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Mammary Gland Sympathetic Innervation Is a Major Component in Type 1 Deiodinase Regulation

Carmen Aceves, Rita Rojas-Huidobro, Nephtali Marina, Maria Teresa Morales, and Flavio Mena

Departamento de Neuroendocrinología, Centro de Neurobiología, Campus Juriquilla, UNAM. Querétaro, México

Recent observations have shown that in lactating rats previously deprived of suckling, either suckling stimulus or ip injection of norepinephrine was capable of increasing mammary deiodinase type 1 (M-D1) mRNA content and enzyme activity. In the present work, we show that intact efferent sympathetic mammary innervation is required to restore both mammary D1 mRNA content and enzyme activity, whereas suckling-induced secretion of catecholamines from the adrenal glands does not seem to participate in M-D1 enzyme regulation. The data also indicate that the sympathetic reflex activation in response to suckling involves two complementary autonomic components: (1) activation, presumably through mammary segmental arrangement affecting neighboring mammary glands; and (2) an individual reflex regulatory mechanism capable of maintaining M-D1 activity within each mammary gland. In addition to these findings, we show that the suckling-induced sympathetic activation of M-D1 activity could be blocked by prior activation of ductal mechanoreceptors. This set of regulatory and counterregulatory mechanisms seems to ensure the optimal control of mammary energetic expenditure according to litter size.

Key Words: Thyroid hormone metabolism; deiodinases; sympathetic innervation; mammary gland; lactation.

Introduction

Mediated through the binding of triiodothyronine (T_3) to specific nuclear receptors, thyroid hormones regulate important processes in the organism, e.g., growth and development, thermogenesis, and energy expenditure *(1,2)*. Metabolically active T_3 is mainly generated by the extrathyroidal enzymatic 5' deiodination of thyroxine, which is catalyzed by deiodinase type 1 (D1) and type 2 (D2). D1 is extremely sensitive to inhibition by propylthiouracil, has a ping-pong catalytic mechanism, and is responsible for generating ≈80% of circulating T_3. D2 has a sequential catalytic mechanism, and its function seems to be the local generation of T_3 for tissue-specific autoconsumption *(3)*. A concerted adaptive response of organ-specific changes in deiodinase activity and sympathoadrenal activity, aimed at preserving the organism's metabolic fitness, has been suggested by previous work *(4)*. We and others have shown that lactation resembles a selective pseudohypothyroidism state in which deiodinase activity is decreased in the liver, kidney, and brown adipose tissue, whereas at the level of the mammary gland, D1 is expressed *de novo* and its activity is directly related to the intensity of lactation *(5–7)*. Moreover, mammary D1 (M-D1) activity may provide the lactating gland with a local large quantity of T_3, which represents more than 80% of the nuclear T_3 content *(8)*. Recent findings have also shown that M-D1 is positively regulated by suckling through β-adrenergic receptors *(9)*. These findings are consonant with previous data showing that the mammary gland is an effector of the autonomic nervous system, in which both milk ejection and secretion may be regulated physiologically by catecholamines (CATs) *(10)*. In the present study, we sought to determine the role played by the adrenals and/or mammary nerve endings on the suckling-induced activation of M-D1. In addition, we analyzed the relation between the number of glands suckled and the degree of M-D1 enzyme restitution. Finally, we conducted a series of experiments to determine whether the suckling-induced M-D1 activity could be inhibited by the activation of ductal mechanoreceptors.

Results

Adrenalectomy

Bilateral adrenalectomies or sham operations (exposure of adrenal glands without ligature) were performed through a dorsal incision under ether anesthesia 48 h before the experimental procedure. The mothers were returned to their pups 2 h after surgery, and they were given saline water to drink ad libitum. On the day of the experiment, the mothers were separated from their pups for 12 h. One group was sacrificed (12 h nonsuckled group [12hNS]) and the

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Author to whom all correspondence and reprint requests should be addressed: Carmen Aceves, Departamento de Neuroendocrinología, Centro de Neurobiología, Campus Juriquilla, UNAM, Apartado Postal 1-1141, Querétaro, Qro. 76001, México. E-mail: caracev@servidor.unam.mx

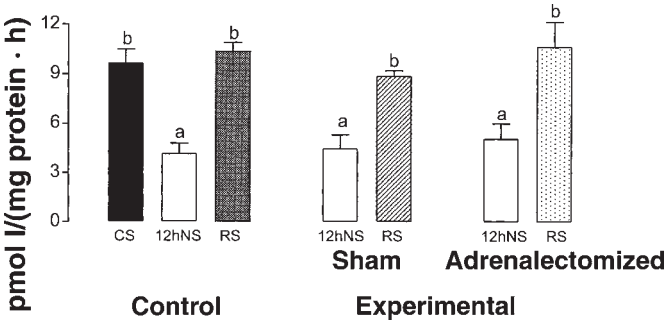

Fig. 1. Effect of 15 min of resuckling on M-D1 activity in conscious intact, sham, and bilaterally adrenalectomized mothers. Pups were removed from their mothers for 12 h. One group of mothers was sacrificed (12 h nonsuckled group [12 h NS]), and the others were resuckled by their pups for 15 min and sacrificed 4 h later (resuckled group [RS]). Control animals included intact mothers in which suckling was continuous (CS). Data are expressed as the mean ± SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$).

other was resuckled by their pups for 15 minutes and sacrificed 4 h later (resuckled group [RS]). As shown in Fig. 1, restitution of M-D1 activity was complete after 4 h in conscious control, sham, and adrenalectomized rats previously nonsuckled for 12 h and then suckled for 15 min.

Suckling Stimulation in Anesthetized Rats

Several groups of intact mothers were used. Each mother was separated from her pups for 12 h. In one set of experiments, mothers were anesthetized with urethane and placed on their backs on a warm slide (32–35°C). Next, the pups (one, two, three, or six pups) were manually placed in contact with the ventral surface of the mother and allowed a 1- or 2-min period for establishing contact and to attach to the nipples. Once suckling was established, the pups suckled for 15 min and then were removed. In a second set of experiments, conscious mothers were returned with their 10 pups allowing random suckling in all the glands for 15 min. In all experiments the mothers were killed 4 h later. Restitution of M-D1 mRNA content and enzyme activity in mammary glands varied according to the number of glands suckled by the pups. Thus, as shown in Fig. 2, whereas complete restoration occurred in all glands when 10 or 6 pups were allowed to suckle, suckling of three glands provoked maximal restoration in the corresponding suckled glands, and only a partial effect was observed in the rest of the glands. Finally, suckling of one or two glands provoked full restoration exclusively in the glands in which the stimulus was applied.

Denervation

The segmental nerve corresponding to the first left abdominal mammary gland (gland no. 7) was exposed and subsequently cut. Denervation or sham operations were performed under ether anesthesia 12 h before the experimental procedure. On the day of the experiment, the rats were anesthetized with urethane and suckling was applied for 15 min by one pup on each pair, the denervated or sham, and the contralateral intact gland. Next, the mothers were killed 4 h later. When suckling was applied by one pup to a denervated gland, restoration of M-D1 mRNA content and enzyme activity did not occur (Fig. 3).

Activation of Ductal Mechanoreceptors

Experiments were designed to determine whether the stimulatory effect of suckling on M-D1 activity could be blocked by prior activation of ductal mechanoreceptors *(11,12)*. To this end, 60 mU of oxytocin (OT) were given to the rats 40 min before applying the 15-min period of suckling with the litter (10 pups). As compared to control rats, which were injected intraperitoneally with saline, activation of mechanoreceptors by OT effectively blocked the suckling-induced restoration of both M-D1 mRNA content and enzyme activity (Fig. 4).

Discussion

This study was based on our recent observation that either suckling or intraperitoneally injected norepinephrine was able to increase mammary D1 mRNA content and its enzyme activity through activation of β-adrenergic receptors *(9)*. Also, a large body of information exists concerning the regulatory influence exerted by the sympathetic system on mammary gland physiology *(10)*. Thus, it was shown that suckling provoked the release of CAT from the adrenals into the circulation and that mammary innervation regulated milk ejection *(13,14)*. Mammary innervation consists of the somatic sensory (afferent) nerves, conveying impulses from tactile cutaneous and parenchyma mechanoreceptors to the central nervous system (CNS), and the sympathetic motor (efferent) nerves, which innervate blood vessels and contractile muscles of the teat and the ducts *(10,15)*. Previous work suggested that alveolar cells are not innervated, but more recent evidence suggests that the sympathetic nerve terminals in the mammary gland participate in the alveolar cell biosynthetic machinery. Thus, besides their presence in epidermis and dermis, around smooth muscle cells and blood vessels in the nipple and in the ducts, sympathetic nerve endings have also been found surrounding the alveoli *(16)*. The alveolar cell possesses β-adrenergic receptors, functionally coupled to adenylate cyclase, whose number and affinity are modified during lactation *(17)*. The content of norepinephrine of mammary gland exhibits changes during the reproductive cycle, and surgical denervation of the gland decreases both the content of norepinephrine and β-receptors *(18,19)*. In the present work, we observed that efferent mammary innervation is responsible for restoring the M-D1 activity and that adrenal glands do not seem to participate. These findings, together with previous data in which we showed that M-D1 activity is confined to the alveolar cell *(20)*, indicate that efferent innervation to the mammary glands is

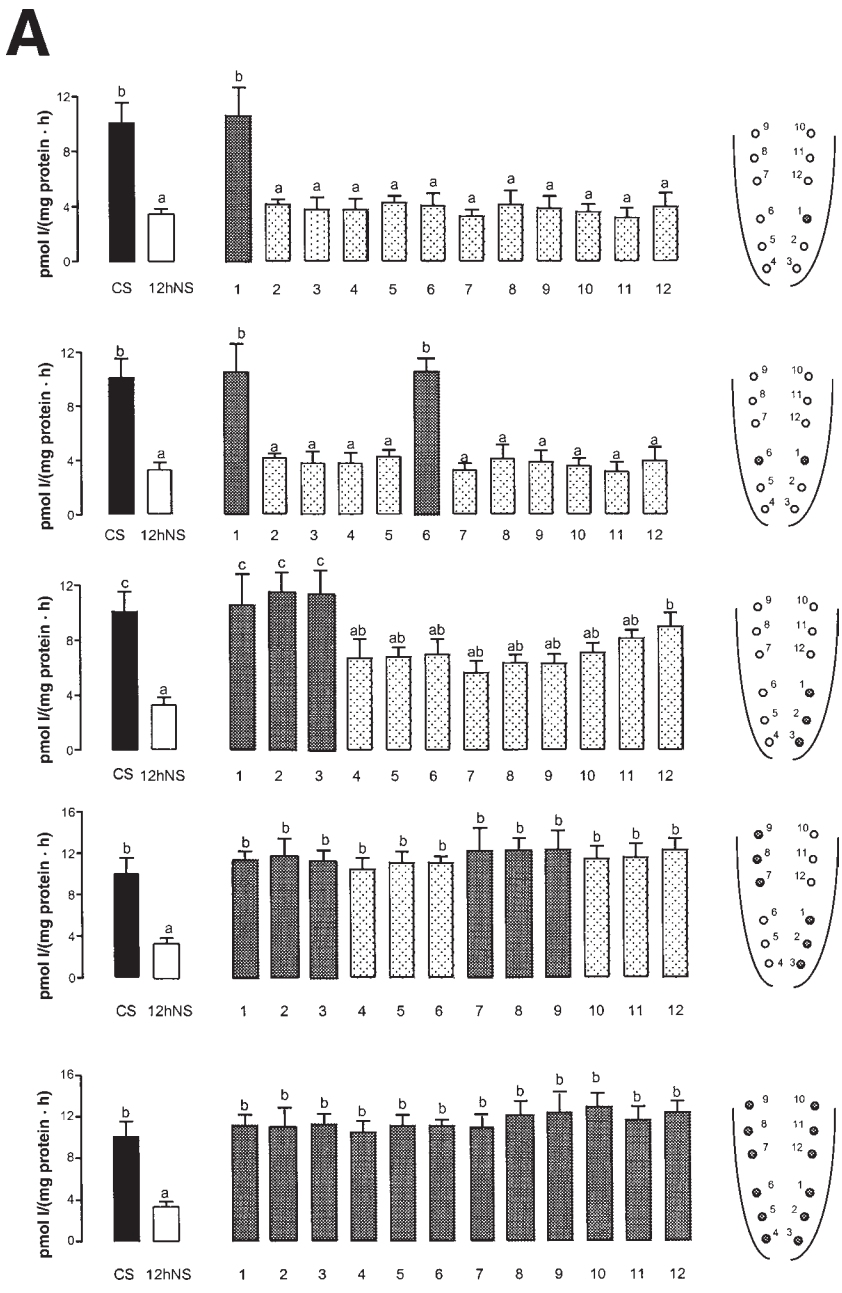

Fig. 2. Effect of differential suckling stimulation on M-D1 enzyme in anesthetized or conscious rats. 12h nonsuckled mothers were stimulated by 1, 2, 3, 6, or 10 pups (see details in Results section). **(A)** Comparison of M-D1 activity between mammary glands that were suckled (solid bar) or not suckled (open and dotted bars). **(Right)** Positions of the nipples that were suckled in each group. Data are expressed as the mean ± SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$).

involved in regulating energetic expenditure of the lactating mammary gland through the generation of local T_3.

Another finding in this study was the observation of a clear relationship between the number of glands suckled and the degree of M-D1 enzyme restitution. Thus, consistent with previous reports that six pups suckling is sufficient for maintaining lactation in all the glands *(21)*, suckling of six glands was as effective as suckling of all glands on the restitution of M-D1 mRNA content and enzyme activity. On the other hand, when three glands were suckled, full restoration of M-D1 enzyme was observed in the glands that were suckled, and only partial restoration occurred in the rest of the glands. Furthermore, when only one or two glands were suckled, enzyme restoration was found in the same gland and no effect was observed in the rest of the glands. Based on the reflexive sympathetic activation elicited by suckling, the present results suggest that the activation of D-1 in the different mammary glands is exerted through segmental arrangements; that a certain threshold, however, exists for these effects, between suckling by three and six pups; and that in addition to their involvement in the segmental effects, mammary neural

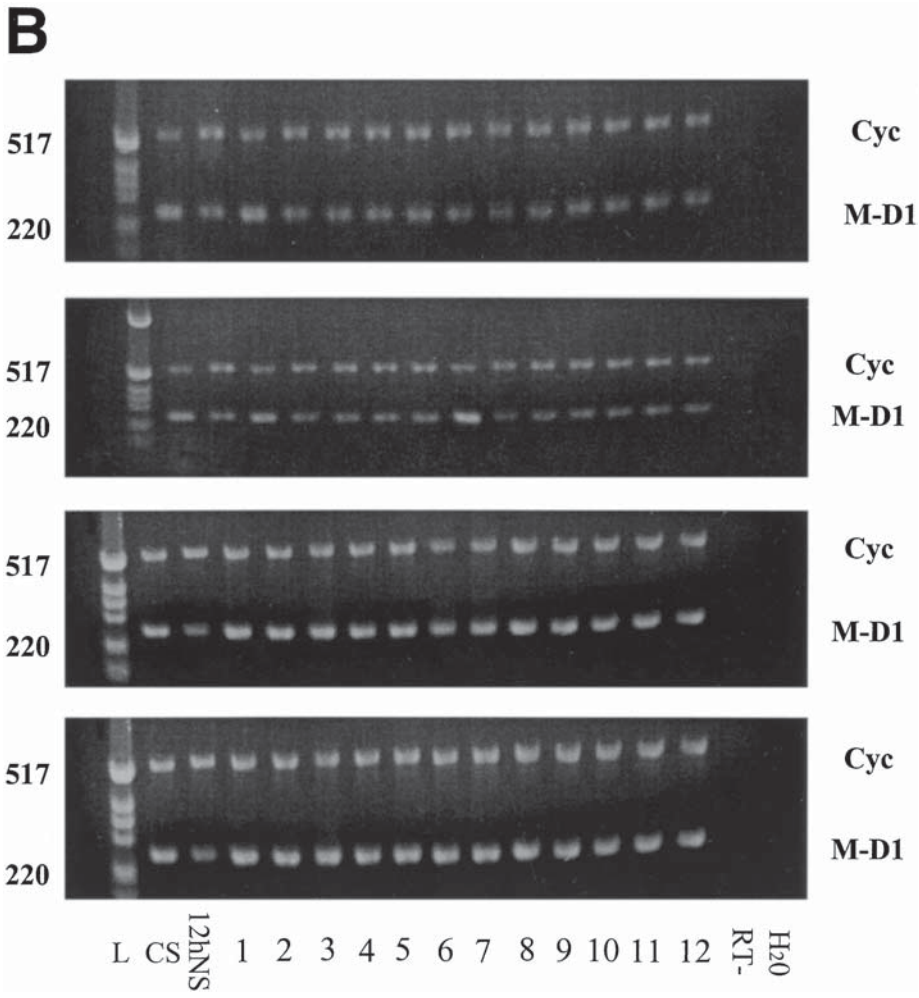

Fig. 2. Effect of differential suckling stimulation on M-D1 enzyme in anesthetized or conscious rats. 12h nonsuckled mothers were stimulated by 1, 2, 3, 6, or 10 pups (see details in Results section). **(B)** Ethidium bromide–stained gel showing reverse transcriptase-polymerase chain reaction (RT-PCR) products for M-D1 (251 bp) and Cyclophilin (Cyc) (521 bp) after 28 cycles of amplification. L, ladder; CS, animals with continuous suckling; 12hNS, mothers whose pups were removed for 12 h; 1–12, mammary glands; RT-, RNA sample and the appropriate oligonucleotide primers, but without the RT; H_2O, water with all the PCR reagents. **(Top to bottom)** M-D1 mRNA content of animals that were suckled by 1, 2, 3, or 6 pups. The experiments were repeated twice with independent RNA samples.

afferents exert a local regulatory mechanism capable of maintaining M-D1 activity on each suckled gland. The existence of these complementary mechanisms may have important survival value for the species, i.e., allowing a differential adjustment of energetic expenditure gland by gland, depending on the size of the litter.

Finally, our finding that suckling was ineffective in restoring M-D1 mRNA and enzyme activity in rats previously injected with 60 mU of OT is consonant with previous data *(12,14)* showing that the activation of mechanoreceptors interrupts sympathetic mammary outflow. These results suggest that under normal conditions, sympathetic activation within the first minutes of suckling simultaneously interrupts milk ejection and induces M-D1 enzyme, leading to maintenance of the high metabolic rate that characterizes the lactating mammary gland. Following this period, ductal mechanoreceptors may be activated and the milk obtained by the pups. In support of this interpretation is the fact that 15 min or fewer of suckling are sufficient for full restoration of M-D1 activity in the mammary glands *(9)*.

In conclusion, we have shown herein that regulation of M-D1 activity is exerted through reflex activation of mammary innervation by suckling. This sympathetic regulation involves segmental and local nervous arrangements, as well as the activation of ductal mechanoreceptors. This set of regulatory and counterregulatory mechanisms seems to ensure the optimal control of the mammary energetic expenditure according to litter size. Further work is required to determine whether the sympathetic reflex is processed at the spinal cord or involves higher levels of the CNS, and to determine the nature of ductal mechanoreceptors.

Materials and Methods

Reagents

Nonradioactive thyronines were obtained from Henning (Berlin, Germany). Radiolabeled rT_3 (sa 1174 μCi/μg) was

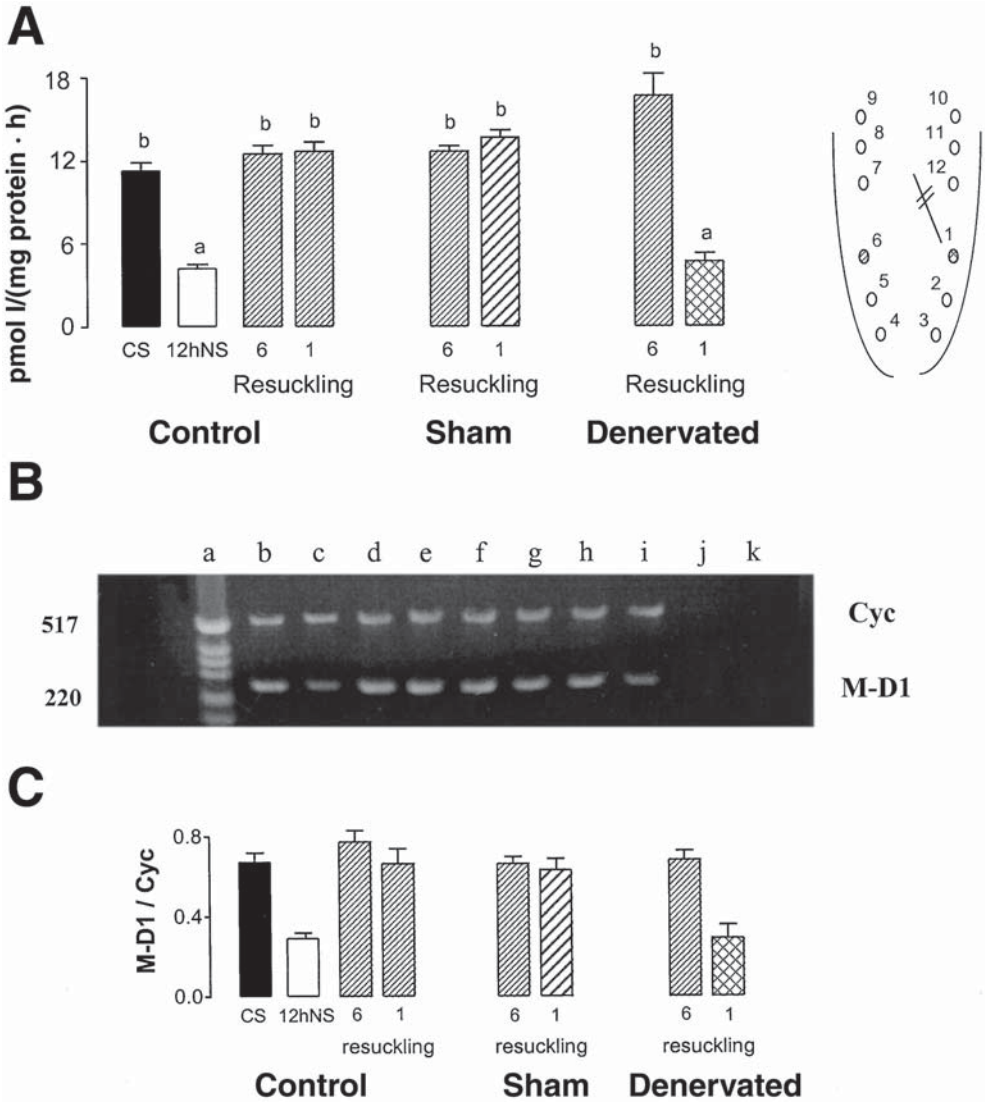

Fig. 3. Effect of 15 min of resuckling on M-D1 activity and mRNA content in intact, sham, or left abdominal mammary-denervated mothers. Anesthetized rats were resuckled for 15 min by one pup on both the denervated or sham and the contralateral intact gland (numbers 1 and 6). The mothers were killed 4 h later. Control animals included mothers in which suckling was continuous (CS), mothers in which the pups were removed for 12 h (12hNS), and 12hNS mothers resuckled by 15 min and killed 4 h later. **(A)** M-D1 enzyme activity. Data are expressed as the mean ± SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$). **(Right)** The position of the nipples that were suckled. **(B)** Ethidium bromide–stained gel showing RT-PCR products for M-D1 (251 bp) and Cyc (521 bp) after 28 cycles of amplification. Lane a, ladder; lanes b–i, experimental animals described above in the same order; lane j, RNA sample and the appropriate oligonucleotide primers, but without the RT; lane k, water with all the PCR reagents. **(C)** Quantitation by densitometry of Polaroid negative of ethidium bromide–stained gel. The experiments were repeated twice with independent RNA samples and the values were normalized to Cyc RNA amplicons (M-D1/Cyc).

purchased from New England Nuclear (Boston, MA). Dithiothreitol (DTT) was obtained from Calbiochem (La Jolla, CA) and OT from Armour (La Jolla, CA). Urethane (ethyl carbamate) was purchased from Cedrosa (Mexico City, Mexico). Oligonucleotides were synthesized by Gibco-BRL (Gaithersburg, MD). All other reagents were of the highest purity commercially available.

Animals

Primiparous rats of the Wistar strain were housed in individuals cages in a room with alternating 14 h of light and 10 h of dark. On postpartum d 1, the size of the litter was adjusted to 10 pups per mother, and all the experimental procedures were conducted on postpartum d 10 ± 1. The rats were fed Purina Lab Chow and tap water ad libitum. Procedures regarding care, administration of treatment, and euthanasia of animals were reviewed and approved by the supporting DGAPA/UNAM committee. Mothers were anesthetized with urethane administered intraperitoneally at a dose of 115–130 mg/100 g, and were killed by decapitation. Two grams of different mammary glands of each individual were dissected; 1 g of tissue was frozen imme-

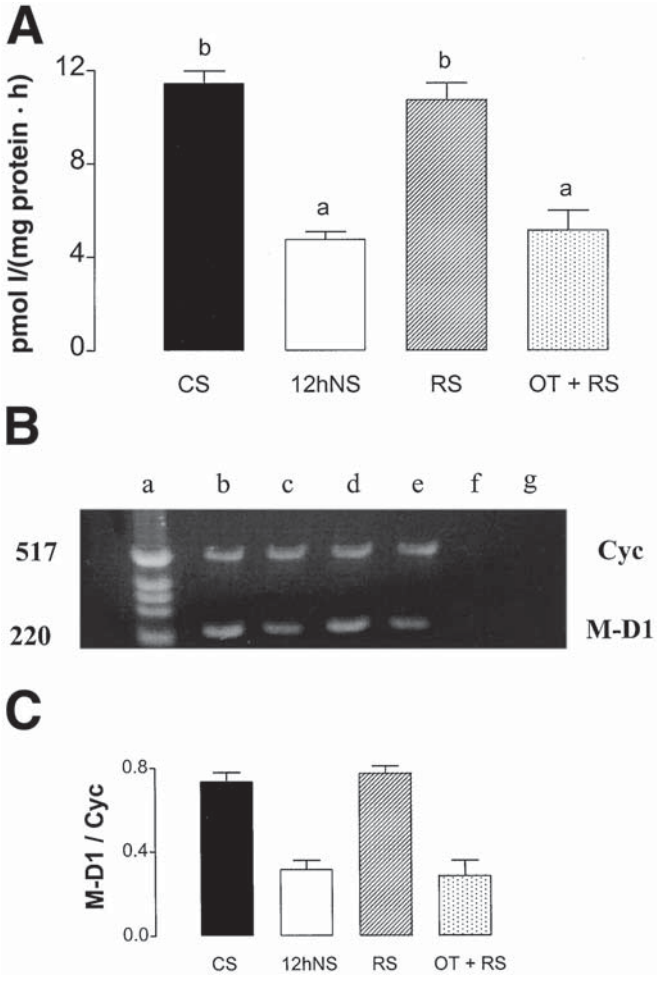

Fig. 4. Effect of 15 min of resuckling in conscious animals in which mammary mechanoreceptors were activated 40 min before resuckling (OT + RS). Animals received saline or 60 mU of OT 40 min before resuckling. Animals were resuckled for 15 minutes and sacrificed 4 h later. **(A)** M-D1 enzyme activity. Data are expressed as the mean ± SD ($n = 4$). Means with different letters are significantly different ($p < 0.05$). **(B)** Ethidium bromide–stained gel showing RT-PCR products for M-D1 (251 bp) and Cyc (521 bp) after 28 cycles of amplification. Lane a, ladder; lanes b–e, experimental animals described above in the same order; lane f, RNA sample and the appropriate oligonucleotide primers, but without the RT; lane g, water with all the PCR reagents. **(C)** Quantitation by densitometry of Polaroid negative of ethidium bromide–stained gel. The experiments were repeated twice with independent RNA samples and the values were normalized to Cyc RNA amplicons (M-D1/Cyc). Abbreviations as in Fig. 1.

diately on acetone–dry ice and 1 g was homogenized in guanidine thiocyanate.

All experiments and all test periods had parallel controls that consisted of rats with continuous suckling and 12hNS rats. Whenever the glands were not resuckled by pups, accumulated milk was removed by ip administration of OT (30 mU) 1 min before sacrifice. This procedure ensures that accumulated milk will not act as a dilution factor when tissue proteins are quantified.

Enzymatic Assay

Mammary D1 activity was determined by a modification of the radiolabeled iodide release method as described elsewhere *(22)* and standardized for mammary gland *(20)*. Mammary glands were homogenized in 10 m*M* HEPES, pH 7.0, with 0.32 *M* sucrose, 1.0 m*M* EDTA, and 10 m*M* DTT. The glands were centrifuged at 2800*g*, 30 min at 4°C. Assay conditions were as follows: 200 μg of protein, 2 n*M* ^{125}I-rT_3, 0.5 μ*M* nonradiolabeled rT_3, and 5 m*M* DTT. After a 3-h incubation, released acid-soluble radioiodide was isolated by chromatography in Dowex 50W-X2 columns. Proteins were measured by the Bradford method (Bio-Rad protein assay, Bio-Rad, Richmond, CA). Results are expressed as pmol of radioiodide released/mg of protein·h.

Reverse Transcriptase Polymerase Chain Reaction

D1 mRNA in lactating mammary glands was identified by using a previously standardized semiquantitative PCR procedure in which an amplicon of the structural protein Cyc was simultaneously amplified *(9)*. Briefly, the RT reaction was primed with oligo (dT) subscript, and a specific M-D1 antisense oligo primer (660-ATC CTG CCT TCC TGT ATC-677) with 5 μg of total mammary RNA that was isolated by a modification of Chrigwin's method *(23,24)*. The PCR reaction was carried out using 5 μL of RT mixture and the following primers: for D1, 377-GCA CCT GAC CTT CAT TTC TT-396 (sense) and 627-CTG GCT GCT CTG GTT CTG-610 (antisense); and for Cyc, 7-AGA CGC CGC TGT CTC TTT TCG-27 (sense) and 527-CCA CAC AGT CGG AGA TGG TGA TC-507 (antisense). The PCR mixture contained 25 pmol of each oligonucleotide primer, 200 μ*M* dNTPs, 1.5 m*M* $MgCl_2$, and 2.5U of *Taq* polymerase (Gibco-BRL) in a 100-μL reaction. Amplification was carried out for 28 cycles of melting at 94°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 1 min. As a control, a reaction mixture containing an RNA sample and the appropriate oligonucleotide primers, but without the RT was included in every experiment. The resultant PCR fragments were 251 bp for M-D1 and 521 bp for Cyc, and were resolved on a 3% agarose gel and visualized using ethidium bromide. The sizes of the bands were confirmed by a restriction-digested pUC plasmid (1-kb DNA ladder, Gibco-BRL). After a Polaroid photograph was taken, the photograph was digitized using a Hewlett Packard Scanner Jet 11CX, and the signals were analyzed by using an editing version of the National Institutes of Health-image program. Values obtained were normalized according to the Cyc mRNA levels detected in each sample.

Statistical Analysis

Data are expressed as the mean ± SD. Differences between experimental groups were analyzed using a one-way analysis of variance and Tukey's HSD test. Differences with a $p < 0.05$ were considered statistically significant.

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