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Authors: Fujino, Yukio, and Fujii, Tomoko

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Hypoglycemia-induced Catecholamine Release from Adrenals in 21-day-old Rats is Blocked by Hexamethonium Pretreatment

Yukio Fujino* and Tomoko Fujii

Department of Pharmacology, Teikyo University School of Medicine, 2-11-1, Kaga, Itabashi-Ku, Tokyo 173-8605, Japan

ABSTRACT—Acetylcholine, released from cholinergic nerve terminals, innervated to adrenal chromaffin cells, evokes catecholamine release from the chromaffin cells. In the previous studies neostigmine, an acetylcholinesterase inhibitor, nicotine and oxotremorine, a muscarinic receptor agonist, hardly induce catecholamine release from adrenal medulla of 21-day-old rats. Not only adrenaline but also noradrenaline is released from the chromaffin cells by insulin-induced hypoglycemia in fasted 21-day-old rats, whereas preferential adrenaline release occurs in fasted 8-week-old rats. The purpose of the present study was to characterize the catecholamine output induced by hypoglycemia in 21-day-old rats. Hexamethonium, a nicotinic receptor antagonist, blocked the adrenaline and noradrenaline release from the chromaffin cells almost completely as judged from measuring the catecholamine content and observing the morphological changes of the chromaffin cells. Nicotine or oxotremorine, injected into the fasted animals, induced catecholamine release. In the multiple steps of the chromaffin granule exocytotic pathway one or several steps are probably inactive in infant rat and these steps become to be fully active by starvation. The nicotinic receptors of the chromaffin cells in infant rats mainly contribute the secretion induced by hypoglycemia. It has been postulated that the nicotinic receptors are primarily concentrated in the synaptic zones of the chromaffin cell membrane and are involved in the physiological stimulation, whereas the extrasynaptic regions contain a mixture of the nicotinic and muscarinic receptors, these are activated by injected secretogogues.

INTRODUCTION

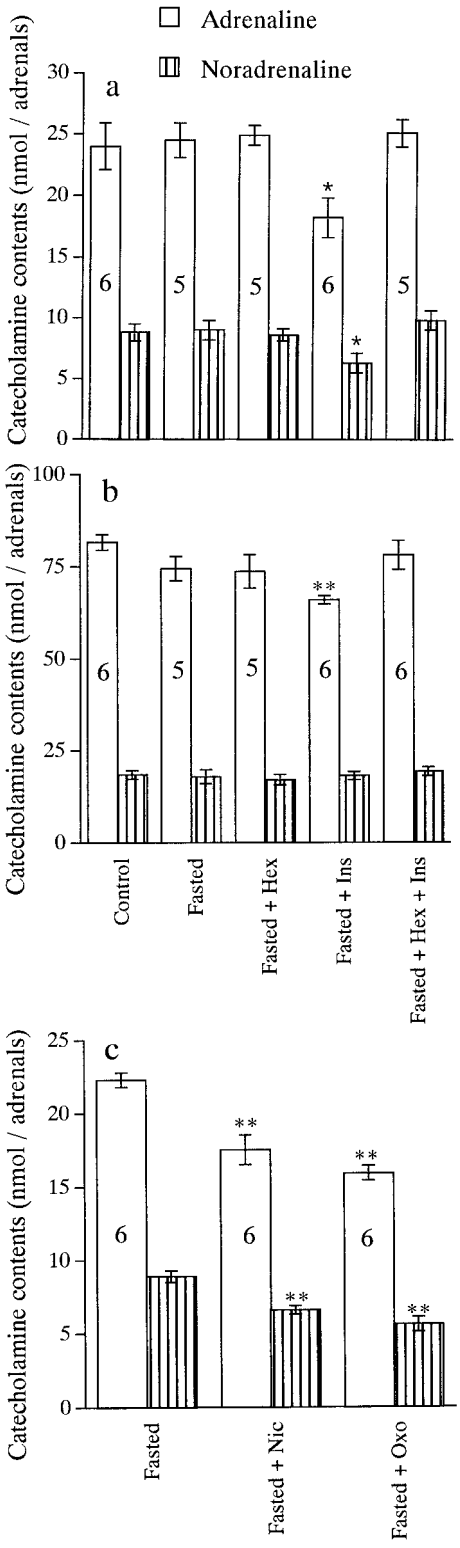
Acetylcholine, released from cholinergic nerve terminals, innervated to adrenal chromaffin cells, evokes catecholamine release from the chromaffin cells. In rats, both muscarinic and nicotinic acetylcholine receptors contribute catecholamine release from the adrenal chromaffin cells in the separate mechanisms (Wakade and Wakade, 1983; Akaike *et al*., 1990; Zhou *et al*., 1991; Chowdhurry *et al*., 1994).

The function of the adrenal chromaffin cells and the cholinergic nerves has been postulated to become mature in 10-day-old rats (Slotkin *et al*., 1980, 1982). In the previous studies we demonstrated that acetylcholine could not exert catecholamine release from adrenal slices prepared from 21-day-old rats (Fujino and Fujii, 1997). In *in vivo* studies neostigmine, a blood-brain barrier impermeable (Maayani *et al*., 1978) acetylcholinesterase inhibitor, nicotine and oxotremorine, a non-selective muscarinic agonist, hardly induced catecholamine release from the adrenals of 21-day-old rats (Fujino and Fujii, 1994, 1997). Not only adrenaline but also noradrenaline is released from the chromaffin cells by insulin-induced hypoglycemia in fasted 21-day-old rats, whereas preferential adrenaline release occurs in fasted 8-week-old rats (Fujino and Fujii, 1995). Insulin-induced hypoglycemia stimulates preferential adrenaline release from adrenal medulla in various animals including rats (Cannon *et al*., 1924; Dunner, 1953; Coupland, 1958; Gagner *et al*., 1985; Vollmer *et al*., 1992). Ω-shaped profiles resulted from acetylcholine release are observed in the cell membrane of the synapse buttons innervated to the chromaffin cells (Fujino and Fujii, 1995). These indicate that the chromaffin cells and the cholinergic nerves innervating the chromaffin cells are at least functionally active in 21-day-old rats, although the responsiveness to several secretogogue stimulation and hypoglycemia is somewhat different from that in young adult rats.

In 8-day-old rats, catecholamine release from the adrenals evoked by hypoxia is almost completely blocked by pre-treatment with nicotinic antagonist (Seidler and Slotkin, 1985). It may be probable that the catecholamine release induced by hypoglycemia in 21-day-old rats is also inhibited by the treatment. In the present study we examined the effect of hexamethonium, a nicotinic receptor blocker, on the release induced by hypoglycemia in 21-day-old and 8-week-old rats. In addi-

* Present address of corresponding author: Kitashinagawa 5-14-12, Shinagawa-ku, Tokyo 141-0001, Japan; Tel./FAX. 03-3442-1962

tion nicotine or oxotremorine was administered to 21-day-old fasted rats. The doses and durations of these secretogogue treatments are sufficient to induce catecholamine release from the adrenal medulla of 8-week-old rats (Fujino and Fujii, 1994, 1997). Catecholamine release from the adrenal chromaffin cells was detected by determination of the catecholamine contents in adrenals. In 21-day-old rats morphological changes of the chromaffin cells, resulted from the release, such as a decrease in the number of chromaffin granules and an increase in the number of vacuoles produced by membrane recycling resulting from exocytosis (Heuser and Reese, 1973; Nordmann *et al.*, 1974), were observed simultaneously by electron microscopy. Noradrenaline-storing chromaffin cells were distinguished from adrenaline-storing cells based on the ultrastructural features of their granules (Coupland *et al.*, 1964).



MATERIALS AND METHODS

Determination of the catecholamine contents in adrenals and serum glucose levels

Male Wistar-Imamichi 21-day-old and 8-week-old rats (The Institute for Animal Reproduction, Ibaraki, Japan) were used after 1 week housing in our animal room for adaptation. The animals were fasted from 4:00 pm to the next morning 10:00 am; Then, 30 mg/kg of hexamethonium chloride, dissolved in saline, or saline was subcutaneously injected into rats. Three U/kg of insulin or saline was subcutaneously injected 5 min after the first injection. Rats were killed by decapitation 30 min after insulin administration. In some experiments 5 mg/kg nicotine or 3 mg/kg oxotremorine, dissolved in saline, was injected subcutaneously into rats and the animals were killed 2 or 5 min after the injection, respectively. Adrenals were dissected immediately from the animals and homogenized with a Teflon pestle homogenizer in the medium composed of 0.3 M sucrose, 10 mM 2-(N-morpholino) ethane sulfonic acid (MES) buffer (pH 5.9), 2 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (PMSF). Catecholamine concentration in the homogenate was analyzed by HPLC as reported previously (Fujino *et al.*, 1994). Serum glucose levels were determined using a commercially available kit (F-kit glucose; Boehringer Mannheim, Germany).

Electron microscopy

Adrenals were dissected rapidly from the animals after decapitation and were cut into two pieces with two razor blades. These were pre-fixed by microwave irradiation by the method of Mizuhira *et al.* (1990) as reported previously (Fujino and Fujii, 1997). These segments were immersed in the fixative for an additional 2 hr at room temperature. Adrenal medulla was isolated with razor blades during this period. These were washed three times with 0.1 M phosphate buffer, pH 7.2, for 10 min. The samples were post-fixed by immersion with 1% O_sO_4 in the phosphate buffer for 1 hr at room temperature, and were washed three times with the buffer for 10 min. These were dehydrated with ascending concentrations of ethanol and n-butyl glycidyl ether (QY-1) and embedded in Quetol 812. Ultrathin sections of 60–90 nm thick were cut, mounted on copper grids. These were stained by 2% uranyl acetate for 7 min and 2.7% lead citrate for 4 min.

Fig. 1. Catecholamine release from adrenal medulla in fasted 21-day-old and 8-week-old rats. (a) Effect of hexamethonium on the release induced by hypoglycemia in 21-day-old fasted rats. Five min after subcutaneous injection of 30 mg/kg of hexamethonium or saline, 3 U/kg of insulin or saline was subcutaneously injected. Rats were killed by decapitation 30 min after insulin or saline administration. (b) Effect of hexamethonium on the release induced by hypoglycemia in 8-week-old rats. The animals were treated as described in (a). (c) Effect of nicotine and oxotremorine on the release in fasted 21-day-old rats. Two or five min after subcutaneous injection of 5 mg/kg of nicotine or 3 mg/kg of oxotremorine, respectively, rats were killed by decapitation. Hex: hexamethonium, Ins: insulin, Nic: nicotine, Oxo: oxotremorine. Bars represent means ± S.E. for number of animals indicated. * $p < 0.05$ and ** $p < 0.005$ vs. control.

Chemicals

EGTA, hexamethonium chloride, MES, nicotine, oxotremorine, PMSF and tannic acid were obtained from Sigma Chem. Co., Missouri, U.S.A. Insulin was the product of Novo Nordics, Denmark. Glutaraldehyde, paraformaldehyde and O_sO_4 were from TAAB Laboratories Equipment Ltd., Berks, England. Quetol 812 and QY-1 were from Nisshin-EM Co. Ltd., Tokyo, Japan. Other chemicals were all analytical or electron microscope grade.

Statistical analyses

Data are expressed as mean ±S.E. To define statistically significant differences in the catecholamine content of adrenals between control and experimental groups, the data were subjected to Student's t-test.

RESULTS

Thirty mg/ kg of hexamethonium was injected to 21-day-old rats. The behavioral activity in the groups received hexamethonium seemed to become almost completely disappeared within 30 sec after the injection. This indicates that the effect of hexamethonium on these animals appeared at least before insulin injection. Three mg/kg of insulin was administered 5 min after hexamethonium treatment. The activity in the group administered insulin only did not seem to become low. Serum glucose concentration in control and fasted rats was 1.10± 0.04 g/l and 0.84±0.03 g/l, respectively ($p<0.005$). As shown in Fig. 1a, the contents of adrenaline and noradrenaline did not change in fasted and hexamethonium-received fasted rats. The contents decreased statistically significantly in the insulin-treated animals. This decrease was almost completely blocked by pre-treatment of hexamethonium (Fig. 1a).

As assessed by electron microscopy, a decrease in the number of chromaffin granules was observed in the adrenaline-storing chromaffin cells of insulin-treated rats (Fig. 2a, b).

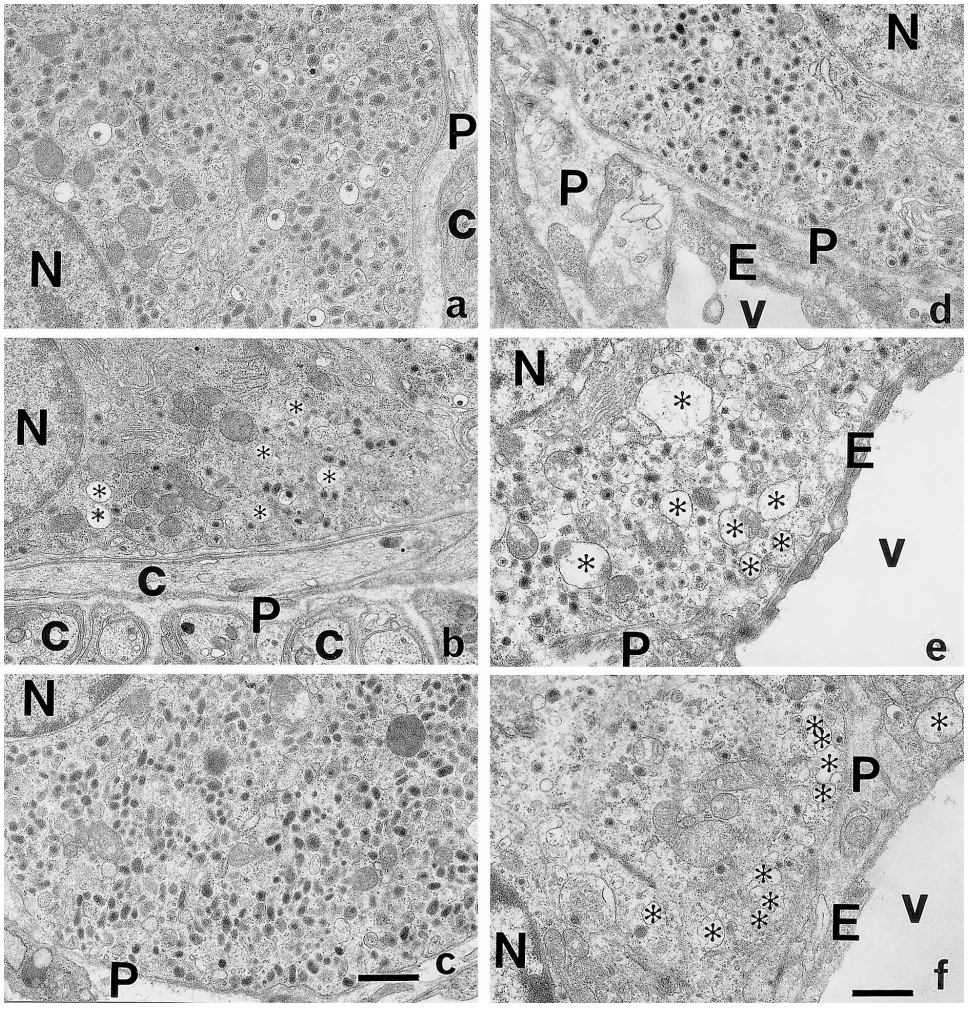


Fig. 2. Electron micrographs of adrenaline-storing chromaffin cells in 21-day-old rats. Panel a: control, Panel b: 30 min after 3 U/kg insulin subcutaneous injection, Panel c: 5 min before insulin injection 30 mg/kg hexamethonium was subcutaneously injected, Panel d: control (fasted), Panel e: 2 min after 5 mg/kg nicotine subcutaneous injection, Panel f: 5 min after 3 mg/kg oxotremorine subcutaneous injection. C: pre-synaptic cholinergic nerve fiber, E: endothelial cell, N: nucleus, P: perivascular space, v: capillary. Note that the number of chromaffin granules is diminished significantly by insulin injection (b) and that the decrease is almost completely blocked by hexamethonium pre-treatment (c). The number is also decreased markedly by nicotine (e) and oxotremorine (f) treatment in the fasted animals. Asterisks in b, e and f indicate vacuoles probably produced by membrane retrieval after exocytosis. Bars in c and f indicate 1 μm.

The number of vacuoles increased in these cells (Asterisks in Fig. 2b). The decrease in the number of chromaffin granules and the increase in the number of vacuoles were almost completely blocked by hexamethonium pre-treatment (Fig. 2c). In the other groups shown in Fig. 1a, any morphological change, resulted from catecholamine output, in the adrenaline-storing cells was hardly found as far as examined (Data not shown).

Although the decrease in the number of chromaffin granules was also found in the noradrenaline-storing chromaffin cells of insulin-received rats, the increase in the number of vacuoles was not observed as far as examined (Fig. 3a, b). About one-third of the cells of insulin-treated rats contained a reduced number of the granules, as far as examined in 3 animals. The decrease was also blocked by hexamethonium pre-treatment (Fig. 3c). In the other groups shown in Fig. 1a, any morphological change, resulted from catecholamine output, in the noradrenaline-storing cells was hardly found as far as examined (Data not shown).

Fig. 1b indicates the effect of hypoglycemia on the catecholamine contents in 8-week-old rats. The behavioral activity in the all groups received hexamethonium seemed to become severely blocked, while the activity in the group administered insulin only became somewhat low. Convulsion was not evoked in the all animals. The adrenaline content in the adrenals tended to decrease in the fasted group. The adrenaline content was statistically significantly decreased by insulin-treatment, whereas the noradrenaline content did not change. This decrease was almost completely blocked by hexamethonium pre-treatment.

The fasted 21-day-old rats were injected with saline or

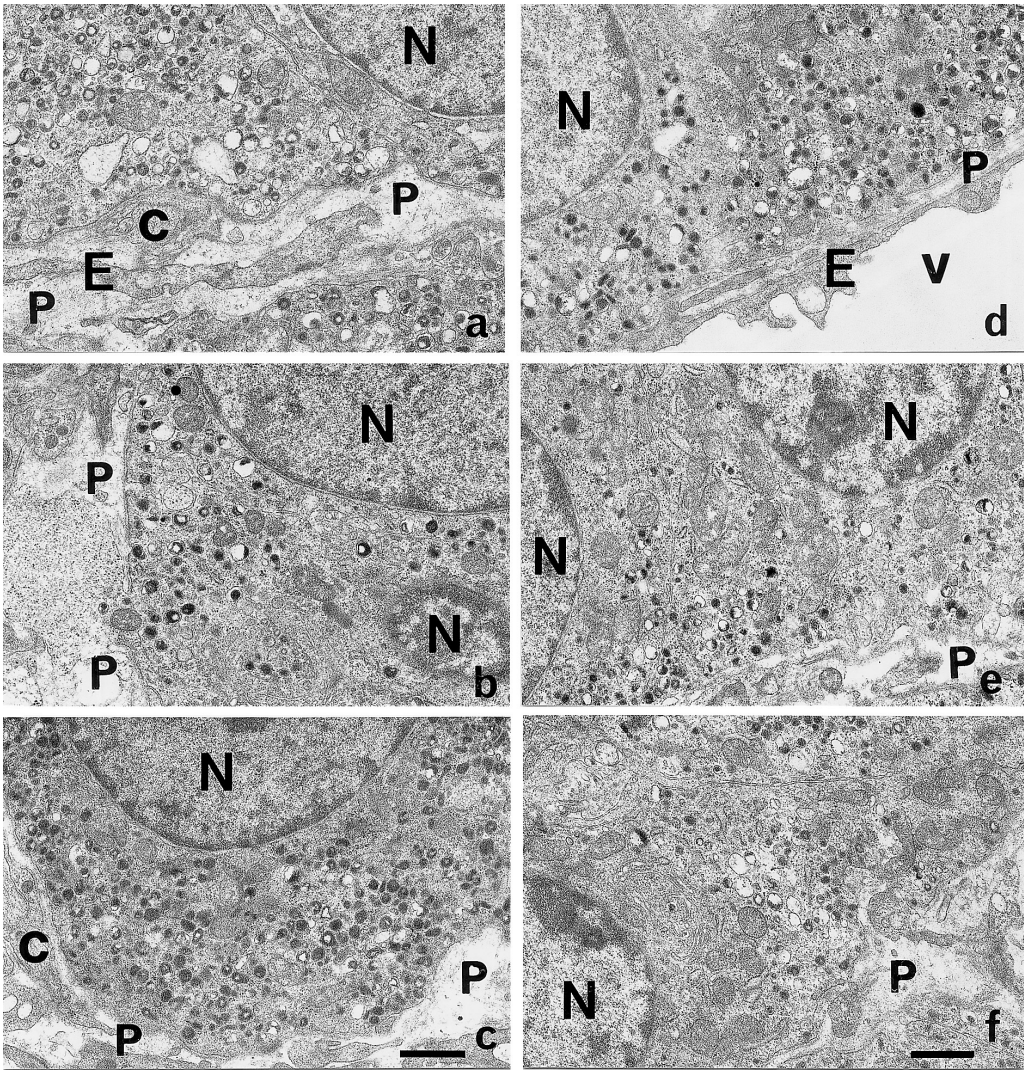


Fig. 3. Electron micrographs of noradrenaline-storing chromaffin cells in 21-day-old rats. Panel a: control, Panel b: 30 min after 3 U/kg insulin subcutaneous injection, Panel c: 5 min before insulin injection 30 mg/kg hexamethonium was subcutaneously injected, Panel d: control (fasted), Panel e: 2 min after 5 mg/kg nicotine subcutaneous injection, Panel f: 5 min after 3 mg/kg oxotremorine subcutaneous injection.. C: pre-synaptic cholinergic nerve fiber, E: endothelial cell, N: nucleus, P: perivascular space, v: capillary. Note that the number of chromaffin granules is diminished significantly by insulin injection (b) and that the decrease is almost completely blocked by hexamethonium pre-treatment (c). The number is also decreased by nicotine (e) and oxotremorine (f) injection to the fasted animals. Bars in c and f indicate 1 μm.

nicotine or oxotremorine. About 20 sec after nicotine injection weak convulsion was initialized and the extent increased gradually up to about 90 sec after the injection. In oxotremorine-treated rats tremor was observed at the almost same time course in the nicotine stimulation. As indicated in Fig. 1c, both the adrenaline and noradrenaline contents in adrenals were decreased statistically significantly by nicotine or oxotremorine treatment. As shown in Fig. 2d, the chromaffin granules of the adrenaline-storing cells in a fasted rat were distributed almost evenly over the cytoplasm. The number of the granules decreased markedly in the nicotine- and oxotremorine-treated animals (Fig. 2e and f). The decrease was found frequently but not all cells responded to the stimulus. About one-third of the cells of nicotine-treated rats and half of oxotremorine-received ones contained a reduced number of the granules, as far as examined in 3 animals for each groups. An increase in the number of vacuoles was observed in the adrenaline-storing cells in the animals received secretogogues (Asterisks in Fig. 2e and f).

Fig. 3d-f indicates the noradrenaline-storing chromaffin cells. Although the decrease in the number of the granules in the noradrenaline-storing cells was observed after nicotine or oxotremorine stimulation, no increase in the number of the vacuoles was found (Fig. 3e and f).

Synapse buttons of cholinergic nerves innervated to the adrenaline- and noradrenaline-storing chromaffin cells were observed in control and insulin-treated rats. The synapse buttons contained a large number of acetylcholine-storing granules, and no synapse button with a reduced number of the granules was observed as far as examined in the control animals (Data not shown). The synapse buttons with a reduced number of the granules were frequently found in the insulin-treated animals. Sometimes the granules were almost completely lost by the treatment (Data not shown).

DISCUSSION

In the present study insulin-induced hypoglycemia exerted the adrenaline and noradrenaline release from adrenals in 21-day-old rats. These results confirm the previous report (Fujino and Fujii, 1995) biochemically and morphologically.

The remarkable decrease in the number of acetylcholine-storing granules in the synapse buttons of the cholinergic nerves innervating the chromaffin cells was frequently observed in insulin-treated fasted rats. This indicates that hypoglycemia induces acetylcholine release from the cholinergic nerve terminals, though the amount of the released catecholamines is unknown at present. Determination of the released acetylcholine in the adrenals is probably difficult, because acetylcholinesterase is present in the adrenal medulla. Acetylcholinesterase immunoreactivity has been found in the adrenal medulla of 2-day-old rats, and this immunoreactivity increases gradually to the adult stage (Holgert *et al*., 1994).

In the previous study acetylcholine failed to induce catecholamine release from adrenal slices prepared from 21-day-old rats (Fujino and Fujii, 1997). In the present study nicotine and oxotremorine exerted the adrenaline and noradrenaline release from the adrenals of 21-day-old fasted rats. The mechanism of the enhancement in the sensitivity of the chromaffin cells to these secretogogues by starvation is unknown at present. It may be probable that the functional mechanism of the chromaffin cells was not changed significantly by starvation and that hypoglycemia solely potentiated the effect of these secretogogues on the chromaffin cells. Additional experiments to characterize the enhancement in the sensitivity are required; however, it is also probable that in the multiple steps of the chromaffin granule exocytotic pathway one or several steps are inactive in infant rats and that these steps become to be fully active by starvation. Elevation of the cytosolic Ca^{2+} concentration of the chromaffin cells, prepared from 7-day-old rats, by muscarinic and nicotinic stimulation has been demonstrated (Oomori *et al*., 1998). This indicates that the function of the muscarinic and nicotinic receptors and the Ca^{2+} channels is mature in infant rats. The elevation of cytosolic Ca^{2+} induces the chromaffin granule docking to cell membrane and the fusion of the granule membrane to the cell membrane. In infant rats one of these processes may be inactive. These processes are very complicated and not fully understood at present.

The adrenaline and noradrenaline release in 21-day-old and the adrenaline release in 8-week-old rats evoked by hypoglycemia were almost completely blocked by pre-treatment of hexamethonium. This suggests that the nicotinic receptors of the adrenal chromaffin cells mainly participate in the catecholamine release induced by hypoglycemia in infant and young adult rats. In the present study not only nicotinic but also muscarinic stimulation evoked the release in fasted 21-day-old rats. It has been postulated that the nicotinic receptors are primarily concentrated in the synaptic zones of the chromaffin cell membrane and are involved in the physiological stimulation of the adrenal medulla, whereas the extrasynaptic regions contain a mixture of the nicotinic and muscarinic receptors, these are activated by injected secretogogues (Wakade and Wakade, 1983). This is probably a reason why the nicotinic antagonist almost completely blocked the release induced by acetylcholine secreted from the cholinergic nerve terminals during hypoglycemia. It has been reported that stimulation of muscarinic receptors results in long lasting catecholamine release from the chromaffin cells (Chowdhurry *et al*.,1994). Although physiological meaning of the muscarinic receptors of the chromaffin cells is unknown at present, they may respond to the other stimulation than the acetylcholine release from the cholinergic nerve terminals, such as the elevation of blood acetylcholine concentration.

We have already reported that serum glucose levels in fasted 21-day-old rats decrease about 1/8 of the control animals 30 min after insulin injection (Fujino and Fujii, 1995). Although the value obtained from the fasted rats in the present study was statistically significantly decreased, the extent of the decrease was less remarkable when compared to that in the insulin-received fasted rats. The system to hold serum glucose level, such as glycogenolysis in liver, may be acti-

vated by starvation.

The previous and present studies indicate that in the postnatal development maturation of the cholinergic nerve function proceeds to the appearance of the responsiveness to acetylcholine in the chromaffin cells. Adrenaline, secreted from the adrenal medulla, increases heartbeats, blood output from heart, diameter of pupils and bronchial tubes, and glycogenolysis in liver. These responses ensure survival in the case of emergency. Suckling rats are protected by their mother from these events and they are not required to secret catecholamines from the adrenals in order to respond to the emergencies within a short time. We speculate that unresponsiveness of the cells to acetylcholine in suckling rats probably keeps their behavioral activity at an unexciting state.

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