycin (10 μM) increased tyrosine hydroxylase activity by 73% over the control, whereas orexin-A produced more than additive effect (by 143% over the control).

3.5. Effects of various protein kinase inhibitors on tyrosine hydroxylase activity in orexin-A-stimulated cells

Next, we examined effects of protein kinase inhibitors on orexin-A-stimulated tyrosine hydroxylase activity. H-7 and KN-62 were used as inhibitors of protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II, respectively. H-7 (10 μM) did not alter the basal activity, but it abolished the stimulatory effect of orexin-A (Fig. 5). Staurosporine (1 μM) (another inhibitor of protein kinase C) also nullified the effect of orexin-A (data not shown). KN-62, however, did not affect basal and orexin-A-stimulated tyrosine hydroxylase activity.

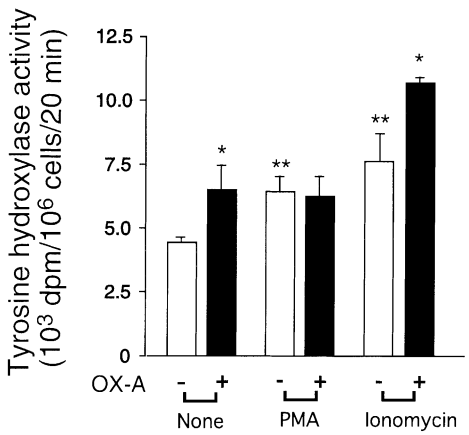

Fig. 4. Effects of protein kinase C activator and a Ca^{2+} ionophore on tyrosine hydroxylase activity in orexin-A-stimulated cells. The cells were exposed to 250 μL of KRP buffer with stimulating agents, PMA (1 μM) or ionomycin (10 μM) in the presence or absence of orexin-A (OX-A) (1 nM), supplemented with L-[1-^{14}C]tyrosine (0.2 μCi). Data are the mean ± SD of five experiments carried out in triplicate. $^{*}P < 0.05$, compared with orexin-A (−); $^{**}P < 0.05$, compared with None, OX-A (−).

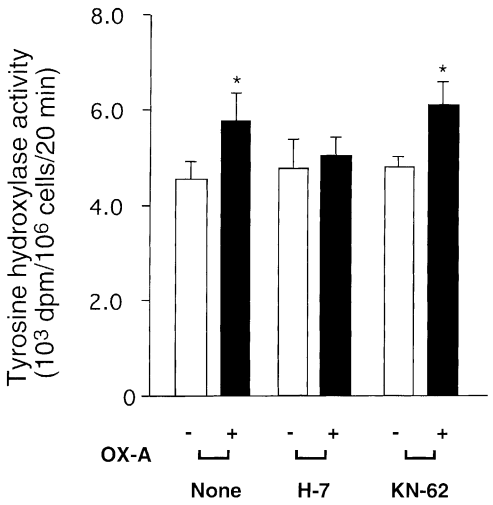

Fig. 5. Effects of various protein kinase inhibitors on tyrosine hydroxylase activity in cells induced by orexin-A. The cells were exposed to 250 μL of KRP buffer with or without inhibitors, H-7 (10 μM) or KN-62 (1 μM) in the presence or absence of orexin-A (OX-A) (1 nM), supplemented with L-[1-^{14}C]tyrosine (0.2 μCi). Data are the mean ± SD of five experiments carried out in triplicate. $^{*}P < 0.05$, compared with OX-A (−).

3.6. Effect of orexin-A on catecholamine secretion

The effect of orexin-A on catecholamine secretion from adrenal medullary cells was examined. Acetylcholine (0.3 mM) increased catecholamine secretion by 613% over basal secretion (Table 1). Orexin-A at any concentrations (10–1000 pM) did not affect basal and acetylcholine-stimulated catecholamine secretion.

3.7. RT-PCR detection of OX_1R but not OX_2R in bovine adrenal medullary cells

To determine the type of OXR in bovine adrenal medulla, OXR mRNA was assayed by RT-PCR. Since none of bovine OXR types have been cloned, we employed the distinctive regions of the human OX_1R and OX_2R segment to design bovine PCR primers for OX_1R and OX_2R. Amplification of bovine adrenal medullary mRNA with these primers resulted in the formation of a single band of PCR product for OX_1R but not for OX_2R (Fig. 6).

Table 1
Effects of orexin-A and/or acetylcholine on catecholamine secretion from cultured bovine adrenal medullary cells

	Catecholamine secretion (μg/4 × 10^6 cells/20 min)	
	ACh (−)	ACh (+)
Control	0.55 ± 0.02	3.92 ± 0.27
Orexin-A		
10 pM	0.53 ± 0.04	3.87 ± 0.38
100 pM	0.55 ± 0.05	4.06 ± 0.32
1000 pM	0.55 ± 0.02	3.98 ± 0.38

The catecholamines secreted were measured. Data are mean ± SD of four to six separate experiments carried out in duplicate. Cells were incubated with or without various concentrations of orexin-A and acetylcholine (ACh) (0.3 mM) at 37° for 20 min.

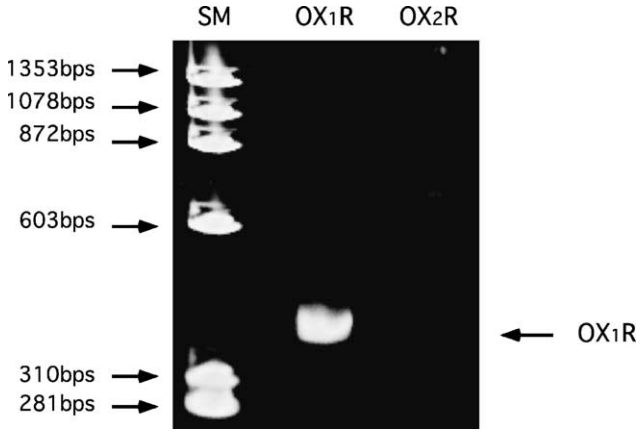

Fig. 6. RT-PCR detection of OX_1R but not OX_2R mRNA in bovine adrenal medullary cells. A PCR product was assayed in bovine adrenal medullary cells using designed from the sequence of human OX_1R and OX_2R. ØX174 DNA-*Hae*III digest size markers are in lane SM (size marker).

4. Discussion

In the present study, we demonstrated that orexin-A stimulates the synthesis of ^{14}C-catecholamines from [^{14}C]tyrosine in cultured bovine adrenal medullary cells. When [^{14}C]DOPA was used as a substrate, orexin-A failed to stimulate ^{14}C-catecholamine synthesis, suggesting that stimulation of catecholamine synthesis induced by orexin-A must occur upstream the DOPA decarboxylase step, and probably at the tyrosine hydroxyl step. Indeed, incubation of cells with orexin-A increased tyrosine hydroxylase activity. Based on these findings, it appears likely that orexin-A activates tyrosine hydroxylase, which, in turn, stimulates catecholamine synthesis in adrenal medullary cells. To our knowledge, this is the first direct evidence to show the stimulatory effect of orexin-A on catecholamine synthesis.

Tyrosine hydroxylase is acutely regulated by various factors [18], including enzyme phosphorylation [19]. A number of *in vitro* and *in situ* experiments have documented that tyrosine hydroxylase can be phosphorylated by multiple protein kinases such as protein kinase A, protein kinase C, and Ca^{2+}/calmodulin-dependent protein kinases [16,25–30]. In the present study, ionomycin (a Ca^{2+} ionophore that may activate Ca^{2+}-dependent protein kinases) had more than additive effect on orexin-A-induced increases in tyrosine hydroxylase activity, while PMA (an activator of protein kinase C) had no additive effect. Furthermore, tyrosine hydroxylase activation induced by orexin-A was nullified by H-7 and staurosporine (inhibitors of protein kinase C), but not by KN-62 (an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II). These findings suggest that orexin-A activates tyrosine hydroxylase activity through a protein kinase C-dependent but not

Ca^{2+}/calmodulin-dependent protein kinase II-dependent pathway.

Relative expression of the type of OXR (OX_1R or OX_2R) in adrenal medulla remains controversial. López *et al.* [20] have reported high levels of OX_1R and OX_2R expression in the rat adrenal medulla by RT-PCR and immunohistochemistry, while only OX_2R mRNA has been found to be expressed at high levels in human pheochromocytomas [21] and the rat adrenal medulla [4]. In the present study, we observed robust expression of OX_1R mRNA in bovine adrenal medulla. We attempted to amplify OX_2R mRNA by RT-PCR using primers designed from the human cDNA, but unfortunately could not detect the corresponding PCR product. The present results, however, do not exclude the possibility of the presence of OX_2R in the bovine adrenal medulla.

Regarding the functional studies of OXRs in the adrenal medulla, there are conflicting reports showing the effects of orexins on catecholamine secretion. In the first study, orexins were found to suppress catecholamine secretion in cultured PC12 cells [4]. On the other hand, orexins were found to stimulate catecholamine secretion from human pheochromocytomas [21] or to have little effect on catecholamine secretion in human adrenal medullary cells [22]. In the present study, orexin-A did not affect catecholamine secretion in bovine adrenal medullary cells. Because orexin receptors are members of a family of seven-transmembrane, G protein-coupled receptors, the receptors are coupled to multiple G proteins such as Gq, Gi, and so on. The orexins cause an increase in cytoplasmic Ca^{2+} levels in neurons cultured from rat medial and lateral hypothalamus [31], and Chinese hamster ovary cells [32] and HEK 293 cells expressing OX_1R through Gq [1]. Taken together, we speculate that orexin-A stimulates catecholamine synthesis through activation of OX_1R coupled with Gq, which may be linked to an activation of protein kinase C but not enough increase in intracellular Ca^{2+} concentrations in bovine adrenal medullary cells. The latter possibility is well supported by the present result that orexin-A was unable to cause catecholamine secretion which is critically dependent on increase in an intracellular Ca^{2+} [33].

Orexins are derived from a precursor, prepro-orexin that is synthesized in the rat brain hypothalamus [1]. These peptides potently stimulate food consumption when administrated in the ventricles [1]. Moreover, orexins regulate the sleep-wake cycle [1] and induce hyperlocomotion and stereotypy through the central dopaminergic system [5]. Hagan *et al.* [34] have reported that orexin-A activates locus caeruleus cell firing and increases dopamine turnover in the medial prefrontal cortex as well as arousal in the rats, suggesting that orexins induce some level of excitation of catecholaminergic neurons in the brain. On the other hand, the serum levels of orexin-A have been found to be 1.94 ± 0.24 pM in healthy humans [14]. Therefore, in the present study it is noteworthy that orexin-A, even at 1–3 pM, stimulates basal as well as acetylcholine-induced activity of tyrosine hydroxylase in adrenal medullary cells. Although the physiological significance of serum orexin-A is not clear, the present findings suggest that in addition to brain functions, orexin-A plays a role in the regulation of catecholamine synthesis in peripheral tissues, including the adrenal medulla. Furthermore, our findings lead us to hypothesize that orexin-A potentiates catecholamine synthesis when produced by stress or emotional excitation, thus mediating stimulation of splanchnic nerves and releasing acetylcholine from the nerves to the adrenal medulla.

In summary, the present study reports that orexin-A stimulates tyrosine hydroxylase activity and catecholamine synthesis, probably via activation of the OX_1R-protein kinase C pathway in cultured bovine adrenal medullary cells. This information should provide us with a better understanding of the mechanism by which orexin-A acts on metabolic homeostasis in peripheral tissues.

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