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Insulin Stimulation of Ornithine Decarboxylase Activity in Developing Rat Heart

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ABSTRACT

Ornithine decarboxylase (ODC; EC 4.1.1.17) is an important enzyme in the synthesis of polyamines and is associated with growth and differentiation. Insulin stimulation of cardiac ODC has been proposed as a marker of the functional completion of the sympathetic pathway to the rat heart. However, earlier studies, using subsaturating substrate concentrations and a single time point measurement after insulin treatment, have been inconsistent concerning the postnatal age at which significant insulin stimulation of ODC occurs. The present study, using a validated near-saturating substrate assay, examines more thoroughly early neonatal insulin induction of cardiac ODC with respect to both the magnitude and the time course of response. Insulin (20 IU/kg s.c.) significantly increased ODC activity at several time points at each postnatal age measured (days 2, 5, 8, 15 and 22), with maximum ODC activity occurring by 2.5 to 3 hr after insulin injection at all ages. Insulin-stimulated ODC activity was increased over control levels by 86, 84, 87, 150 and 127% on days 2, 5, 8, 15 and 22, respectively. These results demonstrate that age is not a variable in the time of peak insulin stimulation of ODC activity and, in contrast to earlier reports, show that significant insulin induction of cardiac ODC activity occurs reliably across ages in the early postnatal period. The inconsistency of earlier studies may be due to a number of factors, including the use of subsaturating enzyme assays only, known to be subject to several types of error.

Insulin affects growth and metabolism in a variety of tissues, an example of which is insulin stimulation of cardiac ODC (EC 4.1.1.17) in rats (Bareis and Slotkin, 1978; Roger and Fellows, 1980). ODC is an important enzyme in the synthesis of polyamines and is associated with growth and differentiation (Russell and Synder, 1968; Russell, 1980). It has also been used as an enzymatic marker of tissue response to pharmacological stimuli in studies of cardiac and SNS ontogeny (Slotkin, 1979; Slotkin and Thadani, 1980; Haddox *et al.*, 1981; Miska *et al.*, 1984). During postnatal development insulin stimulation of cardiac ODC is reported to first occur at about 5 to 8 days of age (Bareis and Slotkin, 1978; Slotkin, 1979). However, the magnitude of increases in insulin-stimulated ODC activity during the first postnatal week have been inconsistent within and between laboratories (Bareis and Slotkin, 1978; Lau and Slotkin, 1979a; Bartolome *et al.*, 1981; Thadani, 1983a,b). These studies used subsaturating concentrations of ornithine in ODC assays in order to detect changes in enzyme affinity for ornithine which have been documented during development and after drug treatment (Lau and Slotkin, 1980). The present study examines in a more rigorous manner the effect of early neonatal insulin exposure on ODC activity in the rat heart, using a saturating ornithine concentration in order to determine if K_m changes alone are responsible for reported activity differences. Also, because the time course of insulin stimulation of heart ODC activity at early postnatal ages has not been reported, ontogenetic changes in the time course might account for some inconsistencies. ODC activity was measured at five ages over the first 3 weeks of neonatal life and evaluated relative to the time course and magnitude of its response after insulin or saline administration.

Methods

Chemicals. L-[1-^{14}C]ornithine monohydrochloride (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA); Tris and sucrose from Schwarz/Mann (Orangeburg, NY); disodium EDTA, citric acid and sodium chloride from Fisher Scientific Co. (Pittsburgh, PA); BSA and sodium fluoride from Sigma Chemical Co. (St. Louis, MO); dithiothreitol from Aldrich Chemical Co. (Milwaukee, WI); ethanolamine and 2-methoxyethanol (Scintillation Grade), pyridoxal 5-phosphate and L-(+)-ornithine monohydrochloride from Eastman Kodak

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ABBREVIATIONS: ODC, ornithine decarboxylase; SNS, sympathetic nervous system; BSA, bovine serum albumin; DFMO, α-difluoromethylornithine; PND, postnatal day.

Co. (Rochester, NY); Scintisol liquid scintillation fluid from Isolab, Inc. (Akron, OH); DFMO from Midwest Research Institute (Kansas City, MO); and insulin (pork, insulin injection USP, regular, 10 U/ml) from E. R. Squibb and Sons, Inc. (Princeton, NJ).

Animals. Date-mated Sprague-Dawley-derived CD rats (approximately 200–250 g) were obtained from the National Center for Toxicological Research animal colony (Jefferson, AR). They were housed individually on a 12-hr light cycle and received laboratory chow (Purina No. 5012) and water *ad libitum*. The day of littering (before 8:30 A.M.) was designated PND 0. On PND 1, all pups were pooled by sex and redistributed randomly to maternal animals, with litters standardized at 10 pups (five of each sex).

Tissue preparation. Pups were selected (balanced with respect to sex) using a randomized design, weighed and injected s.c. (1 ml/kg) with either 0.9% saline or insulin (20 IU/kg). This insulin dose produces maximal ODC stimulation (Bareis and Slotkin, 1978) and elevates serum insulin levels above normal (Roger and Fellows, 1980). At intervals after injection, pups were sacrificed by either decapitation (PNDs 2, 5 and 8) or cervical dislocation (PNDs 15 and 22). The hearts were excised, rinsed in 0.9% saline, blotted, weighed, frozen in liquid nitrogen and stored at −70°C until assayed (Haddox *et al.*, 1981). Hearts were homogenized in about 10 volumes of ice-cold Tris-sucrose buffer (50 mM Tris-HCl, pH 7.2, and 250 mM sucrose) with a Polytron (Brinkmann Instruments, Westbury, NY) homogenizer and centrifuged (Beckman Instruments model L5-75 ultracentrifuge, Type 42.2 Ti rotor) at 40,000 × *g* for 15 min at 4°C. The supernatant fraction was analyzed for protein using the Bio-Rad Laboratories (Richmond, CA) protein assay [based on the Bradford (1976) dye-binding method], BSA standards and a Beckman Instruments model 25 spectrophotometer.

ODC assay. ODC activity was determined by measuring the amount of $^{14}CO_2$ released from L-[1-^{14}C]ornithine by a modification of the method of Russell and Snyder (1968). The supernatant fraction (150 μl) was incubated, with gentle shaking, for 1 hr at 37°C with 25 μl of reaction mixture (50 mM Tris-HCl, pH 7.2; 2.4 mM L-(+)-ornithine monohydrochloride; 40 mM sodium fluoride; 0.4 mM pyridoxal 5-phosphate; and 0.8 mM disodium EDTA) and 25 μl of 0.8 mM L-[^{14}C] ornithine (prepared in 50 mM Tris-HCl, pH 7.2, and 16 mM dithiothreitol). Final concentrations in the incubation mixture were 0.1 mM L-[1-^{14}C]ornithine, 0.3 mM L-(+)-ornithine monohydrochloride, 5 mM sodium fluoride, 0.05 mM pyridoxal 5-phosphate, 0.1 mM disodium EDTA and 2 mM dithiothreitol. Before diluting the L-[1-^{14}C]ornithine, the open vial was gently shaken at room temperature for 1 hr in order to release free $^{14}CO_2$ and minimize background (D. H. Russell, personal communication). Incubation was performed in a 13-ml polypropylene tube capped with a Kontes rubber stopper from which a polypropylene center well was suspended above the incubation mixture. The reaction was terminated by injecting 500 μl of 2 M citric acid through the rubber stopper into the incubation mixture, and the tubes were gently shaken for an additional 30 min at 37°C to allow complete absorption of the evolved $^{14}CO_2$ by a 2:1 (volume) mixture (200 μl) of ethanolamine and 2-methoxyethanol contained in the center well. Assay blanks containing Tris-sucrose buffer instead of supernatant cytosol were subtracted as background (typically < 1% of insulin-stimulated ODC activity). The center well was counted for ^{14}C in 10 ml of Scintisol with a Packard Tri-Carb liquid scintillation spectrometer at 93% efficiency.

In validation studies to determine if measured activity respresented only ODC activity, pups at various ages were injected s.c. (1.5 ml/kg) with either saline or DFMO (500 mg/kg), a specific, irreversible inhibitor of ODC, and were sacrificed 3 hr later (Slotkin *et al.*, 1982). Hearts were removed, frozen, stored, prepared and assayed for ODC as described above.

Statistical methods. Data for each age were collected in three replicates (two at PND 5), with each replicate consisting of two pups per treatment per time point. Within an age, the observed differences in the ODC activities between saline- and insulin-injected pups and among kill times for each injection were tested for statistical significance by an analysis of variance. All hypotheses were tested by the mean square error among pups for the same injection and kill time.

Results

Validation studies demonstrated that ODC activity in frozen hearts remained stable at −70°C in all age groups examined (table 1) and that *in vivo* administration of DFMO, a specific, irreversible inhibitor of ODC, almost completely inhibited $^{14}CO_2$ generation in cytosol except on PND 22 (table 2). Whereas the activity in saline-treated animals fell 5-fold from PND 5 to PND 22, the activity remaining after DFMO inhibition did not show the same pattern with age. Thus, although the activity remaining after DFMO treatment was equivalent on PNDs 5 and 22, the fall in basal ODC with age resulted in a higher percentage of noninhibitable activity on PND 22. The minimal residual activity in the youngest animals after DFMO treatment demonstrates that the ODC activity described in the present study resulted largely from ornithine, rather than nonspecific, decarboxylation.

Figure 1 depicts basal- and insulin-stimulated ODC activity on both a time-from-treatment and age-related basis. Basal activity did not vary significantly at any age tested at any time after saline injection. As expected, the level of basal ODC activity declined progressively with age.

As shown in figure 1, insulin significantly ($P \leq .0002$) increased ODC activity above basal levels at all ages tested, even as early as PND 2. Significant stimulation was seen at several time points after injection, with maximum activity generally occurring by 2.5 to 3 hr. This maximum response, when taken as the average response occurring at 2.5 to 3 hr, represented an increase over control values of 86, 84, 87, 150 and 127% on PNDs 2, 5, 8, 15 and 22, respectively. Insulin-stimulated activity returned to base-line values by 5.5 hr at all ages except PND 2.

TABLE 1

Effect of freezing on cardiac ODC activity

Hearts from separate groups of rats at the indicated ages were either processed immediately for ODC determination or frozen as described under "Methods" and assayed 6 to 7 days later.

Age	ODC Activity[a]	
	Fresh tissue	Frozen tissue
	nmol CO_2/hr/g	
PND		
5	30.3 ± 6.0	27.7 ± 3.6
8	24.7 ± 2.7	25.1 ± 2.6
15	19.1 ± 1.8	18.9 ± 2.2
22	11.2 ± 2.8	9.4 ± 1.2

[a] Values are the mean ± S.E. of seven to eight determinations from independent groups of hearts.

TABLE 2

Effect of DFMO on ODC activity

Rats at the indicated ages were injected with either saline or DFMO (500 mg/kg) in saline, sacrificed 3 hr later and their hearts processed for ODC determinations.

Age	ODC Activity[a]		
	Saline	DFMO	
	nmol CO_2/hr/g		%
PND			
5	35.5 ± 5.1 (8)	1.2 ± 0.4 (4)	3.4
8	24.5 ± 4.5 (8)	0.7 ± 0.1 (4)	2.9
15	10.4 ± 1.6 (8)	0.5 ± 0.3 (5)	4.8
22	7.3 ± 5.9 (8)	1.4 ± 0.2 (4)	19.2

[a] Values are the mean ± S.E. of the number of animals in parentheses.

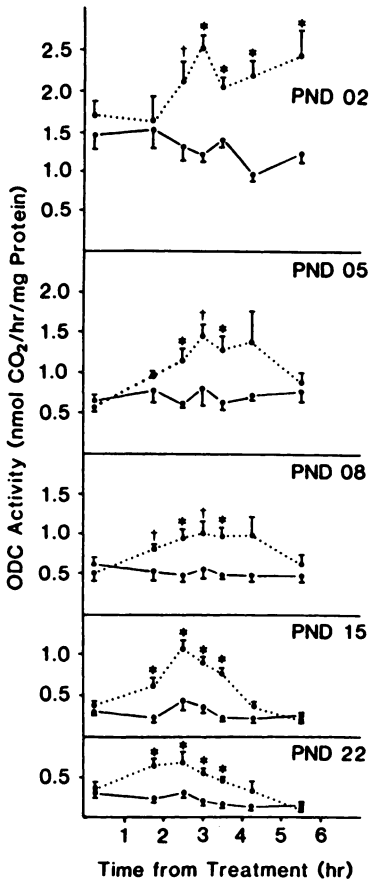

Fig. 1. Time course of cardiac ODC activity in rats treated with saline (——; control) or insulin (· · ·; 20 IU/kg) on PNDs 2, 5, 8, 15 and 22. Each point represents the mean ± S.E. of a minimum of four hearts. * Significant difference ($P < .05$) from corresponding control time point. † Additional insulin time points significantly different from the mean of all control time points.

Discussion

In our validation studies, we demonstrated the presence of ODC in frozen tissue at levels seen in fresh tissue. That this was authentic ODC was shown by the 81 to 97% inhibition of activity by DFMO. The level of activity remaining after DFMO treatment, unlike that left after saline treatment, did not change greatly with age and may have resulted from nonenzymatic or nonspecific enzymatic decarboxylation (Slotkin and Bartolome, 1983). In previous studies on heart ODC, a nonsaturable activity, insensitive to cycloheximide inhibition, was found at ornithine concentrations above 400 μM (Lau and Slotkin, 1979b; Slotkin and Bartolome, 1983). This artifact made ODC activity measurements at this and higher substrate concentrations inaccurate. In our studies, shaking the [^{14}C] ornithine solution for 1 hr before assay released significant amounts of $^{14}CO_2$ which would have otherwise increased blank values. The remaining nonenzymatic decarboxylation in blank assay tubes was only 1% of the activity in insulin-stimulated hearts. Additionally, substrate saturation studies show cardiac ODC activity to be saturable at several early postnatal ages (Miska *et al.*, 1984; S. P. Miska, unpublished data) with K_m values of 0.09 to 0.20 mM, similar to values reported for both crude (Lau and Slotkin, 1979b) and purified (Flamigni *et al.*, 1984) heart ODC. Thus, our data demonstrate that artifacts need not limit study design as, under appropriate conditions, authentic ODC can be assayed at saturating substrate concentrations.

The pattern of basal cardiac ODC activity found in the present study was similar to that reported previously (Anderson and Schanberg, 1972; Bareis and Slotkin, 1978; Thadani, 1983b; Miska *et al.*, 1984). There was a decrease in basal activity of about 5-fold from PND 2 to PND 22. This is indicative of changes in cardiac development and is consistent with alterations in cardiac growth rate and DNA and protein synthesis occurring during this period (Claycomb, 1975, 1977; Miska *et al.*, 1984).

Time course studies of the ontogeny of insulin-stimulated cardiac ODC activity have not been published. Thus, it was postulated that age-dependent differences in the time course of induction might account for some of the variability noted within and between laboratories. However, the present study demonstrated that the time of peak ODC activity after insulin treatment is constant across ages, even as early as PND 2. The stimulation on PND 2 occurred in a manner similar to that on PNDs 5 and 8, in that maximum activity was reached within 2.5 to 3 hr, and activity was about 80% above basal levels. We found, in two to three independent relicates, significant insulin stimulation of ODC not only at all ages tested, but also at several time points at each age. This observation provides greater confidence than single time point measurements can that insulin does stimulate heart ODC at early postnatal ages.

The reproducible stimulation seen at all ages in the present study is in contrast to earlier reports, in which significant stimulation was not uniformly observed across ages. In one study, Bareis and Slotkin (1978) report significant stimulation on day 8, but not day 4. In a subsequent report (Lau and Slotkin, 1979a), significant stimulation was shown to occur on day 4, but not day 2, whereas in another study day 2 values are significantly increased, but not day 4 values (Bartolome *et al.*, 1981). In addition, reports from another laboratory show significant insulin stimulation of ODC activity on days 3, 5 and 7 (Thadani, 1983a) and on days 4, 6 and 8 (Thadani, 1983b). A number of explanations have been advanced to account for these discrepancies, including animal-to-animal variations and non-neuronal "special mechanisms" lost soon after birth (Lau and Slotkin, 1979a; Bartolome *et al.*, 1981). Other possible explanations for these inconsistencies are: 1) the failure to observe a significant response at early ages may be due to inadequate resolving power of the experiment (insufficient number of animals) or to a statistical anomaly (chance occurrence); 2) the maturation process may vary in onset in animals from various laboratories, due to environmental or genetic differences; and 3) the use of subsaturating ornithine concentrations provides less precision in the ODC assay. Enzyme velocity is much more sensitive to changes in substrate concentration at subsaturating *vs.* saturating concentrations, and velocity measurements are therefore subject to more error in subsaturating assays. In all of these previous studies cited, subsaturating concentrations (5–12 μM) of ornithine were used. In a discussion of saturated *vs.* nonsaturated assays, Slotkin and Bartolome (1983) point out other potential pitfalls of using subsaturating ornithine concentrations, such as isotopic dilution by endogenous unlabeled ornithine and product inhibition by endogenous putrescine.

Additional problems in interpretation also exist. Two forms of ODC, having different ornithine affinities, have been described in the rat heart. The high affinity form, seen within the

first few days after birth, is replaced by a lower affinity form during postnatal life (Lau and Slotkin, 1979b; Miska *et al.*, 1984). Although subsaturating assays can detect both forms, the high affinity form will be preferentially measured in a mixture. Changes in the low affinity form have not been well documented, due to assay problems at high substrate concentrations as discussed earlier. Thus, subsaturating assays measure a mixture of the two K_m enzyme forms, as well as a mixture of K_m changes (Lau and Slotkin, 1979b) and V_{max} changes (this paper) induced by insulin. The present study used near-saturating ornithine concentrations, which should be considered when comparing our results with other reports of insulin stimulation of ODC. Under our conditions, only V_{max} changes are measured, that is, altered reaction velocity due to changes in enzyme protein concentration or in catalytic efficiency. Age- or insulin-dependent stimulation *via* K_m changes would not be measured, as the substrate concentration used here is near-saturating for both the high- and low-affinity forms of the enzyme (Miska *et al.*, 1984). In view of these facts, it is possible that only V_{max} stimulation occurs in the youngest insulin-treated animals, as their heart ODC is already in the high-affinity state, whereas both V_{max} and K_m changes occur in older animals, as has been documented for cardiac ODC after isoproterenol treatment (Miska *et al.*, 1984).

The stimulation of cardiac ODC activity by insulin has been suggested as a tool for testing the functional completion of the sympathetic pathway to the heart, with significant stimulation reported to first occur at about 1 week postnatally (Bareis and Slotkin, 1978). This is based on the principle that insulin-induced hypoglycemia stimulates the central nervous system and, ultimately, the heart through the sympathetic pathway (Bareis and Slotkin, 1978). However, nicotine does not signficantly stimulate rat heart ODC until several days after insulin (Bareis and Slotkin, 1978). This suggests that the development of functional sympathetic innervation in the heart is complex and that the use of insulin stimulation of ODC as a marker of completion of this process may be inappropriate. Studies using other methods, such as fluorescence histochemistry and field stimulation of intramural cardiac nerves (Standen, 1978; Mackenzie and Standen, 1980), show regional differences in the timing of cardiac innervation. Thus, these methods may be more suitable for measuring effects on the ontogeny of sympathetic innervation and function in the rat heart.

Furthermore, our data, demonstrating significant insulin stimulation of cardiac ODC at all ages examined, suggests a non-neuronal mechanism for insulin stimulation of V_{max} changes in cardiac ODC. In support of this interpretation, Thadani (1983a,b) has reported insulin stimulation of cardiac ODC on day 3 which is not completely blocked by chlorisondamine, a ganglionic blocking agent. Also, Bartolome *et al.*, (1981) found significant stimulation on day 2 which was not prevented by the *beta* adrenoceptor blocker, propranolol. In these studies, however, low substrate concentrations were used, so it is not known whether the increased enzyme activity found in the presence of inhibitors is due to a V_{max} or a K_m change. Roger and Fellows (1980), in concluding that it was unlikely that early postnatal insulin stimulation of brain ODC was secondary to insulin-induced hypoglycemia, considered several mechanisms, among them direct insulin stimulation, as an explanation. Insulin interacts with target tissues through a membrane receptor, and the rat heart shows a high level of insulin binding (Posner *et al.*, 1974) with characteristics of a specific insulin receptor (Eckel and Reinauer, 1980). Furthermore, Santora *et al.*, (1979) and Wheeler *et al.*, (1980) have characterized insulin binding in embryonic chick heart cells and have proposed a two-receptor model to relate binding and functional responses. Steven and Whitsett (1979) have also demonstrated insulin binding in neonatal hearts of other species. Thus, a receptor mechanism for direct interaction of insulin with myocardial membranes appears to be one possible explanation for stimulation of ODC activity before innervation. Our data suggest that further experiments are necessary to elucidate the mechanism of insulin stimulation of cardiac ODC. Substrate saturation analyses under the assay conditions described here should allow resolution of the high and low K_m forms of the enzyme before and after insulin treatment. Additionally, the use of *beta* adrenoceptor blockers under validated experimental conditions should be considered in such an analysis of both enzyme forms, to determine the role of sympathetic innervation.

In summary, the present study has demonstrated that the rat heart is capable of responding to insulin with increases in ODC activity as early as PND 2. The temporal pattern of response was similar across ages, and the percentage of increase of the response over basal levels almost doubled between the 2nd and 3rd week. These results do not preclude cardiac stimulation through an indirect mechanism. However, if the functional connection of the heart and SNS is incomplete before PNDs 6 to 8, then alternative hypotheses must be considered. The evidence suggests that the developing heart may be able to respond directly to insulin and that the relationship of insulin binding and ODC stimulation should receive further attention.

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References

ANDERSON, T. R. AND SCHANBERG, S. M.: Ornithine decarboxylase activity in developing rat brain. J. Neurochem. **19:** 1471–1481, 1972.

BAREIS, D. L. AND SLOTKIN, T. A.: Responses of heart ornithine decarboxylase and adrenal catecholamines to methadone and sympathetic stimulants in developing and adult rats. J. Pharmacol. Exp. Ther. **205:** 164–174, 1978.

BARTOLOME, J., SCHANBERG, S. AND SLOTKIN, T.: Premature development of cardiac sympathetic neurotransmission in the fetal alcohol syndrome. Life Sci. **28:** 571–576, 1981.

BRADFORD, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein dye-binding. Anal. Biochem. **72:** 248–254, 1976.

CLAYCOMB, W. C.: Biochemical aspects of cardiac muscle differentiation. Deoxyribonucleic acid synthesis and nuclear and cytoplasmic deoxyribonucleic acid polymerase activity. J. Biol. Chem. **250:** 3229–3235, 1975.

CLAYCOMB, W. C.: Cardiac-muscle hypertrophy. Differentiation and growth of the heart cell during development. Biochem. J. **168:** 599–601, 1977.

ECKEL, J. AND REINAUER, H.: Characteristics of insulin receptors in the heart muscle: Binding of insulin to isolated muscle cells from adult rat heart. Biochem. Biophys. Acta **629:** 510–521, 1980.

FLAMIGNI, F., GUARNIERI, C. AND CALDARERA, C. M.: Characterization of highly purified ornithine decarboxylase from rat heart. Biochim. Biophys. Acta **802:** 245–252, 1984.

HADDOX, M. K., WOMBLE, J. R., LARSON, D. F., ROESKE, W. R. AND RUSSELL, D. H.: Isoproterenol stimulation of ornithine decarboxylase blocked by propranolol during ontogeny of the murine heart. Mol. Pharmacol. **20:** 382–386, 1981.

LAU, C. AND SLOTKIN, T.: Accelerated development of rat sympathetic neurotransmission caused by neonatal triiodothyronine administration. J. Pharmacol. Exp. Ther. **208:** 485–490, 1979a.

LAU, C. AND SLOTKIN, T. A.: Regulation of rat heart ornithine decarboxylase: Change in affinity for ornithine evoked by neuronal, hormonal, and ontogenetic stimuli. Mol. Pharmacol. **16:** 504–512, 1979b.

LAU, C. AND SLOTKIN, T. A.: Regulation of ornithine decarboxylase activity in the developing heart of euthyroid or hyperthyroid rats. Mol. Pharmacol. **18:** 247–252, 1980.

MACKENZIE, E. AND STANDEN, N. B.: The postnatal development of adrenoceptor responses in isolated papillary muscles from rat. Pflügers Arch. **383:** 185–187, 1980.

MISKA, S. P., KIMMEL, G. L., HARMON, J. R. AND WEBB, P.: Ontogeny of cardiac ornithine decarboxylase and its *beta* adrenergic responsiveness in the rat. J. Pharmacol. Exp. Ther. **230:** 419–423, 1984.

POSNER, B. I., KELLY, P. A., SHIU, R. P. C. AND FRIESEN, H. G.: Studies of insulin, growth hormone and prolactin binding: Tissue distribution, species variation and characterization. Endocrinology **95:** 521–531, 1974.

ROGER, L. J. AND FELLOWS, R. E.: Stimulation of ornithine decarboxylase activity by insulin in developing rat brain. Endocrinology **106:** 619–625, 1980.

RUSSELL, D.: Ornithine decarboxylase as a biological and pharmacological tool. Pharmacology (Basel) **20:** 117–129, 1980.

RUSSELL, D. AND SNYDER, S. H.: Amine synthesis in rapidly growing tissues: Ornithine decarboxylase activity in regenerating rat liver, chick embryo, and various tumors. Proc. Natl. Acad. Sci. U.S.A. **60:** 1420–1427, 1968.

SANTORA, A. C. II, WHEELER, F. B., DEHAAN, R. L. AND ELSAS, L. J. II: Relationship of insulin binding to amino acid transport by cultured 14-day embryonic chick heart cells. Endocrinology **104:** 1059–1068, 1979.

SLOTKIN, T. A.: Minireview: Ornithine decarboxylase as a tool in developmental neurobiology. Life Sci. **24:** 1623–1630, 1979.

SLOTKIN, T. A. AND BARTOLOME, J.: Ornithine decarboxylase: Marker of neuroendocrine and neurotransmitter actions. Methods in Enzymol. **103:** 590–603, 1983.

SLOTKIN, T. A., SEIDLER, F. J., TREPANIER, P. A., WHITMORE, W. L., LEREA, L., BARNES, G. A., WEIGEL, S. J. AND BARTOLOME, J.: Ornithine decarboxylase and polyamines in tissues of the neonatal rat: Effects of α-difluoromethylornithine, a specific, irreversible inhibitor of ornithine decarboxylase. J. Pharmacol. Exp. Ther. **222:** 741–745, 1982.

SLOTKIN, T. A. AND THADANI, P. V.: Neurochemical teratology of drugs of abuse. *In* Advances in the Study of Birth Defects, ed. by T. V. N. Persaud, vol. 4, pp. 199–234, University Park Press, Baltimore, 1980.

STANDEN, N. B.: The postnatal development of adrenoceptor responses to agonists and electrical stimulation in rat isolated atria. Br. J. Pharmacol. **64:** 83–89, 1978.

STEVEN, J. AND WHITSETT, J. A.: Insulin binding to neonatal human, guinea pig and rat myocardial membranes (Abstract). Pediat. Res. **13:** 482, 1979.

THADANI, P. V.: Prenatal ethanol alters development of cardiac ornithine decarboxylase response to adrenergic agents in rat. I. Continuous exposure. Arch. Int. Pharmacodyn. Ther. **265:** 192–202, 1983a.

THADANI, P. V.: Prenatal ethanol exposure alters development of heart ornithine decarboxylase response to insulin in rat. II. Daily dose. Res. Commun. Chem. Pathol. Pharmacol. **41:** 19–36, 1983b.

WHEELER, F. B., SANTORA, A. C. II AND ELSAS, L. J. II: Evidence supporting a two-receptor model for insulin binding by cultured embryonic heart cells. Endocrinology **107:** 195–207, 1980.

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