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EFFECTS OF α_2-ADRENERGIC AGONISTS ON CARBACHOL-STIMULATED CATECHOLAMINE SYNTHESIS IN CULTURED BOVINE ADRENAL MEDULLARY CELLS

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Abstract—We examined the effects of α_2- and α_1-adrenergic agonists on synthesis of catecholamines in cultured bovine adrenal medullary cells. Clonidine, an α_2-adrenergic agonist, inhibited carbachol-stimulated synthesis of [^{14}C]catecholamines from [^{14}C]tyrosine in a concentration-dependent manner. Clonidine also inhibited carbachol-induced uptake of $^{45}Ca^{2+}$ into cells at concentrations similar to those that inhibited the synthesis of [^{14}C]catecholamines. Other α_2-adrenergic agonists, oxymetazoline and guanfacine, also strongly inhibited carbachol-stimulated synthesis of [^{14}C]catecholamines. α_1- Adrenergic agonists, phenylephrine and norfenefrine, did not affect the synthesis. Tyrosine hydroxylase (EC 1.14.16.2) activity in a soluble fraction of cultured bovine adrenal medullary cells was assayed after gel filtration on a Sephadex G-25 column. Stimulation of the cells with carbachol increased the activity of tyrosine hydroxylase. Clonidine, oxymetazoline, and guanfacine all suppressed the carbachol-induced increase in activity of tyrosine hydroxylase in the cells. These results suggest that α_2-adrenergic agonists inhibit carbachol-stimulated synthesis of catecholamines by suppression of tyrosine hydroxylase activity, probably through the inhibition of Ca^{2+} uptake. However, the involvement of α_2-adrenoceptors in the inhibitory effects of α_2-agonists on catecholamine synthesis is still unsettled, since yohimbine failed to antagonize the inhibitory effect of clonidine on the synthesis in cultured bovine adrenal medullary cells.

Adrenal medullary cells are paraneurons of neural crest origin and share many physiological and pharmacological properties with postganglionic sympathetic neurons. Stimulation of acetylcholine receptors in adrenal medullary cells increases the secretion [1–4] and the synthesis [5, 6] of catecholamines in a Ca^{2+}-dependent manner. We recently demonstrated the presence of α_2-adrenoceptors in bovine adrenal medullary cells [7] and reported that α_2-agonists inhibit carbachol-evoked secretion of catecholamines through the inhibition of Ca^{2+} uptake [8].

In vivo experiments have shown that clonidine inhibits the synthesis of catecholamines in rat heart, submaxillary gland, adrenal gland [9] and brain [10] and mouse brain [11]. However, the mechanism by which clonidine reduces the synthesis of catecholamines, and the effects of other α_2-agonists or α_1-agonists on catecholamine synthesis, have not been well investigated. In the present experiments, we studied the effects of α_2- and α_1-agonists on the synthesis of [^{14}C]catecholamines and on the activity of tyrosine hydroxylase in cultured bovine adrenal medullary cells.

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† Abbreviations: KRP, Krebs–Ringer phosphate; 6MePtH$_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine HCl; and IBMX, 3-isobutyl-1-methylxanthine.

METHODS

Oxygenated Krebs–Ringer phosphate (KRP†) buffer containing 0.5% bovine serum albumin was used throughout unless otherwise specified. It had the following composition (mmol/l): NaCl, 154; KCl, 5.6; $MgSO_4$, 1.1; $CaCl_2$, 2.2; NaH_2PO_4, 0.85; Na_2HPO_4, 2.15; and glucose, 10, adjusted to pH 7.4. L-[U-^{14}C]Tyrosine (504 mCi/mmol) and $^{45}CaCl_2$ (0.5 to 2.0 Ci/mmol) were obtained from Amersham International (Buckinghamshire, England). L-[1-^{14}C]Tyrosine (53.4 mCi/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Phenylephrine HCl, yohimbine HCl, and collagenase (type 1) were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Heat-inactivated calf serum was from the Nakarai Chemicals Co. Ltd. (Kyoto, Japan). Eagle's Minimum Essential Medium was from the Nissui Seiyaku Co. Ltd. (Tokyo, Japan). The following reagents were donated: clonidine HCl (Boehringer, Ingelheim, F.R.G.), guanfacine HCl (Sandoz, Basel, Switzerland), norfenefrine HCl (Grelan Pharmaceutical Co. Ltd., Tokyo, Japan) and oxymetazoline HCl (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan).

Primary culture of bovine adrenal medullary cells. Fresh bovine adrenal glands from the local slaughterhouse were used throughout. Adrenal medullary cells were isolated by collagenase digestion [5, 12]. The cells were maintained in monolayer culture in Eagle's Minimum Essential Medium containing 10% calf serum, aminobenzylpenicillin (60 μg/ml), streptomycin (100 μg/ml), amphotericin B (0.3 μg/ml) and cytosine arabinoside (3 μmol/l) at

a density of 4×10^6 cells/dish (35 mm, Falcon) (Cookeysville, MD, U.S.A.) [5, 13]. Cells were used for experiments between 3 and 7 days of culture.

Uptake of [^{14}C]*tyrosine and synthesis of* [^{14}C]*-catecholamines from* [^{14}C]*tyrosine in adrenal medullary cells.* Cultured cells (4×10^6) were incubated at 37° for the indicated times in 2.0 ml of KRP buffer containing L-[U-^{14}C]tyrosine (final concentration, 20 μmol/l; 2.35×10^6 dpm) with or without carbachol (0.3 mmol/l) and α-agonists. The reaction was terminated by aspirating the incubation medium, after which the cells were washed four times with 1.0 ml of ice-cold KRP buffer. Then cells were scraped in 5 ml of 0.4 mol/l perchloric acid. After extraction, [^{14}C]catechol compounds were isolated from [^{14}C]tyrosine by aluminum hydroxide adsorption. [^{14}C]Catecholamines were separated into the fraction of [^{14}C]dopamine and [^{14}C]norepinephrine plus [^{14}C]epinephrine by ion exchange chromatography on Duolite C-25 columns (H^+ type, 0.4×7 cm) [14, 15]. [^{14}C]Tyrosine and [^{14}C]catecholamines were counted in toluene base scintillator using a Beckman LS-7000 liquid scintillation counter.

Assay of tyrosine hydroxylase activity in the soluble fraction of cultured adrenal medullary cells. In this study, KRP buffer devoid of bovine serum albumin was employed. Cells were incubated at 37° for 10 min with or without carbachol (0.3 mmol/l) and α_2-agonists. Then the incubation medium was aspirated, and cells were immediately frozen on dry ice and scraped in potassium phosphate buffer (30 mmol/l) (pH 6.8) containing NaF (50 mmol/l) and EDTA (1 mmol/l). The suspension was homogenized, and the homogenate was centrifuged at 20,000 *g* for 10 min. The supernatant fraction was subjected to gel filtration on a Sephadex G-25 column to remove catecholamines and other small molecules as previously reported [16]. Activity of tyrosine hydroxylase was determined by a modification of the decarboxylase-coupled assay [17]. Protein was determined according to the method of Bradford [18] with bovine serum albumin as standard. The specific activity of tyrosine hydroxylase is expressed as nmol of $^{14}CO_2$ formed per mg protein per min.

Uptake of $^{45}Ca^{2+}$ *by cultured adrenal medullary cells.* Cells were incubated at 37° for 1 min with 2.0 μCi of $^{45}CaCl_2$ in 2.0 ml of KRP buffer in the presence or absence of carbachol and clonidine. The reaction was terminated by aspiration of medium, and cells were rapidly washed four times with 1 ml of ice-cold Ca^{2+}-free KRP buffer containing hexamethonium (1 mmol/l) [13]. Then cells were detached and solubilized in 1 ml of Triton X-100 (10%). Radioactivity was counted in a liquid scintillation counter.

Measurement of cyclic AMP. Cells were preincubated with 3-isobutyl-1-methylxanthine (IBMX) (0.3 mmol/l) at 37° for 10 min, and then incubated for another 5 min with or without test compounds in the presence of IBMX (0.3 mmol/l). After aspiration of the medium, cells wer rapidly scraped in ice-cold 7% trichloroacetic acid (TCA) and centrifuged. The TCA in the supernatant fraction was extracted with diethyl ether, and the aqueous extract was allowed to stand for 20 min at 4° with 2.5% $ZnSO_4$ and 75 mmol/l $Ba(OH)_2$. After centrifugation, the supernatant fraction was applied to an ion exchange column (AG 50W × 4, H^+ type). Cyclic AMP was assayed using a cyclic AMP assay kit (Amersham International).

Statistics. All values are expressed as the mean ± SD. Statistical analysis was carried out using Student's *t*-test.

RESULTS

Time courses of [^{14}C]*tyrosine uptake and* [^{14}C]*-catecholamine synthesis in cultured bovine adrenal medullary cells.* [^{14}C]Tyrosine in the incubation medium was rapidly taken up by the cells. After 10 min of incubation, the uptake of [^{14}C]tyrosine reached a plateau that continued for up to 30 min (the duration of the test, Fig. 1a). The uptake of [^{14}C]tyrosine was not affected by α_2- and α_1-agonists employed in this study (data not shown).

The synthesis of [^{14}C]catecholamines (norepinephrine plus epinephrine and dopamine) was almost linear for at least 30 min (Fig. 1b), as previously reported in isolated bovine adrenal medullary cells [5, 15].

Effect of clonidine on synthesis of [^{14}C]*catecholamines and uptake of* $^{45}Ca^{2+}$. Carbachol increased the synthesis of [^{14}C]catecholamines about 2-fold over the basal synthesis of [^{14}C]catecholamines during a 30-min incubation period (Fig. 2a). Spontaneous release of newly synthesized [^{14}C]cat-

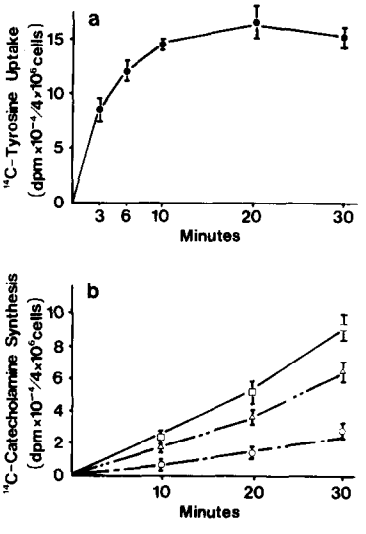

Fig. 1. Time courses of [^{14}C]tyrosine uptake (a) and [^{14}C]catecholamine synthesis (b) in cultured bovine adrenal medullary cells. Cells (4×10^6) were incubated at 37° for the indicated times in 2.0 ml of KRP buffer containing L-[U-^{14}C]tyrosine (20 μmol/l, 2.35×10^6 dpm). (a) [^{14}C]Tyrosine uptake by the cells was measured (see Methods) and expressed as dpm of [^{14}C]tyrosine per 4×10^6 cells. Each point shows the mean of four experiments, with the standard deviations (SD) indicated by the vertical bars. (b) [^{14}C]Catecholamines formed in the cells were measured as described in Methods. Key: (○——○) norepinephrine plus epinephrine, (△ - - - -△) dopamine, and (□——□) total catecholamines. Data are the mean ± SD of four to six experiments.

echolamines into control medium was 0.4%, and carbachol-evoked release was 5.5% of total [^{14}C]catecholamines after 30 min of incubation (data not shown). Clonidine (≦100 μmol/l) had no effect on the basal synthesis of [^{14}C]catecholamines but inhibited carbachol-stimulated synthesis of [^{14}C]-catecholamines in a concentration-dependent manner. However, 1 mmol/l of clonidine reduced the basal synthesis of [^{14}C]catecholamines and abolished the stimulatory effect of carbachol on the synthesis of [^{14}C]catecholamines. Clonidine, at each concentration, inhibited carbachol-stimulated synthesis of [^{14}C]norepinephrine plus [^{14}C]epinephrine, as it inhibited carbachol-stimulated synthesis of [^{14}C]dopamine (data not shown).

Extracellular Ca^{2+} has an important role in the stimulation of catecholamine synthesis caused by cholinergic agonists in adrenal medullary cells [5, 6]. Carbachol caused a rapid uptake of $^{45}Ca^{2+}$ by the adrenal medullary cells, which reached a plateau within 1 min, as reported previously [13]. Therefore, we incubated the cells for 1 min to investigate the effect of clonidine on carbachol-evoked uptake of $^{45}Ca^{2+}$. As shown in Fig. 2b, clonidine inhibited carbachol-induced $^{45}Ca^{2+}$ uptake with a concentration–response curve similar to that of carbachol-stimulated synthesis of [^{14}C]catecholamines. High K^+ (56 mM), which is known to activate voltage-dependent Ca^{2+} channels and to cause uptake of Ca^{2+} [13], also increased the synthesis of [^{14}C]catecholamines. However, this increase in catecholamine synthesis induced by high K^+ was not suppressed by clonidine (100 μmol/l) (data not shown).

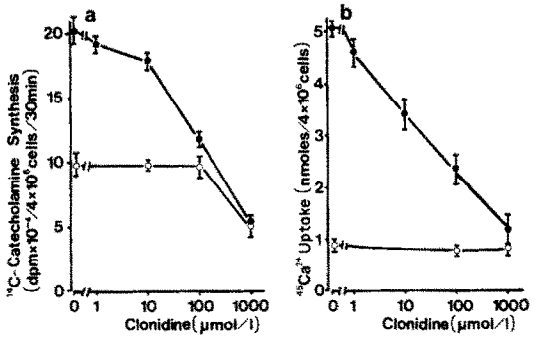

Fig. 2. Concentration–inhibition curves of clonidine for (a) basal and carbachol-stimulated [^{14}C]catecholamine synthesis and (b) $^{45}Ca^{2+}$ uptake. (a) In the presence or absence of various concentrations of clonidine, basal (○——○) and carbachol (0.3 mmol/l)-stimulated (●——●) syntheses of [^{14}C]catecholamines were measured at 37° for 30 min. [^{14}C]Catecholamines are shown as total [^{14}C]catecholamines. Data are the mean ± SD of four to six experiments. (b) Cells were incubated with $^{45}Ca^{2+}$ (2 μCi) at 37° for 1 min in 2.0 ml of KRP buffer in the presence (●——●) or absence (○——○) of carbachol (0.3 mmol/l), and the effects of various concentrations of clonidine on $^{45}Ca^{2+}$ uptake were examined. Ca^{2+} uptake is expressed as nmol per 4 × 10^6 cells, being calculated from its initial specific activity in the incubation medium. Values obtained at 4° were subtracted. Data are the mean ± SD of four experiments.

Effect of clonidine on the level of cyclic AMP in the cells. Quite recently, Pocotte *et al.* [19] reported that carbachol causes an increase in cyclic AMP level in cultured bovine adrenal medullary cells. As shown in Table 1, carbachol (0.3 mmol/l) increased the cyclic AMP level about 2.8-fold. We then examined the effect of clonidine on the level of cyclic AMP in carbachol-stimulated cells. Clonidine (100 μmol/l) had little effect on the basal level but significantly suppressed the carbachol-induced increase in cyclic AMP level.

Effects of α_2- and α_1-agonists on the synthesis of [^{14}C]catecholamines. We compared the effects of α_2- and α_1-agonists on [^{14}C]catecholamine synthesis. Guanfacine (50 μmol/l) and oxymetazoline (50 μmol/l), selective α_2-agonists, had little effect on the basal synthesis of [^{14}C]catecholamines, but they strongly inhibited carbachol-stimulated synthesis of [^{14}C]catecholamines (Fig. 3). On the other hand, norfenefrine (100 μmol/l) and phenylephrine (100 μmol/l), selective α_1-agonists, had no effect on the basal and carbachol-stimulated synthesis of [^{14}C]catecholamines.

Effect of yohimbine on synthesis of [^{14}C]catecholamines. Next we examined whether the inhibitory effect of clonidine on carbachol-stimulated synthesis of [^{14}C]catecholamines was abolished by yohimbine, a selective α_2-adrenergic antagonist. Yohimbine, by itself, however, inhibited the basal and carbachol-stimulated synthesis of [^{14}C]catecholamines and augmented the inhibitory effect of clonidine on carbachol-stimulated synthesis of [^{14}C]catecholamines (Fig. 4). Other α-adrenergic antagonists, phentolamine (30 μmol/l) and phenoxybenzamine (30 μmol/l), also inhibited the carbachol-induced increase in [^{14}C]catecholamine synthesis and did not antagonize the inhibitory effect of clonidine on the synthesis (data not shown).

Effects of α_2-agonists on activity of tyrosine hydroxylase in cultured bovine adrenal medullary cells. Since α_2-agonists inhibited carbachol-stimulated synthesis of [^{14}C]catecholamines in the cells, we measured the activity of tyrosine hydroxylase in the soluble fraction of cells that had been stimulated by carbachol in the presence or absence of α_2-agonists. Carbachol increased the activity of tyrosine

Table 1. Effect of clonidine on the level of cyclic AMP in cultured adrenal medullary cells

	Cyclic AMP (pmol/4 × 10^6 cells)
Control	12.4 ± 1.1 (8)
Clonidine	14.4 ± 0.6 (3)
Carbachol	34.4 ± 3.3 (5)
Carbachol + clonidine	25.0 ± 5.7* (8)

Cells were preincubated with IBMX (0.3 mmol/l) at 37° for 10 min and then incubated for another 5 min with or without carbachol (0.3 mmol/l) and clonidine (100 μmol/l) in the presence of IBMX (0.3 mmol/l). Cyclic AMP in the cells was measured as described in Methods. Data are the mean ± SD, with the number of experiments given in parentheses.

* $P < 0.05$, compared with carbachol alone.

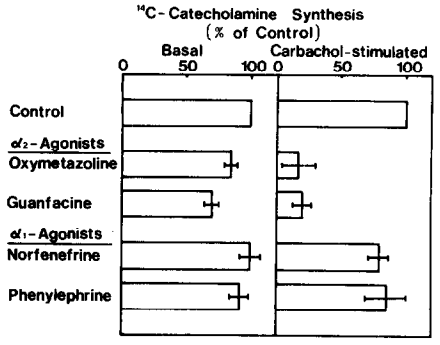

Fig. 3. Effects of α_2- and α_1-agonists on basal and carbachol-stimulated [^{14}C]catecholamine synthesis. Cells were incubated in the same manner as shown in the legend of Fig. 2a in the presence or absence of carbachol (0.3 mmol/l) and α_2-agonists (50 μmol/l) or α_1-agonists (100 μmol/l). [^{14}C]Catecholamine synthesis is expressed as the percent of control (basal [^{14}C]catecholamine synthesis, 95,900 ± 1,800 dpm per 4 × 10^6 cells per 30 min; carbachol-stimulated increase in [^{14}C] catecholamine synthesis, 110,000 ± 5,900 dpm per 4 × 10^6 cells per 30 min) (see Fig. 2a). Data are the mean ± SD of four experiments.

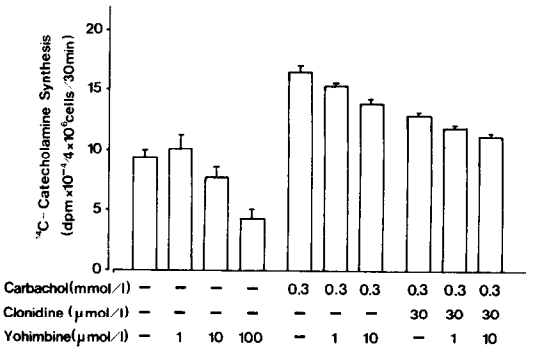

Fig. 4. Effect of yohimbine on synthesis of [^{14}C]catecholamines. Cells were preincubated at 37° for 10 min with or without yohimbine (1.0 to 100 μmol/l) and then incubated at 37° for 30 min in 2.0 ml of KRP buffer containing L-[U-^{14}C]tyrosine in the presence or absence of carbachol (0.3 mmol/l), clonidine (30 μmol/l) and yohimbine (1.0 to 100 μmol/l). Data are the mean ± SD of three to five experiments

hydroxylase about 2-fold over the basal activity of the enzyme (Table 2). Clonidine (100 μmol/l), guanfacine (50 μmol/l) and oxymetazoline (50 μmol/l) significantly inhibited the carbachol-induced increase in tyrosine hydroxylase activity, although these α_2-agonists had no effect on the basal activity of the enzyme.

DISCUSSION

In the present study, we demonstrated that the α_2-agonists, clonidine, guanfacine and oxymetazoline, inhibited carbachol-stimulated synthesis of [^{14}C]-catecholamines, whereas the α_1-agonists, phenylephrine and norfenefrine, had no effect in cultured bovine adenal medullary cells. These results confirm and extend previous findings [9–11] that clonidine reduces the synthesis of catecholamines in central or peripheral noradrenergic neurons *in vivo*. We also observed that clonidine inhibited carbachol-induced uptake of $^{45}Ca^{2+}$ with a concentration–response curve similar to that of carbachol-stimulated [^{14}C]catecholamine synthesis. We previously reported that guanfacine and oxymetazoline, α_2-agonists, inhibit carbachol-evoked $^{45}Ca^{2+}$ uptake by isolated bovine adrenal medullary cells [8]. Since stimulation of catecholamine synthesis induced by cholinergic agonists is dependent on the presence of extracellular Ca^{2+} [5, 6], it appears that α_2-agonists may inhibit carbachol-stimulated synthesis of catecholamines by suppression of Ca^{2+} uptake.

Our observation that α_2-agonists selectively inhibited carbachol-stimulated catecholamine synthesis would seem to suggest that α_2-adrenoceptors [20, 21] are involved in the inhibition of cat-

Table 2. Effects of α_2-agonists on basal and carbachol-stimulated activity of tyrosine hydroxylase in cultured adrenal medullary cells

	Tyrosine hydroxylase activity (nmol $^{14}CO_2$ formed/mg protein/min)	
	Basal	Carbachol
Control	0.425 ± 0.041 (8)	0.863 ± 0.068 (6)
Clonidine	0.394 ± 0.040 (6)	0.566 ± 0.069* (6)
Guanfacine	0.424 ± 0.005 (3)	0.434 ± 0.024* (4)
Oxymetazoline	0.427 ± 0.001 (3)	0.469 ± 0.023* (4)

Cells were incubated at 37° for 10 min with or without clonidine (100 μmol/l), guanfacine (50 μmol/l) or oxymetazoline (50 μmol/l) in the presence or absence of carbachol (0.3 mmol/l). Tyrosine hydroxylase was obtained from cultured cells, and its activity was assayed *in vitro* (see Methods). The incubation mixture (final volume 100 μl) contained 10 μmol potassium phosphate buffer (pH 6.8), 0.5 μmol ascorbate, 0.5 μmol EDTA, 6500 units catalase (Boehringer Mannheim), 0.01 μmol 6MePtH$_4$ (Calbiochem-Behring), 0.01 μmol L-[1-^{14}C]tyrosine (0.1 μCi) and enzyme. The reaction was allowed to proceed at 30° for 8 min. Data are the mean ± SD, with the number of experiments given in parentheses.

* $P < 0.05$, compared with carbachol alone.

echolamine synthesis. To assure that α_2-agonists inhibit the synthesis of [^{14}C]catecholamines via activation of α_2-adrenoceptors, we examined whether yohimbine, a selective α_2-antagonist, reversed the inhibitory effect of clonidine. In cultured bovine adrenal medullary cells, however, by itself, yohimbine had an inhibitory effect on the basal and carbachol-stimulated synthesis of [^{14}C]catecholamines and did not antagonize the inhibitory effect of clonidine. Since recent reports have shown that yohimbine exhibits diverse properties which are not related to those of α_2-antagonists [22, 23], we examined the effects of other α-antagonists on the clonidine-induced inhibition of catecholamine synthesis. Phenoxybenzamine and phentoalamine also inhibited the carbachol-produced increase in [^{14}C]catecholamine synthesis and failed to reverse the inhibitory effect of clonidine. These results seem to suggest that α_2-adrenoceptors may not mediate the inhibitory effects of α_2-agonists on catecholamine synthesis. Since clonidine did not inhibit a high K^+-evoked increase in catecholamine synthesis, it may be that clondine and other α_2-agonists act as cholinergic antagonists in cultured bovine adrenal medullary cells.

Quite recently, Powis and Baker [24] observed that clonidine inhibits carbachol-evoked catecholamine secretion and that the inhibitory effect of clonidine is not antagonized by phentolamine and phenoxybenzamine, nor by yohimbine, in isolated bovine adrenal medullary cells. They considered that the inhibitory effect of clonidine on catecholamine secretion was not due to activation of α_2-adrenoceptors. However, our previous experiments [7] clearly demonstrated that in membrane fractions prepared from bovine adrenal medullary cells there exists a saturable, reversible and high affinity binding of [^{3}H]clonidine. The binding of [^{3}H]clonidine to membrane fractions was inhibited by the α-antagonists yohimbine and phentolamine (unpublished observations). These results suggest that yohimbine and phentolamine share common binding sites with clonidine. Our observations may be consistent with the presence of α_2-adrenoceptors. Further, all α-antagonists could not be evaluated equally as α_2-antagonists to be used, because, by themselves, they inhibited carbachol-stimulated synthesis of catecholamines. Further experiments will be required to determine whether α_2-adrenoceptors function in adrenal medullary cells.

It has been well documented that stimulation of adrenal medullary cells results in an increase in catecholamine synthesis [25] that is associated with an activation of tyrosine hydroxylase, an enzyme catalyzing the rate-limiting step in the synthesis of catecholamines [26–28]. Recent *in vitro* studies have shown that tyrosine hydroxylase is activated and phosphorylated by several Ca^{2+}-dependent protein kinases, Ca^{2+}/calmodulin-dependent protein kinase [29, 30] and Ca^{2+}/phospholipid-dependent protein kinase [31, 32]. Moreover, in bovine adrenal medullary cells [6, 19] and rat pheochromocytoma PC12 cells [16, 33, 34] both the *in situ* phosphorylation and activation of tyrosine hydroxylase are stimulated by acetylcholine or depolarizing agents that are dependent on the presence of extracellular Ca^{2+}. Mestikawy *et al.* [35] reported that tyrosine hydroxylase activation in depolarized central dopaminergic terminals involves a Ca^{2+}-dependent phosphorylation process. These investigations suggest that tyrosine hydroxylase activation is triggered by the uptake of Ca^{2+}, a prerequisite for the activation of Ca^{2+}-dependent protein kinase(s). In our experiments, the increase in activity of tyrosine hydroxylase caused by carbachol was observed even after separation of the enzyme on a Sephadex G-25 column, suggesting that tyrosine hydroxylase was activated continuously, probably by an increase in phosphorylation of the enzyme. Since α_2-agonists suppressed carbachol-evoked activation of tyrosine hydroxylase, it seems that α_2-agonists may inhibit carbachol-produced activation of tyrosine hydroxylase by interfering with Ca^{2+} uptake which eventually may lead to an inhibition of the enzyme phosphorylation.

On the other hand, there have been many reports suggesting that cyclic AMP also plays an important role in the regulation of catecholamine synthesis. Tyrosine hydroxylase is activated and phosphorylated by cyclic AMP dependent protein kinase *in vitro* [36–39] and by cyclic AMP derivatives *in situ* [40–43]. Since clonidine and other imidazolines have been reported to inhibit the norepinephrine-elicited accumulation of cyclic AMP in brain slices [44], it seems that a change of cyclic AMP level may be involved in the clonidine-induced inhibition of tyrosine hydroxylase activity. In our experiments, clonidine suppressed the carbachol-induced increase in the level of cyclic AMP in adrenal medullary cells. These results suggest that the inhibitory effects of α_2-agonists on tyrosine hydroxylase activity may also be mediated by the decrease in cyclic AMP level. Pocotte *et al.* [19] reported that the carbachol-induced rise in cyclic AMP is dependent on extracellular Ca^{2+}. They suggested that the rise in cyclic AMP evoked by carbachol results from Ca^{2+}-dependent stimulation of adenylate cyclase caused by the rise in intracellular Ca^{2+}. In our experiments, it seems that the inhibitory effect of clonidine on the level of cyclic AMP may be mediated by the decrease in Ca^{2+} uptake.

In conclusion, our observations suggest that α_2-agonists but not α_1-agonists inhibit carbachol-stimulated synthesis of catecholamines by inhibition of tyrosine hydroxylase activation and that it is a probable consequence of α_2-agonist-induced decrease in Ca^{2+} uptake in cultured bovine adrenal medullary cells. Our results may be helpful in understanding the pharmacological actions of therapeutically employed α_2-agonists.

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