Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 77-83

# INHIBITORY EFFECT OF OKADAIC ACID ON CARBACHOL-EVOKED SECRETION OF CATECHOLAMINES IN CULTURED BOVINE ADRENAL MEDULLARY CELLS

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Received November 20, 1990

**Summary** We examined the effect of okadaic acid on catecholamine secretion caused by carbachol in cultured bovine adrenal medullary cells. Treatment of cells with 100 nM okadaic acid for 3-24 hr produced an inhibition of catecholamine secretion stimulated by carbachol. The half-maximal and maximal inhibition of secretion was observed at 40 nM and 300 nM okadaic acid for 24 hr, respectively. Okadaic acid also inhibited veratridine- and high $K^+$-induced secretion but not ionomycin-induced secretion. Okadaic acid strongly suppressed $^{45}Ca^{2+}$ influx and slightly inhibited $^{22}Na^+$ influx in carbachol-stimulated cells. These results suggest that okadaic acid inhibits carbachol-evoked secretion of catecholamines mainly by suppression of $Ca^{2+}$ influx in adrenal medullary cells. 

Okadaic acid isolated from a black sponge, Halichondria okadai, is a polyether derivative of a $C_{38}$ fatty acid (1,2), and inhibits protein phosphatase 1 and 2A in vitro (2,3). In intact cells, it produces a great increase in phosphorylation of several proteins, i.e., myosin light chain (4), acetyl CoA carboxylase (5) and tyrosine hydroxylase (6), and modulates a variety of cellular functions, i.e., smooth muscle contraction (7), fatty acid biosynthesis (5) and catecholamine synthesis (6).

Adrenal medullary cells are paraneurons of neural crest origin. Previously we reported that stimulation of nicotinic acetylcholine receptors in adrenal medullary cells by carbachol

Abbreviations used: DFA, dimethylformamide; KRP, Krebs-Ringer phosphate.

0006-291X/91 $1.50

evokes $Ca^{2+}$ influx by increasing $Na^{+}$ influx and the subsequent increase in cellular $Ca^{2+}$ triggers catecholamine secretion (8). On the other hand, enhanced phosphorylation of several proteins are reported to accompany catecholamine secretion in the cells stimulated by various secretagogues such as acetylcholine or nicotine (9,10). Phorbol esters (11) and forskolin (12) enhanced the secretion of catecholamines by stimulating protein kinase C and cyclic AMP-dependent protein kinase, suggesting that protein kinases participate in the regulation of catecholamine secretion.

Little is known, however, about a role of protein phosphatases in the process of stimulus-secretion coupling. In the present study, we examined the effect of okadaic acid on catecholamine secretion in cultured bovine adrenal medullary cells. We report here the inhibitory effect of long treatment with okadaic acid on carbachol-evoked secretion of catecholamines.

**Materials and Methods**

Bovine adrenal medullary cells were isolated, and maintained in monolayer culture ($4x10^6$ cells/dish, Falcon 35 mm) in Eagle's minimum essential medium containing 10% calf serum and antibiotics (13,14). Oxygenated Krebs-Ringer phosphate (KRP) buffer was used throughout. It had the following composition (mM) : NaCl 154, KCl 5.6, $MgSO_4$ 1.1, $CaCl_2$, $NaH_2PO_4$ 0.85, $Na_2HPO_4$ 2.15, and glucose 10, adjusted to pH 7.4.

Catecholamine secretion was examined as reported previously (14). Briefly, after treatment of cells with okadaic acid, cultured cells were washed with KRP buffer and incubated at 37° C for 5 min with or without carbachol and other secretagogues in 2 ml of KRP buffer. Catecholamines secreted into the medium were adsorbed to aluminum hydroxide and estimated by the ethylenediamine condensation method (15) using a fluorescence spectrometer (Hitachi Model 650-10S) with an excitation wave length of 420 nm and an emission of 540 nm.

Influx of $^{22}Na^{+}$ and $^{45}Ca^{2+}$ was measured as reported previously (8). Cells were incubated with 1.5 μCi of $^{22}NaCl$ or $^{45}CaCl_2$ at 37° C for 5 min in 1 ml of KRP buffer in the presence or absence of carbachol. $^{22}Na^{+}$ and $^{45}Ca^{2+}$ in the cells were counted by a Beckman LS-7000 liquid scintillation counter.

Chemicals were obtained from the following sources: okadaic acid, Moana BioProducts (USA); carbachol, veratridine and collagenase, Sigma (USA); ionomycin, Calbiochem (USA); $^{45}CaCl_2$ (0.5-2.0 Ci/mmol), Amersham International (UK); $^{22}NaCl$ (6-17 Ci/mmol), New England Nuclear (USA); Eagle's minimum essential medium, calf serum and other chemicals, Nacalai Tesque (Japan). Okadaic acid was dissolved in 100% dimethylformamide (DFA). Cells were treated with 0.5% dimethylformamide (DFA)(control) or okadaic acid in 0.5% DFA in culture.

## Results and Discussion

Okadaic acid (100 nM) produced an inhibition of catecholamine secretion evoked by carbachol in a time-dependent manner (Fig. 1). The significant inhibition of secretion by okadaic acid was detectable after treatment for 3 hr ($p<0.05$, compared to 0 hr) and the maximal inhibition was after treatment for 12-24 hr. Catecholamine content in cells treated with 100 nM okadaic acid for 24 hr was slightly increased (65.3 ± 3.9 μg in 0.5% DFA-treated cells; 72.3 ± 4.1 μg in cells treated with 100 nM okadaic acid). Treatment of cells with okadaic acid for 24 hr caused a concentration-dependent inhibition of catecholamine secretion (Fig. 2). The half-maximal and maximal inhibition was observed at 40 nM and 300 nM okadaic acid, respectively. Basal secretion

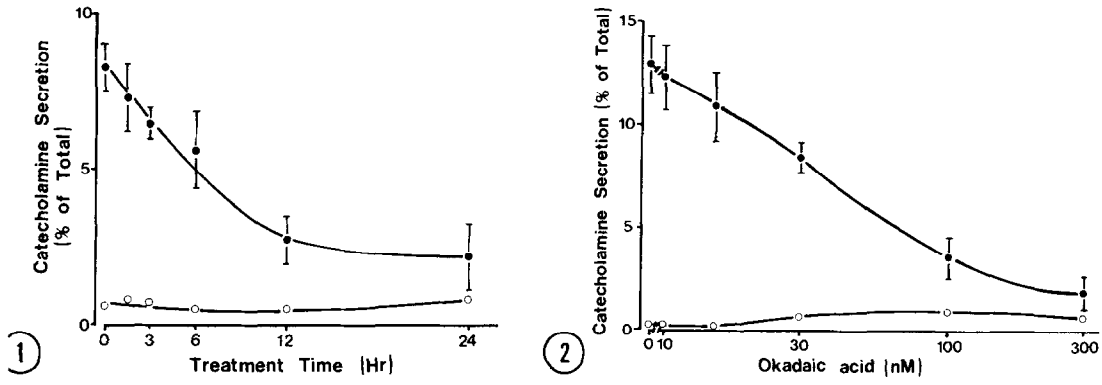


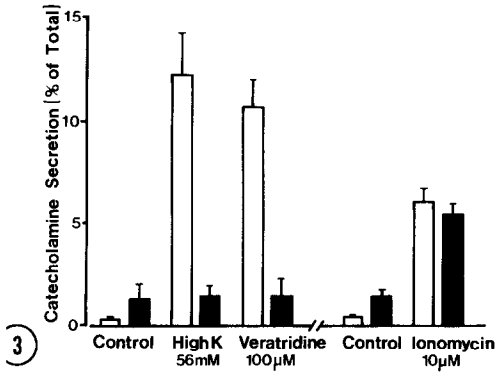

**Figure 1. Time course of okadaic acid-induced inhibition of catecholamine secretion caused by carbachol.** Cultured cells (4 x $10^6$) were treated with 0.5% DFA or 100 nM okadaic acid in 0.5% DFA for the period indicated. After treatment, cells were washed 3 times with KRP buffer and incubated at 37° C for 5 min in 2 ml of KRP buffer in the presence ( ● ) or absence ( ○ ) of 0.3 mM carbachol. Catecholamines secreted into the medium were measured as described in Materials and Methods and expressed as % of total catecholamines. Values obtained at 4° C were subtracted. Data are the mean of 4 experiments and the standard deviations are expressed by the vertical bars. Statistical analysis was carried out using Student's *t* test.

**Figure 2. Concentration-response curve for inhibition of carbachol-evoked secretion of catecholamines caused by okadaic acid.** Cells were treated for 24 hr with various concentrations of okadaic acid (0-300 nM) in 0.5% DFA. After treatment, cells were incubated at 37° C for 5 min in the presence ( ● ) or absence ( ○ ) of 0.3 mM carbachol. Catecholamines secreted were measured as described in Fig. 1. Data are means ± SD of 3-6 experiments.

was slightly increased at high concentrations of okadaic acid (30-300 nM).

In cultured bovine adrenal medullary cells, several investigators have demonstrated that the cells have at least three distinct types of ionic channels: (i) nicotinic acetylcholine receptor-associated ion channels responsible for carbachol-induced $Na^+$ influx (8,16), (ii) voltage-dependent $Na^+$ channels responsible for veratridine-induced $Na^+$ influx (8,16) and (iii) voltage-dependent $Ca^{2+}$ channels (8,17) which is activated by high $K^+$. Our previous study suggests that either carbachol-induced $Na^+$ influx or veratridine-induced $Na^+$ influx contributes to $Ca^{2+}$ influx via voltage-dependent $Ca^{2+}$ channels whereas high $K^+$ gates voltage-dependent $Ca^{2+}$ channels without $Na^+$ influx (8). Calcium ionophore, ionomycin, is known to directly evoke $Ca^{2+}$ influx and catecholamine secretion (18) via bypassing $Ca^{2+}$ or $Na^+$ channels. We examined the effect of okadaic acid on catecholamine secretion stimulated by these secretagogues. As shown in Fig. 3, okadaic acid (100 nM, 24 hr) inhibited the stimulatory effects of veratridine and high $K^+$ (56 mM) but not of ionomycin on catecholamine secretion. These results suggest that in carbachol-stimulated cells okadaic acid may act on acetylcholine receptors or ionic channels in plasma membrane rather than on intracellular action site of $Ca^{2+}$.

To investigate the inhibitory site of okadaic acid in carbachol-evoked catecholamine secretion, we measured the influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ caused by carbachol. Okadaic acid (100 nM, 24 hr) strongly inhibited carbachol-evoked $^{45}Ca^{2+}$ influx and slightly $^{22}Na^+$ influx (Fig. 4A and B). These results suggest that okadaic acid inhibits carbachol-evoked secretion mainly via suppression of $Ca^{2+}$ influx. The mechanism of okadaic acid-induced inhibition of $Ca^{2+}$ influx is, at present, not clear. It is unlikely that the inhibitory effect of okadaic acid is due to



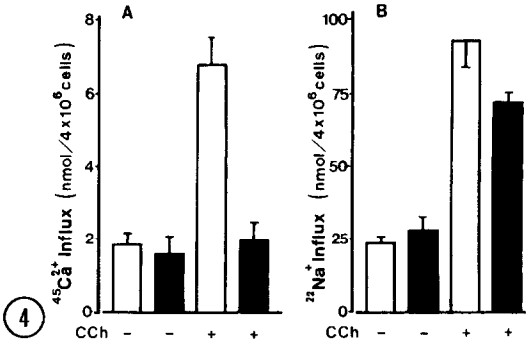




**Figure 3. Effect of okadaic acid on various secretagogues-induced secretion of catecholamines.** Cells were treated with 0.5% DFA (□) or 100 nM okadaic acid (■) in 0.5% DFA for 24 hr. Cells were then incubated with or without 0.3 mM carbachol, 0.1 mM veratridine and high $K^+$ (56 mM) at 37° C for 5 min or 10 μM calcium ionophore ionomycin for 10 min. Catecholamines secreted were measured. Data are means ± SD of 4 experiments.

**Figure 4. Effect of okadaic acid on carbachol-evoked influx of $^{45}Ca^{2+}$(A) and $^{22}Na^{+}$(B) to cells.** Cells were treated with 0.5% DFA (□) or 100 nM okadaic acid (■) for 24 hr. Cells were incubated at 37° C for 5 min in 1 ml of KRP buffer containing 1.5 μCi of $^{22}NaCl$ (3.3 x $10^6$ dpm, 4-6 Ci/mmol) or 1.5 μCi of $^{45}CaCl_2$ (3.3 x $10^6$ dpm, 0.5-2 Ci/mmol) in the presence or absence of 0.3 mM carbachol (CCh). Influx of $Na^+$ and $Ca^{2+}$ was measured as described in Materials and Methods and was expressed in nmol/4 x $10^6$ cells, being calculated from the initial specific radioactivity of $^{22}Na^+$ and $^{45}Ca^{2+}$ in the medium. Values obtained at 4° C were subtracted. Data are means ± SD of 4-6 experiments.

its cytotoxicity, because (i) okadaic acid inhibited carbachol-evoked $^{45}Ca^{2+}$ influx in preference to $^{22}Na^+$ influx, (ii) it selectively suppressed carbachol-, veratridine- and high $K^+$-induced secretion but not ionomycin-induced secretion and (iii) it had a slightly stimulatory effect on catecholamine content. Recently, dihydropyridine-sensitive $Ca^{2+}$ channels from rabbit skeletal muscle are reported to be phosphorylated by cyclic AMP-dependent protein kinase in vitro (19). Okadaic acid is demonstrated to increase the $Ca^{2+}$ current probably by phosphorylation of slow inward $Ca^{2+}$ channels in isolated guinea-pig cardiac myocytes (20). These data and the present results give a possibility that long treatment of adrenal medullary cells with okadaic acid modulates the function of $Ca^{2+}$ channels or may produce a loss of $Ca^{2+}$ channels via prolonged phosphorylation of

the channels. Recent study (21) reported the presence of N- and L-type of voltage-sensitive $Ca^{2+}$ channels by measuring the binding sites for $^{125}$I-$\omega$-conotoxin GVIA and [$^{3}$H]-nitrendipine to plasma membranes from bovine adrenal medullary cells. We are currently studying whether okadaic acid changes these binding sites in the cells. The present results also suggest that protein phosphatases may play an important role in the regulation of stimulus-secretion coupling.

**Acknowledgment**

The authors would like to thank Mr. Katsuhiko Furumi for experimental assistance and helpful discussion.

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