Crucial role of milk-borne insulin in the development of pancreatic amylase at the onset of weaning in rats

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Kinouchi, Toshi, Kyoko Koizumi, Tamotsu Kuwata, and Takaji Yajima. Crucial role of milk-borne insulin in the development of pancreatic amylase activity at the onset of weaning in rats. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1958–R1967, 1998.—The development of pancreatic amylase activity was examined in rats fed in regular cages or in special cages, designed so the pups could not reach solid food to prevent weaning. In both groups, the amylase activity in zymogen granules increased in rat pups aged 14 days, peaked at 18 days, and thereafter remained at a 1.6-fold higher level than at 14 days of age. An increase in the plasma concentration of immunoreactive insulin preceded the increase of amylase activity, whereas the plasma concentration of C-peptide, indicating the secretion rate of endogenous insulin, remained unchanged. The administration of insulin at 20 ng/ml (the physiological concentration) in the milk formula caused an increase in the plasma insulin concentration of 17-day-old pups. In addition, increased pancreatic amylase activity was observed in 17-day-old rats raised on milk formula to which insulin was added. We propose that the increase of amylase activity at the beginning of weaning is dependent on the milk-borne insulin and not on the dietary change in rats.

dietary change; digestive function; milk formula; artificial rearing

MAMMALS ENCOUNTER a significant change in the composition of their diet at the time of weaning, coinciding with alterations in their digestive functions. Rat pups start to chew a solid diet at 15 days of postnatal age, and the gradual change from an exclusively milk diet to a solid diet rich in carbohydrates begins at 17 days of age (8). In addition to the changes in the proportions of nutrients, the nature of the carbohydrates changes: namely, milk has lactose as the principal carbohydrate, whereas a solid diet has starch (5, 8). The activity of pancreatic amylase, the main enzyme in complex carbohydrate digestion, is low in the preweaning rat pancreas and increases sharply at the time of weaning (8, 14, 16, 27). The factors that cause the development of pancreatic amylase remain to be elucidated.

In adult rats, the pancreas adapts to individual nutrients; e.g., a prolonged high-carbohydrate intake leads to an increased concentration of amylase in the pancreas (4). It is therefore possible that the development of pancreatic amylase during the 3rd wk of life in rats is the result of dietary changes (14, 27). However, there is growing evidence that the initiation of ontogenic changes in various gastrointestinal functions, such as disaccharidases, may not be dependent on the dietary change at the time of weaning (8), suggesting that weaning may not be responsible for the development of amylase activity (3, 15). We are interested in whether the dietary change in weaning rats influences the developmental changes of pancreatic amylase.

Even if dietary changes are the cause of the increase in pancreatic amylase during the weaning period, hormonal regulation may also participate in the increase in amylase activity (1, 5, 17). It is well known that insulin is the most important regulator of pancreatic amylase expression (13) and a potential mediator of the pancreatic amylase adaptation to a high carbohydrate diet in adult rats (4). Because the plasma insulin concentration is low in preweaning rats and begins to increase in the third postnatal week (5), insulin may play a key role in the ontogenic changes of pancreatic amylase. The developmental changes in the rat plasma insulin concentration during the third postnatal week have not yet been compared with those of pancreatic amylase.

A number of hormones and trophic factors have been found at high concentrations in mammalian maternal milk (11, 22, 31). In suckling rats, the permeability of the gut to macromolecules is a recognized phenomenon (6, 19, 20, 24, 28–30), and there is evidence that intact large peptides in milk, such as insulin or epidermal growth factor, pass across the intestinal epithelium into the systemic circulation (11, 19). As shown in the present study, rat milk also contains insulin at a high concentration, suggesting that the milk may be a source of insulin for suckling rats. It is not yet known whether insulin in the milk is absorbed from the gut in rat pups or whether it has any effect in peripheral organs, including the pancreas.

The aim of the present study was to explore the factors that regulate the developmental changes of pancreatic amylase in weaning rats. We examined the association between the development of amylase activity and the dietary change and/or hormonal regulation. We also investigated the potential role of insulin in milk in the development of pancreatic amylase activity.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley rats were purchased from J apan SLC (Shizuoka, J apan) and housed in separate cages under controlled temperature (25 ± 2°C), humidity (55 ± 2% relative humidity), and light (lights on 0700–1900) conditions. The day of birth is referred to as day 0. The litter sizes were adjusted to 10 pups at 3 days of age to maintain a standard nutritive status. During lactation, the dams were fed ad libitum with powdered food (CA-1; J apan Clea, Tokyo,
Japan). Because the suckling rats begin to nibble their mothers' food around 15 days of age, from 12 days of age the pups, male and female, were randomly divided into two groups of equal average body weight. The pups in one group and their dams were placed in regular cages, and the pups in the other group were placed in special cages designed so that the rats could have access to the mothers' food. The litter sizes were maintained at 8–10 pups. The special cage consists of a first floor where the pups are kept and a second floor where the mother's powdered food was placed. The structure of the first floor of the special cage was identical to that of the regular cage, and the second floor covered one-third of the area of the first floor, at a height of 22 cm. The mothers could freely go up and down between the floors, but the pups could not climb to the second floor. From 14 days of age, a net was laid on the first floor of each cage to prevent the pups from eating the mothers' feces. No visible sign of the powdered food, such as a change in color or the presence of particles, was found in the gastric contents of the rats reared in the special cages until they were 23 days old. The pups' body weight curves were updated daily. The experimental protocol was approved by our Institute's Committee for Research on Experimental Animals and was conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (1985).

Maternal rat milk and milk formula for rats. On the day when all pups were removed for experiments, maternal rat milk was divided into 50-ml sterilized bottles and then stored frozen at −20°C. When all pups were removed for experiments, the dams were killed by draining the blood with a syringe from the abdominal aorta under anesthesia with ether within 30 min after separation from the dams or the last administration of the milk formula in the morning (0900–1100), except in the experiments on the time course (0900–1500). The blood was collected in tubes containing heparin. Plasma was separated and stored at −40°C until assayed. For the preparation of zymogen granules, the pancreas was quickly removed and homogenized in an ice-cold homogenization buffer: 0.25 M sucrose, 5 mM MOPS, and 0.1 mM MgSO₄, pH 7.0. The homogenate was centrifuged at 150 g for 10 min to remove unbroken cells and nuclei. The supernatant was recentrifuged at 3,800 g for 10 min. The white layer at the bottom was suspended in 0.15 M Tris·HCl buffer (pH 8.2) containing 0.01 M CaCl₂ and then briefly sonicated to break the membranes of the zymogen granules. The suspension was centrifuged at 3,000 g for 5 min, and the resultant supernatant was frozen at −40°C until used for the determination of the pancreatic enzyme activity. For the measurement of the insulin content, the pancreases were immediately frozen after removal and stored at −80°C until assayed.

Assays. Blood glucose concentrations were measured with a Glucose C11-Test Wako (Wako) based on a mutarotase-glucose oxidase method. Trypsin activity was determined with benzoyl-arginine p-nitroanilide (Sigma, St. Louis, MO) as a substrate, after activation with enterokinase at 37°C for 40 min. Crystalline porcine trypsin (Sigma) was used as a standard. One unit of amylase activity was defined as 1 μmol p-nitroaniline liberated per minute at 37°C. Amylase activity was determined with a DiaColor-Amy kit (ToyoBo, Osaka, Japan) based on a method involving 2,4-dichlorophenyl β-maltopentoside as a substrate. One unit of amylase activity was defined as 1 μmol d-glucose oxidase method. Trypsin activity was determined with benzoyl-arginine p-nitroanilide (Sigma, St. Louis, MO) as a substrate, after activation with enterokinase at 37°C for 40 min. Crystalline porcine trypsin (Sigma) was used as a standard. One unit of amylase activity was defined as 1 μmol p-nitroaniline liberated per minute at 37°C. Crystalline porcine amylase (Wako) was used as a standard. The protein concentration was determined with Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard. The amounts of insulin in plasma, milk, and tissue were assayed with a rat insulin radioimmunoassay system (Amersham, Buckinghamshire, UK). This assay kit is based on a specific and sensitive radioimmunoassay and is recommended for blood and tissue samples. Because the milk formula was shown to contain <0.2 ng/ml of insulin, which was possibly due to ingredients from the bovine milk, and good recovery was obtained when rat insulin was added to the milk formula and dams' milk, the insulin concentrations in the milk are precisely measured by this assay system. The total plasma concentrations of corticosterone were determined with a rat corticosterone 125I assay system (Amersham) based on a specific and sensitive radioimmunoassay. The amounts of C-peptide in plasma and milk were assayed with a C-peptide radioimmunoassay kit (Linco Research, St. Louis, MO) specific for rat C-peptide. Good recovery was obtained when rat C-peptide was added to the milk formula and dams' milk, indicating that the C-peptide concentrations in the milk are precisely measured by this assay system.

Statistical analysis. Comparisons between groups were carried out by means of an unpaired Student's t-test or analysis of variance followed by the Tukey test for multiple comparisons, when appropriate.
RESULTS

Growth. In the present study, rat pups were raised in regular cages (normal weaning group) or in special cages so that they had no access to the mothers’ food (nonweaning group). The dams and normal weaning rat pups were fed a commercial powdered diet that was 57% carbohydrate. No difference in the growth rates of dams was observed between the two groups. There was no significant difference in the growth rates between the nonweaning pups and the normal weaning pups (Fig. 1A). These results indicated that the pups and their dams were not stressed by the special cages; the special cage therefore appears to be an appropriate system for investigating the effects of the dietary change at the time of weaning. These cages were thus used to investigate the development of pancreatic amylase during the third postnatal week and its correlation with the dietary change.

Developmental patterns of pancreas weight, amylase, and trypsin activity in pancreatic zymogen granules. As shown in Fig. 1B, the wet weight of the pancreas increased gradually in the two groups until 18 days of age and thereafter the growth rate of the pancreas weight increased more sharply. No significant difference was observed in this parameter between the two groups, suggesting that the dietary change at the time of weaning does not affect the increase in pancreas mass. We determined the developmental pattern of amylase activity in zymogen granules in both groups during the third postnatal week. The amylase activity, at a low level during the first two postnatal weeks (data not shown), increased constantly after 14 days of age (Fig. 2A) and peaked at 18 days of age (P < 0.05, compared with 14-day-old rats). Thereafter, the amylase activity in pancreatic zymogen granules decreased, but remained constant at a level 1.6-fold higher than that at 14 days. After 22 days of age, the amylase activity increased again (44.7 ± 3.8 U/mg protein, normal weaning rats at 22 days old). During the third week of life, the amylase development showed no significant difference between the normal weaning group and the nonweaning group. These results suggested that the ontogenic changes of amylase activity in pancreatic zymogen granules during the early part of weaning are not dependent on the dietary change, i.e., the weaning itself. In other words, these data indicated that there are other regulatory factors that cause the ontogenic increase of amylase activity in preparation for weaning. In contrast, the trypsin activity in the zymogen granules reached the adult level (0.689 ± 0.088 U/mg protein in 2-mo-old rats) at 14 days of age and then remained at a high level (Fig. 2B). Addition-
ally, in the pancreatic homogenates, the developmental patterns of amylase and trypsin activity in the two groups were similar to those in the zymogen granules (data not shown).

Plasma concentrations of hormones. It has been well documented that the expression of pancreatic amylase is affected mainly by glucocorticoid and insulin (13, 18). We quantified plasma corticosterone, the main secreted glucocorticoid in rats, by a radioimmunoassay method. As shown in Fig. 3A, the circulating concentrations of corticosterone increased gradually throughout the third week of life, but the increase in corticosterone was not in parallel with the change in amylase activity in either group.

Insulin is another possible regulatory factor. Previous investigations suggested the involvement of insulin as a mediator of pancreatic development in early weaning (1, 5). As shown in Fig. 3B, the plasma insulin concentration rose constantly from a level of ~0.7 ng/ml after 14 days of age, peaked at a level of ~1.5 ng/ml at 17 days (P < 0.05, compared with 14-day-old rats), and decreased abruptly at 18 days. The plasma insulin level in 19-day-old rats returned to the level seen in 14-day-old rats and then increased gradually. Thus the plasma insulin level in preweaning rats changed in parallel with the amount of amylase activity, and the developmental curve of the plasma insulin level shifted to the left 1 day before the amylase curve. These results suggest that the development of amylase activity may be influenced mainly by insulin rather than glucocorticoid. Additionally, the prevention of weaning did not modify the plasma concentrations of corticosterone or insulin, as well as the enzyme activity of amylase.

Endogenous insulin. We then explored the factors that regulated the developmental change of plasma insulin. We speculated that the developmental increase in plasma insulin could be due to the developmental increase in insulin in the pancreas, the increase in a stimulator of insulin release, or the increase of pancreatic insulin release. As shown in Fig. 4A, the amount of pancreatic insulin per body weight increased gradually during the 3rd wk, but the transient increase that peaks at 17 days of age was not observed in the amount...

![Fig. 3. Developmental patterns of the circulating concentrations of corticosterone (A) and insulin (B) in normal weaning rats and nonweaning rats. Blood was collected from the abdominal aorta within 30 min after separation from the dams. Plasma concentrations of the hormones were determined with specific radioimmunoassays. Results are means ± SE for 4 animals at each time point.](image)

![Fig. 4. A: amounts of pancreatic insulin per body weight in normal weaning rats and nonweaning rats. B: concentrations of glucose in the plasma in normal weaning rats and nonweaning rats. Blood was collected from the abdominal aorta within 30 min after separation from the dams. C: plasma concentrations of C-peptide in normal weaning rats and nonweaning rats. Results are means ± SE for 4 animals at each time point.](image)
of pancreatic insulin. The change in the amount of plasma glucose, which is the main regulator of pancreatic insulin release, during the experimental period was determined. An age-dependent change in the plasma glucose level was not observed (Fig. 4B), suggesting that the change in the plasma insulin level in weaning rats (Fig. 3B) is not dependent on the plasma glucose. We then determined the plasma levels of C-peptide, the production of which would be accompanied by the endogenous insulin production. The plasma C-peptide concentrations were maintained at a constant level from 14 to 21 days of age (Fig. 4C). These results implied that the increase in plasma insulin, which peaked at 17 days of age, could not be attributed to the endogenous insulin. In addition, the plasma glucose level, the insulin concentration in the pancreas, and the plasma C-peptide concentration causing the change of amylase activity were not affected by the dietary change during the 3rd wk of life. Therefore, the participation of milk-borne insulin, the exogenous insulin for pups, was assessed.

Milk-borne insulin and plasma insulin concentrations. High concentrations of insulin are found in the colostrum and milk of several species (11, 21, 31), suggesting that milk may be a source of insulin for sucklings. In the present study, the insulin concentration in the dams’ milk was first measured because there are no available data on the insulin levels in rat milk. Figure 5 shows the ontogenic changes in the insulin content of the dams’ milk. The insulin concentration was increased at 13 days postpartum and then plateaued at a level of ~15 ng/ml. It is possible that the insulin in the mothers’ milk is responsible for the increase and maintenance of amylase activity in rat pups. However, it is not yet known whether insulin in milk is absorbed from the gut in rat pups or whether the absorbed insulin affects neonatal development. For the determination of whether insulin in milk elevates the pups’ plasma concentration of insulin, rat pups were intragastrically administered overnight the milk formula with or without rat insulin. As shown in Fig. 6A, at 14 days of age, the plasma insulin level in the pups administered the milk formula without insulin was not different from that observed in mother-reared (MR) rats and no elevation of plasma insulin by the administration of 20 ng/ml of rat insulin was observed. In the 17-day-old rats administered the milk formula without rat insulin, the plasma level of insulin was as low as that in 14-day-old rats. Moreover, the administration of 20 ng/ml of insulin in the milk formula caused a significant increase in the plasma insulin concentration at 17 days old. No difference in the plasma C-peptide level between the cannulated groups and the MR group at 14 and 17 days of age was observed (Fig. 6B), suggesting that the insulin release from the pancreas was not altered by the milk-borne insulin or by the surgical manipulation. The plasma concentrations of glucose in the rats administered the milk formula with or without insulin (151 ± 6, 154 ± 2 mg/ml plasma, respectively) did not differ significantly from that of 17-day-old MR rats (Fig. 4B).

The elevation of plasma insulin by the intragastric administration of insulin was examined further in 17-day-old rats. Figure 7A shows the time course of the increase in the insulin concentration in plasma. The experiments were performed after the overnight administration of a milk formula to which insulin was not added to prevent the effect of the insulin in the maternal milk. The plasma level of insulin in the control rats that were intragastrically administered the milk formula was lower than that in the MR rats throughout
the experimental period. The administration of 20 ng/ml of insulin in the milk formula caused a significant increase in the plasma insulin concentration (1.5-fold increase over the control, $P < 0.05$) at 3 h after the first administration, and then the level of insulin remained unchanged up to 6 h. Next, the dose dependency of the increase in the plasma insulin level by insulin administration was estimated. Blood samples were collected 6 h after the first administration. When 5 ng/ml of insulin was administered intragastrically to suckling rats, an elevation of the insulin concentration in plasma occurred, but the elevation was not significant (Fig. 7B). The administration of 20 ng/ml of insulin, corresponding to the level in the dams’ milk, to suckling rats led to a significant increase in the plasma insulin level compared with that in the control rats ($P < 0.05$). When 80 ng/ml of insulin was administered, the plasma insulin level increased even more (1.6-fold higher compared with the control), although the administration of insulin failed to increase the plasma insulin level to that in the 17-day-old MR rats. These findings indicate that milk-borne insulin induces an elevation of the plasma insulin level in preweaning rats.

Effect of milk-borne insulin on pancreatic amylase activity. Finally, we investigated whether milk-borne insulin is a modulator of amylase activity in the third postnatal week with the use of a rat artificial rearing (AR) technique (10). An intragastric cannula was implanted in rat pups at 12 days of age, and the pups were given milk formula with (insulin-plus group) or without (insulin-minus group) 20 ng/ml of rat insulin until 17 days of age. As shown in Table 1, the body and pancreas weights in the two groups were not significantly different from those of the MR rats. In the insulin-minus group, the plasma level of insulin was as low as that in the 14-day-old MR rats, and the plasma insulin level in the insulin-plus group was 1.6-fold higher compared with the insulin-minus group, corresponding to the results in the rats administered insulin overnight (Fig. 7). The developmental increase in amylase activity was abolished in the insulin-minus group; the amylase activity in their zymogen granules was as low as that in the 14-day-old MR rats. Furthermore, in the AR rats raised on the milk formula with insulin, the amylase activity was significantly higher (1.5-fold) than that of the insulin-minus group. There was no significant difference in the concentrations of the plasma glucose; C-peptide; or corticosterone, a stress-responsive hormone; the daily growth rates after 13 days of age; or pancreatic trypsin activity between the two AR groups and the MR rats. These results indicated that the effects of stresses attributable to the infusion system and the operation on the AR rats were kept at a minimum and showed that the development of the AR rats was not delayed at all. The results suggested that

![Figure 7](http://ajpregu.physiology.org/)

**Fig. 7.** A: time course of the increase in insulin concentration in plasma after insulin administration in rat pups at 17 days of age. Rat pups were administered milk formula with 0 or 20 ng/ml of rat insulin at the speed of 0.30 ml/h, and blood samples were collected at 0, 1, 3, and 6 h after the first administration. B: concentration-dependent effect of insulin administration on the plasma insulin concentration. Rat pups at 17 days of age were administered milk formula with 0.5, 20, or 80 ng/ml of rat insulin at 0.30 ml/h, and blood samples were collected at 6 h after the first administration. Results are means ± SE for 8 animals. *$P < 0.05$, compared with control rats by an unpaired Student’s t test (A) or analysis of variance followed by the Tukey test for multiple comparisons (B).

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### Table 1. Effect of insulin contained in milk on the pancreatic amylase activity in rat pups at the time of weaning

<table>
<thead>
<tr>
<th>Animals</th>
<th>Weight</th>
<th>Plasma</th>
<th>Pancreatic Enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body, g</td>
<td>Pancreas, mg</td>
<td>Glucose, mg/dl</td>
</tr>
<tr>
<td>MR rats</td>
<td>31.9 ± 0.7</td>
<td>72.3 ± 7.5</td>
<td>152 ± 2</td>
</tr>
<tr>
<td>AR rats</td>
<td>30.5 ± 1.7</td>
<td>78.3 ± 4.7</td>
<td>146 ± 3</td>
</tr>
<tr>
<td>Insulin –</td>
<td>30.5 ± 0.2</td>
<td>74.0 ± 2.1</td>
<td>151 ± 7</td>
</tr>
<tr>
<td>Insulin +</td>
<td>30.5 ± 1.7</td>
<td>78.3 ± 4.7</td>
<td>146 ± 3</td>
</tr>
</tbody>
</table>

Results are means ± SE for 4 animals. An intragastric cannula was implanted into rat pups in the artificially reared (AR) groups at 12 days old, and the rat pups were given milk formula with (insulin +) or without (insulin –) 20 ng/ml rat insulin until 17 days of age. MR, mother reared. *$P < 0.05$, compared with the insulin – group by an unpaired Student’s t-test.
milk-borne insulin is crucial in the development of pancreatic amylase in naturally weaned rats.

**DISCUSSION**

Developmental alterations in the digestive functions are essential for the dietary change at the time of weaning. The functional development of the exocrine pancreas results in an increase in an animal’s abilities to synthesize digestive enzymes and secrete them. Because the enzyme secretion by the rat pancreas increases markedly during the third postnatal week (3, 27), the changes in the pancreatic contents of digestive enzymes during this period are of significance. In particular, the increase in amylase activity is important in rats because the most significant dietary change at the time of weaning is the increase in complex carbohydrate intake. The results of the present study showed the development of amylase activity in pancreatic zymogen granules, which was characterized by the finding that the amylase activity increased from 14 days, peaked at 18 days, and then decreased slowly (Fig. 2A). No visible sign of solid food was found in the gastric contents of the nonweaned pups at 18 days of age, whereas in the rats raised in the regular cages, the major part of the gastrointestinal contents consisted of solid food at 19 days of age. It stands to reason that the amylase activity increased before the age of 18 days, when the rapid dietary transition began. Although several studies have demonstrated the development of pancreatic amylase during the rat weaning period (3, 8, 27), the mechanisms underlying the initiation of the ontogenic changes of amylase are unknown.

A relationship between the development of digestive enzymes and the changes in the diet composition has been documented (3, 14, 15, 17). These studies obtained conflicting results regarding the effect of weaning on the development of digestive functions. This may be because there has been no appropriate model system for the assessment of natural weaning with which suckling rats can grow without any stress (such as early forced weaning, the feeding of abnormal milk, and intermittent feeding). In this respect, the present system using the bilevel cage is suitable for examining the developmental process of the digestive functions on natural weaning. In the present study, the effects of the deprivation of solid food on the development of pancreatic exocrine enzymes and of plasma hormones that regulate this development were investigated using the special cages. The results resolved the controversy regarding the presence or absence of feeding controls of the digestive functions in weaning rats; that is, the amylase development during the third postnatal week is not under the control of nutritional changes, and there are other mechanisms by which pancreatic amylase increases with the dietary alteration of natural weaning.

Possible roles of insulin and corticosterone as mediators of the maturation of pancreatic amylase have been proposed (5, 8, 17), but the relationship between the development of pancreatic amylase during natural weaning and the changes of these hormone concentrations in plasma was by no means clear. In the present experiments, the close relationship between the plasma insulin level and pancreatic enzyme activities suggested the involvement of insulin as a stimulatory factor of pancreatic development in the early stage of weaning. Interestingly, the increased plasma insulin did not affect the plasma glucose level during the 3rd wk. Issad et al. (9) reported that an insulin-resistant state in plasma glucose metabolism was present in suckling rats that disappeared after weaning. The elevation of the plasma insulin level at the onset of weaning would be important as a stimulant of developmental processes, such as pancreatic amylase development, rather than the regulator of the plasma glucose level. Accumulating evidence indicates that the intestinal maturation at the beginning of the weaning period is controlled by plasma corticosterone (8, 31), a possible regulatory factor for pancreatic amylase (17, 18). Although corticosterone may participate in amylase development, the decrease in amylase activity after 19 days (Fig. 2A) and the present AR experiments (Table 1) indicated that amylase development during the third postnatal week may be mediated mainly by plasma insulin. Despite the marked decrease in the plasma insulin concentration at 18 days, amylase activity was maintained at a relatively high level, probably due to the activated mechanism of amylase synthesis and the slow second rise in the plasma insulin. The second increase in plasma insulin could also be important for the acute increase in amylase activity after 22 days.

The present data indicated that the secretion of endogenous insulin by the endocrine pancreas might remain unchanged during the 3rd wk of life in MR rats (Fig. 4). In addition, the release of endogenous insulin in 14- or 17-day-old rats administered milk formula with or without insulin might not differ from that in MR rats (Fig. 6). These results suggested that the increase in the plasma insulin level, which peaked at 17 days of age, may be attributable to exogenous insulin, i.e., milk-borne insulin, rather than the endogenous insulin secreted by the endocrine pancreas. Nevertheless, the endogenous insulin may also be important for the pancreatic amylase development. Lee et al. (16) showed that streptozotocin treatment of neonatal rats led to an impairment of their pancreatic amylase development. The increased plasma insulin based on both the constant secretion of endogenous insulin and the additional exogenous insulin may induce amylase development at weaning. Alterations of plasma insulin concentration could also occur as a result of altered rates of insulin clearance. In the present study, however, in rats administered insulin-deficient milk formula, the plasma insulin concentrations at 17 days of age were as low as those at 14 days of age in contrast with the increased values of the MR rats, suggesting that the age-dependent decrease in the clearance rates of plasma insulin did not occur from 14 to 17 days of age. The gradual increase in the plasma insulin concentration after 19 days could possibly be attributed to the decreasing rate of insulin clearance, because the plasma C-peptide level remained unchanged at the same time.
Plasma to that in MR rats or the pancreatic amylase concentration of insulin failed to increase the insulin level in gastrointestinal epithelium in weaning rats. In regard to the present results with C-peptide, intragastrically chang in plasma insulin in weaning rats. In this respect, the insulin in rat milk in the present study was relatively low at 10 days postpartum and then increased just before 15 days when the plasma concentration of insulin began to increase. The present study showed that 5 ng/ml of insulin did not sufficiently increase the plasma insulin level, whereas 20 ng/ml of insulin, corresponding to the level in rat milk, significantly increased the plasma insulin (Fig. 7B). These results might indicate that the elevation of the milk-borne insulin level at 13 days postpartum is important for the increase in plasma insulin in rat pups. The insulin concentration in milk remained unchanged thereafter, in contrast to insulin-like growth factor (IGF)-I, the level of which in rat milk was reported to decrease sharply at 20 days postpartum (11).

The results of the present study clearly demonstrated that intragastrically administered insulin caused an increase in the plasma insulin concentration in weaning rats. The absorption of intact large peptides, such as epidermal growth factor (6, 12, 30), IGF-I (12, 23, 32), immunoglobulin G (29), and nerve growth factor (28), from the gut during the suckling period is a widely recognized phenomenon. Indeed, it has been demonstrated by others that intragastrically administered insulin may be absorbed from the gut in several species, including rats (19, 20, 24), but the doses of administered insulin in these experiments were over 100-fold higher than that found in rat milk. Attributable to the difficulty in removing specific substances from milk, pharmacological doses of each factor, whose effect exceeds that of the factor contained in milk, were usually needed to examine the functional role of milk-borne substances in suckling rats. In this respect, the AR technique would be suitable and useful (10), because the artificial milk formula prepared in our laboratory contains scarcely any hormones and its composition can be controlled. The present data obtained with the AR technique showed that the oral intake of a physiological dose of insulin in milk elevates the plasma insulin level during the beginning of the weaning period in rats and can account for the characteristic change in plasma insulin in weaning rats. In regard to the present results with C-peptide, intragastrically administered insulin might be absorbed across the gastrointestinal epithelium in weaning rats.

In the present experiments, the intragastric administration of insulin failed to increase the insulin level in plasma to that in MR rats or the pancreatic amylase activity in zymogen granules as a result. A possible explanation is that rat milk contains protease inhibitors, important factors in the effective transport of large peptides from the milk to the blood in suckling rats (25, 29). Other studies showed that milk-borne peptides such as IGF-I remained stable in the gastrointestinal tract (26) and were protected against endoluminal degradation by several milk antiproteases (25). Similarly, it is possible that milk-borne insulin is protected by protease inhibitors contained in milk in MR rats. After 18 days of age, endoluminal insulin could be expected to be digested, owing to the increase in pancreatic enzyme activities (27). Further experiments are necessary to clarify the putative actions of protease inhibitors for the effective absorption of insulin.

It is well known that some bioactive substances in milk are selectively transported intact across the gastrointestinal epithelium (6, 28) and that other proteins are taken up through ileal nonspecific endocytosis in suckling rats (6, 11, 28). In the present study, we did not examine whether insulin is selectively or nonselectively absorbed, or the sites of absorption of insulin. However, we found that the insulin concentration in milk remained unchanged after 13 days (Fig. 5), although the plasma insulin level increased constantly from 14 to 17 days (Fig. 3B); in addition, insulin absorption was observed at 17 days but not at 14 days (Fig. 6). These results may indicate that a specific pathway for insulin appears at the onset of weaning. Because an excess uptake of insulin would have a hypoglycemic effect on pups and because insulin would be precious for the mothers, insulin absorption could be efficiently and strictly controlled via such a specific pathway. The presence of insulin receptors on the intestinal epithelium during the suckling period was recently demonstrated (2, 21), raising the possibility that the receptor is correlated with the transport of insulin across the gastrointestinal epithelium.

Taken together, the present results suggested that milk-borne insulin plays a crucial role in the development of pancreatic amylase. The increase in plasma insulin concentration attributable to endogenous insulin may begin before or after 20 days of age, although the beginning of weaning occurs before then. Therefore, the exogenous milk-borne insulin, as a substitute for endogenous insulin, might stimulate the amylase development of the exocrine pancreas. Pancreatic amylase activity decreased in response to the decrease in plasma insulin after 18 days, but was maintained at a relatively high level. This series of processes is most likely essential for a smooth weaning process. After 21 days of age, the insulin release from the pancreas could increase and concomitantly pancreatic amylase could be expected to begin to change adaptively in response to dietary carbohydrate (4, 27).

Perspectives

Milk contains a significant number of substances in addition to insulin that are known to possess biological
activity (11, 22, 31), whereas infant milk formulas would contain only a small amount of these substances because of ingredients from bovine milk. These substances might have roles in neonatal growth and development (11); studies of the physiological functions of milk-borne substances are therefore interesting and important. For this purpose, infant rats could be suitable as a model of human infants, because the rat AR system has an advantage in that the physiological requirements of milk-borne substances for maturational events in various organ systems can be examined by means of the exclusion and readition of the substances (10). Philipps et al. (23) recently showed, with the use of an AR technique, that the administration of IGF-I to infant rats may increase the serum level of IGF-I and modulate gastrointestinal tract growth (23). Nevertheless, the questions of what role the milk-borne substances, including insulin, play in functional developmental processes and how other substances may collaborate with the effect of certain substances on the developmental processes (especially in tissues milk does not directly contact) remain to be answered. To improve infant milk formulas by supplementation with the substances normally found in breast milk, the functions of these substances should be further elucidated with the use of the AR system.

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