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# Nonneurogenic Hypoxia Sensitivity in Rat Adrenal Slices

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**A change in the intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) level induced by hypoxia was detected in rat adrenal slices by use of fura-2/AM. After hypoxic stress, an increase in $[Ca^{2+}]_i$ was observed only in the adrenal medulla. This increase was inhibited by nifedipine, but not modified by the cholinergic receptor blockers. The hypoxia-induced increase in $[Ca^{2+}]_i$ was observed in all postnatal developmental stages to a similar extent, whereas the nicotine and high $K^+$ sensitivities increased along with postnatal development. A 10 nM ryanodine enhanced the hypoxia-induced $[Ca^{2+}]_i$ increase in adult but not in neonatal rat slices. These results suggest the existence of an oxygen-sensing mechanism in adult rat adrenals even after sympathetic innervation. Hypoxic responses seemed to be similar both in neonate and in adult rat adrenals and were triggered by the influx of $Ca^{2+}$ via L-type voltage-sensitive $Ca^{2+}$ channels. However, the sustained $[Ca^{2+}]_i$ increase caused by hypoxia might depend on postnatal development and be triggered by $Ca^{2+}$-induced $Ca^{2+}$ release (CICR).** 

***Key Words:*** **hypoxia; rat adrenal; slices; postnatal development; ryanodine; mecamylamine; chromaffin cells; CICR; innervation; intracellular $Ca^{2+}$.**

Catecholamines released from the adrenal gland are crucial for protective responses to various stresses such as hypoxia. It has been reported that prior to the development of sympathetic nerve function, adrenal catecholamines play a predominant role in enabling the neonate to survive in the hypoxic state and that this nonneurogenic mechanism is replaced by a neurogenic one in accordance with the development of sympathetic innervation after the end of the first postnatal week in the rat (1). On the other hand, prolonged deprivation of neural input in adult rat adrenals led to the reemergence of nonneurogenic capabilities, such as catecholamine secretion induced by hypoxia (2). These reports suggest the direct responsiveness of the adrenomedullary chromaffin cells (AMCs) to the hypoxic stimulus before innervation, and the reappearance of nonneurogenic hypoxia sensitivity after denervation. Recently, several researchers reported the existence of an oxygen-sensing mechanism in isolated AMCs. However, the age-dependent changes in oxygen-sensing ability in the AMCs were equivocal among these researchers. Thompson *et al.* (3) reported that the AMCs from rats at postnatal day 13 to day 20 did not show the oxygen sensitivity but that those from postnatal day 1–2 rats did. However, we reported that AMCs prepared from 7-week-old rats showed hypoxia-induced responses, such as suppression of $K^+$ channels, an increase in intracellular calcium ($[Ca^{2+}]_i$), and enhancement of catecholamine release (4). In addition, Lee *et al.* (5) reported that AMCs in short-term culture (up to 1 day) from adult rats showed the hypoxia sensitivity. These data suggest the existence of nonneurogenic hypoxia sensitivity in AMCs even after innervation. In explanation of the discrepancy between *in vivo* studies and those using isolated AMCs, it is often stated that isolated cultured cells are somehow similar to developing cells and dissimilar to mature ones. On the other hand, there are surprisingly few reports about the oxygen-sensing mechanism in AMCs *in situ.* Experiments using isolated glands or its slices might serve as a bridge between *in vivo* and *in vitro* studies. Adams *et al.* (6) developed a retrograde adrenal perfusion system using isolated fetal sheep adrenal glands

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and compared hypoxia-stimulated catecholamine release before and after innervation. They found that the nonneurogenic catecholamine response to hypoxia persisted even after the completion of splanchnic innervation. Therefore, it is very important to examine the oxygen-sensing mechanism in AMCs *in situ*.

Here, we examined the $[Ca^{2+}]_i$ change induced by hypoxia in slices of adrenal gland from neonatal to adult rats to elucidate the effects of innervation from the central nervous system. The effects of sympathetic ganglion blockers on the hypoxic responses confirmed the nonneurogenic nature of the responses in adult rat adrenal slices. Though the hypoxic response of the adrenal medulla did not depend on the maturation, modifiers of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) revealed a difference in the contribution of CICR to the hypoxic response between neonatal and adult. Finally, the $[Ca^{2+}]_i$ increase caused by hypoxia in the slices was compared with that in isolated AMCs.

## METHODS

*Preparation of slices.* The experimental protocols were approved by the Ethical Committee on Animal Care and Use from Osaka Bioscience Institute, Japan. Male Wistar rats (postnatal day 1 to 10 weeks old) were anesthetized with diethyl ether. The adrenal glands were excised immediately and removed carefully from the surrounding fat tissues in ice-cold buffer solution containing 120 mM NaCl, 20 mM $NaHCO_3$, 10 mM Na–Hepes, 5 mM $CaCl_2$, 2.7 mM KCl, and 10 mM glucose equilibrated with 95% $O_2$–5% $CO_2$ mixture (pH 7.4). The adrenal glands were then covered with 3% agar, and sectioned at a 150-$\mu$m thickness in ice-cold buffer solution by use of a microslicer (DTK-1000, DOSAKA, Kyoto, Japan). The sections were allowed to recover at room temperature for 1 h in the above-mentioned buffer solution.

*Preparation of AMCs.* The procedure for isolation and cultivation of the cells was carried out as described previously (4). Adrenal glands were obtained from 7-week-old male Wistar rats, and the chromaffin cells were isolated from the medulla by enzymatic digestion using collagenase. The isolated cells were plated on poly-L-lysine-coated coverslips, and the yield was estimated at $1–2 \times 10^4$ cells per gland.

*Measurement of $[Ca^{2+}]_i$.* Fura-2/AM was used as a $Ca^{2+}$ indicator (7). Since the insolubility of fura-2/AM is a problem, especially in slice experiments, 1/10 volume of cremophore EL was added to a stock solution of fura-2/AM (1 mM in dimethyl sulfoxide) before diluting the indicator in buffer solution to increase the solubility of the fura-2/AM (8). The slices were incubated with 10 $\mu$M fura-2/AM at 37°C for 30 min, then washed and kept in the buffer solution at room temperature to allow the acetoxymethyl (AM) group to be removed by enzymatic hydrolysis by endogenous esterase. After 15 min, the slice was set in a siliconized chamber (flexyperm; Heraeus-Biotechnology, U.S.A.) and held in position by a U-shaped platinum wire with nylon strings. The method for loading fura-2/AM into cultured chromaffin cells was described previously, and the cells that showed a $[Ca^{2+}]_i$ increase of >8% during a 3-min period of hypoxia were counted as hypoxia-sensitive cells (4). Fluorescence images were collected by a CCD camera (C2400-80; Hamamatsu Photonics, Hamamatsu, Japan), every 5 s by using excitation light with wavelengths of 340 and 380 nm. The solution was constantly perfused at a rate of 1.5 ml $min^{-1}$. During the challenge with the hypoxic solution, the oxygen concentration in the measuring chamber was monitored by an oxygen electrode. The final $PO_2$ in the chamber was 35–40 mmHg (5%). During hypoxia, $N_2$ gas was streamed over the surface of the measuring chamber. High $K^+$ solution contained 90 mM NaCl, 50 mM KCl, 10 mM K-Hepes (pH 7.4), 5 mM $CaCl_2$, and 10 mM glucose.

*Image analysis.* $[Ca^{2+}]_i$ was calculated as the ratio of the fluorescence intensities (F340/F380) by use of a digital image processor (ARGUS-50/CA; Hamamatsu Photonics). All data were expressed as the relative ratio value (F340/F380). We tried to obtain a calibration curve by using 10 $\mu$M ionomycin and EGTA as we had done in the case of isolated AMCs (4) in accordance with the method by Williams and Fay (9). The slope of the ratio versus $[Ca^{2+}]_i$ in slices was similar to that in cultured cells over the range between $10^{-7}$ to $4 \times 10^{-6}$ M $Ca^{2+}$. However, the slope obtained with slices was not steep over the range less than $10^{-7}$ M $Ca^{2+}$ compared with that for the cultured cells. Therefore, we chose the relative ratio rather than the absolute value of $[Ca^{2+}]_i$. The difficulty in obtaining a $Ca^{2+}$ calibration curve for slices might have been due to the thickness of the slices to control the $[Ca^{2+}]_i$ even in the presence of $Ca^{2+}$ ionophore and EGTA.

*Chemicals.* Mecamylamine, ryanodine, nifedipine, nicotine, cremophor EL, and poly-L-lysine were obtained from Sigma (St. Louis, MO). Fura-2/AM was from Dojin Chemical Institute (Kumamoto, Japan); collagenase (type I), from Worthington Biochemical Corp. (Lakewood, NJ); and imperatoxin inhibitor, from Latoxan (Valence, France).

*Statistical analysis.* Data were presented as the mean ± SEM and analyzed by use of nonparametric Wilcoxon test unless otherwise noted. A $P$ value of $<0.05$ was regarded as significant.

## RESULTS

### *Hypoxia Sensitivity in Adult Rat Adrenal Slices*

The images of $[Ca^{2+}]_i$ in the adult (8-week-old) rat adrenal slices were obtained. The resting level of $[Ca^{2+}]_i$ in the adrenal medulla was much lower than that in the adrenal cortex (data not shown). After 3 min of hypoxic stress, many parts of the adrenal medulla showed an increase in $[Ca^{2+}]_i$, and this increase was clearly shown by subtraction of the ratio image obtained before from that obtained after the stimulation (Fig. 1A). In the adrenal medulla, the region responsive to hypoxia was broad, not localized in some particular region such as the center of the medulla or border between the medulla and cortex. Compared with the adrenal medulla, the cortex showed only a small change due to hypoxic stimulus. When the average value of the increase in $[Ca^{2+}]_i$ (△ ratio of fluorescence: F340/F380) was calculated from different 10 regions of 50-$\mu$m diameter after subtraction of the drift of the baseline, the elevation in the medulla or cortex was 0.086 ± 0.004 or 0.024 ± 0.004 (mean ± SEM, $n = 10$), respectively; and this difference was significant ($P < 0.05$). To confirm the nonneurogenic response to hypoxia in adult slices, hypoxic stimulus was applied under the presence of sympathetic ganglion blocker, 100 $\mu$M mecamylamine. The $[Ca^{2+}]_i$ increase induced by hypoxia was not affected by mecamylamine, whereas that by 100 $\mu$M nicotine was completely suppressed (data not shown).

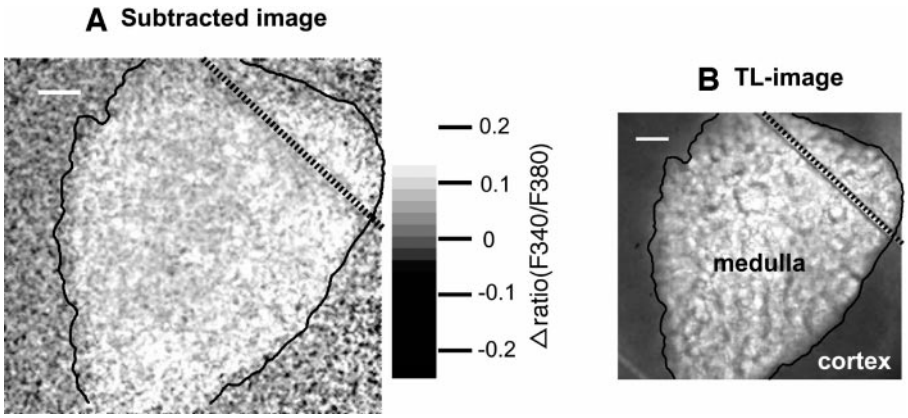


**FIG. 1.** Image of $[Ca^{2+}]_i$ in the adult rat adrenal slice. (A) The change in $[Ca^{2+}]_i$ induced by hypoxia in the adrenal slice from 8-week-old rat is shown. This image was obtained by subtracting the image of 10 s before stimulation from that 3 min after stimulation. After subtraction, the image was normalized and smoothed. The $[Ca^{2+}]_i$ was obtained as the Δratio of the fluorescence intensities (F340/F380). The traces of the edge between adrenal medulla and cortex are presented by black lines, and were obtained from the transmitted light (TL) image (B). The interrupted line shows the nylon strings used to keep the slice in position. Scale bar, 100 $\mu$m.

*Hypoxia Sensitivity before and after Innervation*

Next we compared the hypoxia-induced responses before and after innervation. Slices from postnatal day 5 (P5) rats before innervation, and postnatal day 11 (P11) ones just after innervation, were used (Fig. 2). In both slices, hypoxia, nicotine, and high $K^+$ responses were shown in the medulla, whereas the cortex showed little change in response to these stimuli. As shown in Fig. 2, the basal $[Ca^{2+}]_i$ in the adrenal medulla at P11 was much lower than that at P5. The average values at P0–P5 and P11–P48 were 1.22 ± 0.06 and 1.01 ± 0.05 ($n$ = 10), respectively, which were significantly different by the nonparametric Mann–Whitney test ($P < 0.05$). On the other hand, the $[Ca^{2+}]_i$ increase elicited by hypoxia was similar in both slices. After the return to the control solution, the $[Ca^{2+}]_i$ level in the P5 slice recovered quickly and almost completely; whereas the recovery in the P11 slice was slow and incomplete. Moreover, the response to nicotine or high $K^+$ was smaller in the P5 slice than in the P11 one.

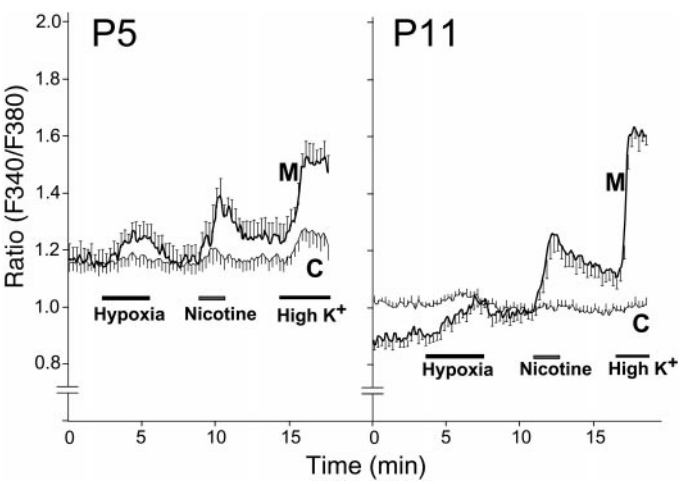


**FIG. 2.** Time-dependent changes in $[Ca^{2+}]_i$ increase induced by several stimulations before and after innervation. The time-dependent changes in the slices from postnatal day 5 (P5), before innervation, and those from postnatal day 11 (P11), after innervation, are shown. The $[Ca^{2+}]_i$ increase is expressed as the ratio of F340/F380. The value of each slice is expressed as the mean ± SEM of 5 different regions, each with a diameter of 15 $\mu$m. M is adrenal medulla, and C is adrenal cortex. After the first trial of hypoxic stimulation, 100 $\mu$M nicotine and 50 mM $K^+$ were applied sequentially.

More quantitative analysis of the developmental changes in hypoxic responses was performed by using the average △ratio of fluorescence (F340/F380) in the medulla (Fig. 3). For each developmental stage, the average ± SEM values were calculated from more than 6 slices obtained from at least 3 different animals. We calculated the average of peak values from more than 10 positions in each slice, since hypoxia sensitivity was distributed broadly in both adult and neonatal adrenal slices. In slices from 1–2-day-old to 6–11-week-old rats, the responses to hypoxia in the adrenal medulla were almost the same; whereas the responses to high $K^+$ showed a gradual increase with development. The nicotinic response of slices from 1–2-day-old rats was even one-fourth of that of adult animals, but it showed a progressive increase that became statistically significant at 8–11 days, which period was after innervation.

*Contribution of CICR to the Hypoxic Response in the Adrenal slices and the Cultured AMCs from Adult Rats*

Before innervation (P1–P5), the relative recovery, which was expressed as the percentage of recovery at 3 min after the end of the hypoxia from the peak height of the increased relative ratio value (F340/F380), was 86.6 ± 2.3% ($n$ = 5), whereas that after innervation

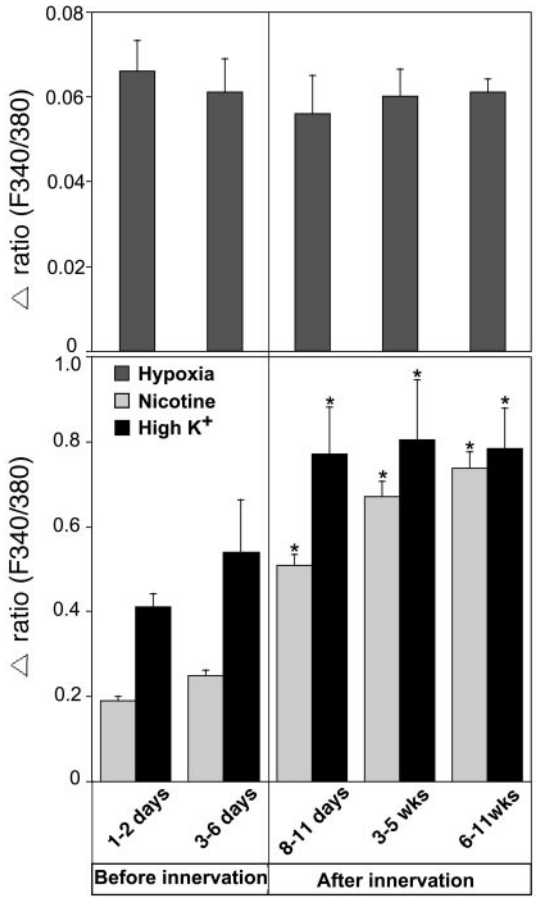


**FIG. 3.** Changes in $[Ca^{2+}]_i$ increase induced by hypoxia, nicotine, or high $K^+$ before and after innervation. The mean ± SEM of the △ratio of F340/F380 in the adrenal medulla of the slices ($n$ = 6–12) in each developmental stage is shown. The value of each slice was calculated as the average value of △ratio of fluorescence before and after stimulation from 10 different regions, each of 50 $\mu$m diameter. Hypoxic responses are shown in the upper panel, and the increases elicited by nicotine or high $K^+$ are in the lower panel. *$P < 0.05$, $[Ca^{2+}]_i$ increase is significantly different by nonparametric Wilcoxon test from the value for postnatal day 1–2.

(P11 to 4 weeks) was 53.4 ± 1.8% ($n$ = 8). The recovery from hypoxic stress was much slower after completion of innervation (Fig. 2), suggesting that secondary $Ca^{2+}$ mobilization from intracellular $Ca^{2+}$ stores might be dependent on postnatal development. There are two pathways for the release of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores. One is inositol 1,4,5-triphosphate ($IP_3$)-dependent $Ca^{2+}$ release, and the other is calcium-induced calcium release (CICR). In the slices prepared from all ages, there was no change in the hypoxic response by the application of 50 $\mu$M cinnarizine, which is a blocker of $IP_3$-dependent $Ca^{2+}$ release (data not shown). Ryanodine stimulates CICR when used at a low (10 nM) concentration, but blocks it at high (10–100 $\mu$M) concentrations (10). The adrenal slices prepared from 8-week-old rats showed a large and slow increase in $[Ca^{2+}]_i$ and little recovery from the sustained phase of $[Ca^{2+}]_i$ increase caused by hypoxia after 10 nM ryanodine treatment, whereas 10 $\mu$M ryanodine did not modify the increase in $[Ca^{2+}]_i$ and allowed recovery following the end of the hypoxic challenge (Fig. 4A). The slices from day 1 rats did not show significant enhancement in the hypoxic response by 10 nM ryanodine (Fig. 4B). On the other hand, the hypoxic response by the AMCs cultured from 7-week-old rats was amplified by 10 nM ryanodine as in the case of the slices from 8-week-old rat adrenals (Fig. 4C). This enhancement in the adult rat adrenal slices was suppressed by 10 $\mu$M nifedipine, a blocker of L-type voltage-dependent $Ca^{2+}$ channels (Fig. 4D). In the slices from neonatal rats, the $[Ca^{2+}]_i$ increase caused by the hypoxic challenge was also blocked by 10 $\mu$M nifedipine (data not shown).

The data shown in Fig. 5 clearly indicate the postnatal developmental changes in the contribution of CICR. The enhancement of the hypoxic response by the

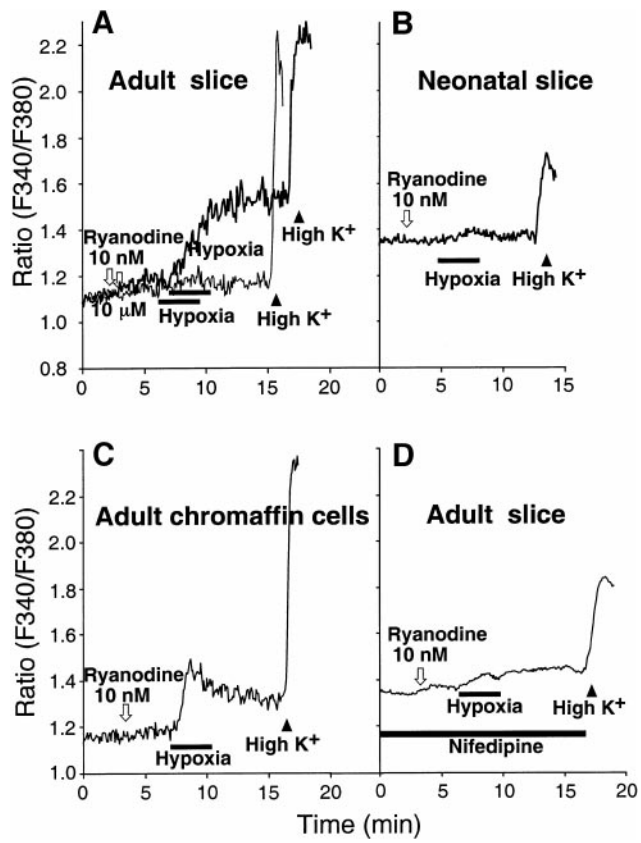


**FIG. 4.** Effects of ryanodine on the hypoxic responses. Calcium-induced calcium release (CICR) agonist (10 nM ryanodine) or antagonist (10 $\mu$M ryanodine) was applied before the hypoxic challenge to adrenal slices from 8-week-old rats (A). The slice from 1-day-old rat was challenged with hypoxic stress in the presence of CICR agonist (B). (C) The average response of 10 AMCs isolated from a 7-week-old rat is shown. In D, 10 $\mu$M nifedipine was applied at time 0 to a slice from a 8-week-old rat. The white arrow indicates the start of ryanodine application, the black arrowhead indicates high $K^+$, and the black bar indicates hypoxia treatment.

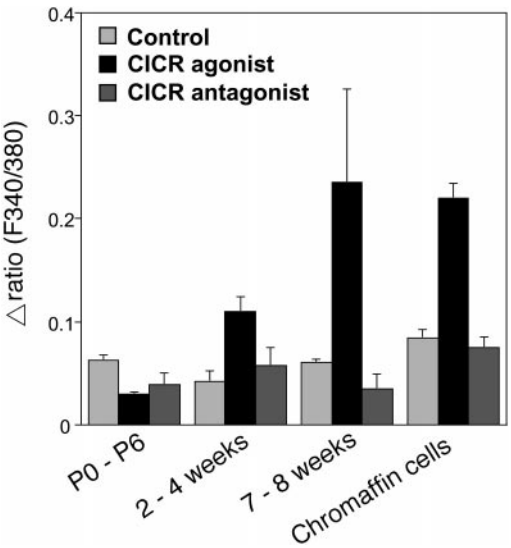


**FIG. 5.** Postnatal developmental changes in the effects of ryanodine. Effects of ryanodine on the hypoxic response were examined in the slices from before (P0–P6) and after (2–4 and 7–8 weeks) innervation and in AMCs isolated from 7-week-old rats. Control values show the response to hypoxia without ryanodine or imperatoxin inhibitor. The low concentration (10 nM) of ryanodine was used as a stimulator of both slices and AMCs. We used 10 $\mu$M ryanodine as a CICR blocker for the slices, and 100 nM imperatoxin inhibitor as ones for AMCs. The AMCs were incubated with imperatoxin inhibitor 30 min before experiments, since cell permeability to the imperatoxin inhibitor was low. The hypoxic challenge was applied under ryanodine or imperatoxin inhibitor treatment, and the maximum changes in $[Ca^{2+}]_i$ (△ratio F340/F380) are presented as the mean ± SEM ($n$ = 3, slices; $n$ = 21–25, AMCs).

agonist of CICR, 10 nM ryanodine, was observed in the adrenal slices from rats 2–4 and 7–8 weeks of age but not in those from P0–P6 animals. Under the application of 10 nM ryanodine approximately 80% of the AMCs from adult rats (20/25) responded to the hypoxic challenge, and the increment in ratio was 0.176 ± 0.021 (mean ± SEM, $n$ = 25). On the other hand, an antagonist of CICR, 100 nM imperatoxin inhibitor, decreased the fraction of cells responsive to hypoxia to 29% (6/21); and the increment in ratio was 0.021 ± 0.001 (mean ± SEM, $n$ = 21). In the slice experiments, 10 $\mu$M ryanodine was used as a CICR antagonist, owing to the low permeability of the imperatoxin inhibitor. In either adult or neonatal slices, 10 $\mu$M ryanodine did not modify the peak value of the hypoxia response.

## DISCUSSION

In rat adrenals, the splanchnic nerve control becomes complete by postnatal days 7–10 (11). Seidler and Slotkin (1) reported that in *in vivo* experiments only neonatal rats showed nonneurogenic hypoxia sensitivity and that this nonneurogenic response disappeared in accordance with the completion of the innervation. However, in this study, we clearly showed that the increase in $[Ca^{2+}]_i$ was induced by hypoxia even in adult rat adrenal slices. This increase was not diminished by the nicotinic antagonist, mecamylamine. Kajiwara *et al.* (12) reported that in slices of rat adrenal glands postsynaptic nicotinic receptors but not muscarinic receptors were responsible for the excitatory postsynaptic potentials. Therefore, the existence of hypoxia sensitivity even when the nicotinic antagonists were applied suggests that the nonneurogenic hypoxia-sensing mechanism still remained after the development of innervation. The decrease in $O_2$ up to 35–40 mmHg might be a severe condition for the slices, but we applied only a short time (3 min) hypoxic stimulation, and observed the recovery of the $[Ca^{2+}]_i$ after the reperfusion with normoxic solution, suggesting continuation of the normal physiological condition during the experiments. Figures 2 and 3 clearly showed that the $[Ca^{2+}]_i$ increase to hypoxia was similar in all ages from neonatal (postnatal day 1) to adult (postnatal week 10), that the responsiveness to nicotine or high $K^+$ developed after birth, and that the recovery in $[Ca^{2+}]_i$ level from hypoxic challenge was slow and incomplete in the slices after innervation.

The enhancement of the hypoxic response by 10 nM ryanodine, an agonist of calcium-induced calcium release (CICR), was observed only in the adult but not in the neonatal adrenal slices (Figs. 4 and 5). These results suggest that the sustained $[Ca^{2+}]_i$ increase by hypoxia seen in the adult might be mediated by CICR. In adult AMCs, the contribution of CICR to the spontaneous $[Ca^{2+}]_i$ fluctuations (13) or to the change in $[Ca^{2+}]_i$ induced by cell depolarization (14) was also reported. Morita *et al.* (15) reported that CICR contributes to the maintenance of the sustained $[Ca^{2+}]_i$ rise during cell stimulation in the adult bovine AMCs. In neonatal AMCs, however, there is no report concerning the contribution of CICR. Morita *et al.* (15) also reported that in adult AMCs, ACh stimulated the synthesis of cyclic ADP-ribose, which is an endogenous ligand of CICR. Based on this report and our results, there is a possibility that the CICR mechanism develops along with the innervation. Further investigation is needed to clarify the relationship between innervation and the development of the CICR mechanism.

It is often believed that cultured cells have the same features as undeveloped cells. However, our findings showed that cultured AMCs from adult rats had similar sensitivity to ryanodine as slices from adult rats, whereas neonatal slices were insensitive to it. By the application of 10 nM ryanodine, the fraction of hypoxia-sensitive cells increased to 80% from 50%, and the peak height of the $[Ca^{2+}]_i$ increase was approximately 3 times higher than that of the control (Fig. 5). This increment in hypoxic response was not observed in the presence of an antagonist of CICR in either cultured AMCs or the slices from adult rats (Fig. 5). In contrast to our results, Thompson *et al.* (3) reported the

loss of hypoxia sensitivity in AMCs isolated from juvenile rat after completion of innervation. This discrepancy might be caused by the difference of the isolation methods for cells. We used only collagenase for digestion, while Thompson *et al.* used collagenase and trypsin. Our results strongly suggest that the cultured AMCs retain their sensitivity to the CICR agonist and to hypoxic stimulation.

Earlier we reported that hypoxic responses of isolated AMCs from adult rats were completely dependent on the influx of extracellular $Ca^{2+}$ (4). By application of the L-type $Ca^{2+}$ channel blocker nifedipine (10 $\mu$M), catecholamine release and the $[Ca^{2+}]_i$ increase induced by hypoxia disappeared. In slices from both neonatal and adult rats, the $[Ca^{2+}]_i$ increase caused by the hypoxic challenge was blocked by nifedipine, an inhibitor of extracellular $Ca^{2+}$ influx; and in the presence of this inhibitor, the enhancement of the hypoxic response by an agonists of CICR (10 nM ryanodine) was blocked (Fig. 4D). These results suggest that the hypoxic responses are triggered by the influx of extracellular $Ca^{2+}$ in the slices as well as in the isolated AMCs from adult rats. Our results from both isolated AMCs and adrenal slices strongly suggest the existence of a nonneurogenic hypoxic response in the adult rat adrenal medulla and its developmental change in the sustained phase of the $[Ca^{2+}]_i$ increase caused by hypoxia.

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