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The Relationship Between Adenosine Diphosphate-Ribosylation and Mammary Gland Differentiation

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Poly(adenosine diphosphate [ADP]-ribosyl)ation, although associated with differentiation in many systems, exhibited a reciprocal relationship with mammary gland differentiation, and both the synthetic and degradatory pathways complemented each other in this regard. Poly(ADP-ribosyl)synthetase activity declined during pregnancy and lactation, while poly(ADP-ribose) degradatory activity rose late in pregnancy and peaked during lactation. In explant cultures, similar changes occurred and appeared to be under separate hormonal control; prolactin suppressed the synthetase activity, whereas insulin stimulated the poly(ADP-ribosyl)glycohydrolase activity. This latter effect may be mediated by a decline in cAMP levels for the following reasons: the glycohydrolase is known to be inhibited by cAMP, insulin decreased cAMP concentrations in mammary explants by 70%, and cholera toxin blocked the effects of insulin on poly(ADP-ribose) degradation. This reciprocal relationship between poly(ADP-ribosyl)ation and mammary gland differentiation is further supported by pharmacological studies: in the presence of insulin, cortisol, and prolactin, an inhibitor of the synthetase stimulated α-lactalbumin three-fold over hormone stimulation alone. However, this inhibitor was unable to induce differentiation in the absence of prolactin. Therefore, although there is a close association between a decline in enzyme activity and mammary differentiation, the data are insufficient to support a causal relationship.

Key words: poly(ADP-ribosyl)ation, mammary gland, histone Hl, HMG proteins, prolactin, insulin

Secondary induction is often easier to effect than primary induction: it may be faster [1,2], greater [3], more sensitive to the inducer [2,4], or less specific in the type of inducer required [5]. Although in some systems this phenomenon could be explained by higher levels of hormone receptors during secondary induction [6], this explanation is not adequate in the mammary gland, where the differential induction characteristics appear to be more readily attributable to alterations in chromatin structure [7]. There are several ways in which either DNA or nuclear proteins can be covalently modified to produce these structural changes, and the purpose of this paper

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is to evaluate the relationship of one of these adenosine diphosphate(ADP)-ribosylation, to mammary differentiation. ADP-ribosylation occurs primarily on the poly(ADP-ribosyl)synthetase itself (self-modification) and on the DNA linker proteins [8], is associated with chromatin relaxation and transcriptional potential [9–11], and can be induced by hormones, such as the sex steroids [12–14]. Since many of these hormones are also important in mammary development [15], the investigation of ADP-ribosylation in this system seems warranted.

MATERIALS AND METHODS

Materials

Ovine prolactin (oPRL-15) was kindly provided by the Hormone Distribution Program, NIADDK, and crystalline porcine insulin (lot 615-08E-220) was a gift from Eli Lilly Co. (Indianapolis, IN). Cortisol, HEPES, casein (purified powder), rennet, UDP-galactose, arginine methyl ester, dibutryl cAMP, cholera toxin, calf thymus DNA, and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Medium 199 with Hanks' salts was obtained from Grand Island Biological Co. (Grand Island, NY), 3-methoxybenzamide (3-MBA) and 3-methoxybenzoate were obtained from Aldrich Chemical Co. (Milwaukee, WI), and all electrophoresis reagents were purchased from BioRad (Richmond, CA). Collagenase (lot 40S239) was obtained from Cooper Biomedical, Inc. (Malvern, PA), and UDP-[6-^{3}H]galactose (16.3 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL). [Adenine-2,8-^{3}H]nicotinamide adenine dinucleotide (22 Ci/mmole), [adenylate-^{32}P]nicotinamide adenine dinucleotide (196 Ci/mmole) and [G-^{3}H]lysine (7.5 Ci/mmole) were purchased from ICN (Irvine, CA), while [5-^{3}H]uridine (26.4 Ci/mmole), [^{33}P]orthophosphoric acid, carrier-free, and protosol were obtained from New England Nuclear Corp. (Boston, MA).

Animals and Organ Culture

Virgin mice (C3H/HeN), 5–7 weeks old, were purchased from Microbiological Associates (Bethesda, MD) and either aged an additional 6 weeks before sacrifice (virgin) or immediately bred with males of the same strain. The latter mice were used 5–7 days (early pregnant), 10–12 days (midpregnant) or 15–18 days (late pregnant) into their pregnancies. Lactating mice were 1 week postpartum.

Mice were sacrificed by cervical dislocation, and explants were prepared from the fourth pair of mammary glands as previously described [16]. Explants were then cultured on siliconized lens paper in medium 199 containing 20 mM HEPES (pH 7.6) and the following hormones at 1 μg/ml, unless otherwise noted: insulin (I), cortisol (F), and prolactin (P). The tissue was incubated under air at 37°C, and the medium was changed daily.

Poly(ADP-ribosyl[PADPR])synthetase Activity

PADPR-synthetase activity was assayed in an epithelial cell-enriched fraction [17] isolated from either explants or fresh glands, as required by the individual experiment. The cells were homogenized with only three strokes, and a crude nuclear pellet was obtained from the 700g-precipitate [18]. The nuclei were suspended in 200 μl of assay buffer containing [^{3}H]NAD^{+} [19] and incubated for 5 min at 37°C. The reaction was stopped, and the histone Hl and the high mobility group (HMG) proteins

were extracted by making the solution 5% (wt/vol) in perchloric acid. The other proteins and nucleic acids were precipitated by centrifugation at 13,000g for 2 min and reextracted with another 200 μl-aliquot of 5% (wt/vol) perchloric acid. The Hl and HMG proteins were recovered by combining the supernatants, making it 0.1 M in HCl, and adding six volumes of acetone. The precipitate was washed once with acidified acetone and either redissolved in protosol for counting in a toluene-based scintillation cocktail or dried for electrophoresis [20].

For two-dimensional electrophoresis, the nuclei were incubated with [^{32}P]NAD$^+$, and the final dried precipitates were redissolved in 0.9 M acetic acid containing 6 M urea and 5% (vol/vol) β-mercaptoethanol. The samples were subjected to electrophoresis in capillary tubes containing 15% (wt/vol) polyacrylamide in 0.9 M acetic acid and 6 M urea. These gels were then incorporated into 18% (wt/vol) slab gels containing 0.38 M glycine, 0.1% (wt/vol) SDS, and 50 mM Tris HCl, pH 6.8 [20,21]. Labeled proteins were located by radioautography, excised, dissolved in hydrogen peroxide, and counted.

Poly(ADP-ribose) Degradation

[^{3}H]Poly(ADP-ribosyl)histone Hl (PADPR-histone Hl) was synthesized in a reaction mixture containing 100 mM Tris HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM [^{3}H]NAD (2 mCi/mmol), calf thymus DNA (0.1 mg/ml), histone Hl (0.1 mg/ml) and PADPR-synthetase (1 μg/ml) for 10 min. The histones were extracted into 5% (wt/vol) perchloric acid and precipitated by making the supernatant 0.2 M in HCl and adding six volumes of acetone. The precipitate was washed with acidified acetone and dried. The PADPR-synthetase was purified from mouse mammary glands by the method of Ito et al [22].

The degradation of [^{3}H]PADPR-histone Hl was assayed in an epithelial cell-enriched fraction [17] by the method of Miwa et al [23] except that [^{3}H]PADPR-histone Hl (50,000 cpm/sample) was substituted for [^{14}C]poly(ADP-ribose). Total degradation of this polymer involves two enzymes: (1) the PADPR-glycohydrolase, which cleaves all of the ribosyl-ribose linkages in the polymer, and (2) the ADP-ribosyl histone hydrolase, which only removes the final monomer from the acceptor protein [24]. As such, this assay more closely reflects the PADPR-glycohydrolase activity.

cAMP Assay

cAMP was measured in a mammary epithelial cell-enriched fraction [17] by a commercial radioimmunoassay kit (New England Nuclear, Boston, MA), according to the accompanying instructions. All values were corrected for losses during sample preparation.

Other Assays

For the determination of casein synthesis, mammary explants were exposed to a 4-hr pulse of [^{33}P]orthophosphoric acid (50 μCi/ml), and the amount of phosphoprotein synthesized was measured by the calcium-rennin precipitation method [16]. Casein synthesis was then estimated by correcting this value for synthesis of noncasein phosphoprotein in explants cultured with the radioisotope but without prolactin (P) [25]. Lactose synthetase and galactosyltransferase activities were determined in mammary explants as described previously [26], except that the homogenization buffer

contained 1% Triton X-100. α-Lactalbumin was assayed by a modification [27] of the method of Fitzgerald et al [28]. For the measurement of total RNA synthesis, explants were pulsed with [^{3}H]uridine (2 μCi/ml) for 4 hr and processed by the method of Falconer et al [29]. The synthesis of DNA linker proteins was determined by exposing the explants to a continuous, 3-day pulse of [^{3}H]lysine (1.5 μCi/ml), preparing an epithelial cell-enriched fraction [17] and extracting these proteins in perchloric acid, as described above. Protein was measured by the method of Lowry et al [30] with bovine serum albumin as standard; and epithelial DNA was determined by the method of Burton [31] with calf thymus DNA as standard.

Since lactose synthetase activity, α-lactalbumin, and casein are unique markers for the mammary epithelium, they can be measured in the whole explant. However, because PADPR-synthetase is ubiquitous, enzyme activity was only measured in an epithelial cell-enriched fraction.

RESULTS

3-MBA is an NAD^+ analog and a potent inhibitor of PADPR-synthetase, the enzyme responsible for the initiation, elongation, and branching of ADP-ribose chains [8]. If ADP-ribosylation is an essential prerequisite for mammary differentiation, its inhibition should block the appearance of milk-specific components. In fact, Figure 1 shows the opposite results occur: in the presence of IFP, 3-MBA significantly stimulates lactose synthetase activity and casein synthesis approximately 200% and

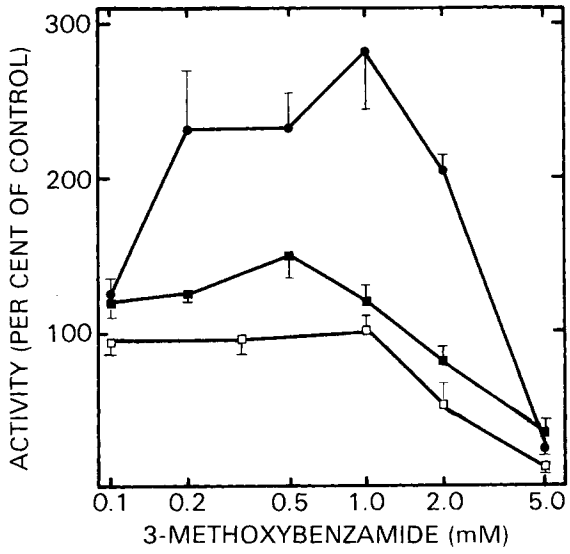

Fig. 1. Effect of 3-methoxybenzamide (3-MBA) on lactose synthetase activity (●), casein synthesis (■), and total RNA synthesis (□). Mammary gland explants from virgin mice were cultured in IFP and 3-MBA for 3 days before assay. The control values (100%) were as follows: lactose synthetase activity, 105 ± 12 pmol lactose formed/mg wet tissue·30 min; casein synthesis, 2,663 ± 148 cpm phosphate incorported/mg wet tissue·4h; and total RNA synthesis, 6,250 ± 581 cpm uridine incorporated/mg wet tissue· 4 hr. All data are expressed as the mean ± 1 SEM for either five (lactose synthetase activity and casein synthesis) or three experiments (total RNA synthesis).

50%, respectively ($P < .05$). The elevation of lactose synthetase activity is due to the stimulation of the regulatory component, α-lactalbumin, from 8.3 $\pm$ 1.7 ng/mg wet tissue to 25.8 $\pm$ 2.9 ng/mg; the activity of the catalytic subunit, galactosyltransferase, is unaffected by 3-MBA. However, there was no differential effect on the different casein species, as judged by SDS gel electrophoresis of the calcium-rennin precipitates. These effects require P, since differentiation does not occur in the presence of only IF and 3-MBA; these effects are also specific for lactose synthetase activity and casein synthesis, since total RNA synthesis is unchanged except above 1 mM 3-MBA, where the drug is toxic (Fig. 1). The inactive analog of 3-MBA, 3-methoxybenzoate, is without effect.

Recently, a mono(ADP-ribosyl)synthetase has been identified in higher eukaryotes; it attaches a single ADP-ribose unit to an arginine [32], whereas the PADPR-synthetase initiates its chains on carboxyl groups [33]. Since both enzymes utilize NAD^+ as a donor, both are negatively affected by 3-MBA and, therefore, the inhibition of either enzyme might account for the above results. However, only the mono(ADP-ribosyl)synthetase is inhibited by arginine methyl ester. This ester, in concentrations as high as 10 mM, has no effect on lactose synthetase activity or casein synthesis (data not shown); concentrations higher than 10 mM are toxic. Therefore, it appears that it is the inhibition of the PADPR-synthetase that is responsible for the enhancement of mammary differentiation.

Since the use of 3-MBA represents an artificial perturbation of this system, experiments were performed to determine if the enzyme was naturally regulated in the mammary gland. There are several ways that PADPR-synthetase activity can be measured: (1) the use of permeabilized cells allows labeled NAD^+ to enter an otherwise "intact" cell [19]; however, there is always some loss of cytoplasmic contents following permeabilization. (2) Labeled adenosine can enter intact cells, but it requires several hours to be converted to NAD^+ and its incorporation into other macromolecules requires extensive purification of the substrates in order to recover the ADP-ribosylated nuclear proteins [34]. Furthermore, by prelabeling the ADP-ribosylated proteins, any subsequently observed change in the amount of labeled protein cannot be exclusively ascribed to alterations in the rate of synthesis, since fluctuations in degradation could also affect the total, labeled protein. (3) Assaying the enzyme in homogenates entails the danger of damaging the DNA and activating the enzyme, which has been implicated in DNA repair as well as differentiation [24]. In this study, cells are gently homogenized (three strokes), and a crude nuclear pellet is assayed; the yield and integrity of the nuclei do not differ among the experimental groups. More rigorous homogenization (between five and ten strokes) results in a ten-fold increase in enzyme activity (from 28.1 $\pm$ 2.0 after three strokes to 329 $\pm$ 12 nmol ADP-ribose incorporated/mg epithelial protein · 5 min), and the hormonal effects described below become blunted. This effect also corresponds to a loss of nuclear integrity as followed by light microscopy. Finally, incubation periods were set at 5 min so that only initial rates of label incorporation would be measured; the resulting activity should primarily reflect synthesis over degradation. Therefore, this assay technique appears to be a valid method for the determination of PADPR-synthetase activity.

Employing this assay, enzyme activity was monitored during mammary differentiation *in vivo* and *in vitro*. Table I shows that enzyme activity is high in mammary epithelium from virgin and early pregnant mice but declines to one-third the original

TABLE I. Poly(ADP-ribosyl)synthetase in Mammary Epithelial Cells From Mice in Different Reproductive States*

Experimental group	Poly(ADP-ribosyl)synthetase activity (pmol ADP-ribose incorporated/10^7 cells)
Virgin	47.4 ± 7.3[a]
Early pregnant	41.9 ± 2.6[a]
Midpregnant	17.9 ± 2.1[b]
Late pregnant	14.2 ± 3.3[b]
Lactating	12.1 ± 4.6[b]

*Enzyme activity was determined in an epithelial cell-enriched fraction prepared from fresh mammary glands. All data are expressed as the mean ± 1 SEM for three experiments; all values with the same superscript do not differ significantly from each other, whereas those with different superscripts do ($P < .002$).

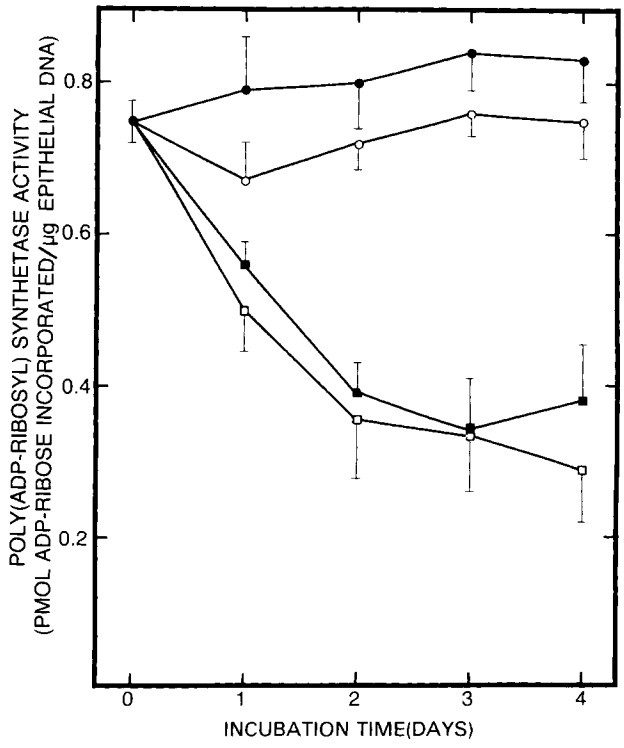

Fig. 2. Effect of hormones on poly(ADP-ribosyl)synthetase activity. Mammary gland explants from virgin mice were cultured in I (●), IF (□), IP (■), or IFP (□). At the indicated times, an epithelial cell-enriched fraction was isolated, and the enzymes were assayed; all data are expressed as the mean ± 1 SEM for three experiments.

value by late pregnancy. In culture, the enzyme activity remains relatively constant in epithelium cultured in media lacking P but is halved when P is present (Fig. 2).

This decrease in activity might reflect a hormone-induced suppression of endogenous substrate levels such that these proteins become limiting. However, Table II shows that P actually stimulates the synthesis of the DNA linker proteins 2.5-fold.

Finally, the DNA linker proteins labeled by PADPR-synthetase were identified on two-dimensional polyacrylamide gel electrophoresis (Table III; Fig. 3). Although ADP-ribosylation has also been reported to occur on both the enzyme itself and on certain core histones, the linker proteins have been more closely associated with differentiation than the other two groups of substrates [35]. Furthermore, ADP-ribosylation of the linker proteins has recently been shown to be hormonally regulated in another system [34,36]; therefore, this study also concentrated on these proteins. Histone H1 and HMG 1/2 are the most heavily modified, containing 20–30% and 65–70% of the label, respectively; and the labeling of both of these groups is hormonally modulated. HMG 14/17, which appear to reside at the nucleosome-linker junction, incorporate very little ADP-ribose (5%), and this value does not fluctuate in response to hormones.

TABLE II. Effect of Hormones on the Synthesis of DNA Linker Proteins in Mammary Epithelium*

Experimental group	DNA linker protein synthesis (cpm lysine incorporated/μg epithelial protein)
I	166 ± 29[a]
IF	266 ± 34[b]
IP	420 ± 16[c]
IFP	422 ± 18[c]

*Mammary gland explants from virgin mice were cultured in the above hormones and [^{3}H]lysine (1.5 μCi/ml). After 3 days, the H1 and HMG fraction was isolated from an epithelial cell-enriched fraction and counted. All data are expressed as the mean ± 1 SEM for four experiments; all values with the same superscript do not differ significantly from each other, whereas those with different superscripts do ($P < .05$).

TABLE III. Indentification of Substrates for Poly(ADP-ribosyl)synthetase in Mammary Epithelial Cells*

Experimental group	DNA linker protein (cpm/μg epithelial DNA)			
	H1	HMG 1/2	HMG 14/17	Total
Time zero	670 ± 98[a]	1537 ± 245[c]	113 ± 16[e]	2320 ± 264[f]
I	606 ± 95[a]	1518 ± 221[c]	105 ± 11[e]	2229 ± 241[f]
IF	625 ± 89[a]	1389 ± 220[c]	95 ± 8[e]	2109 ± 237[f]
IP	276 ± 38[b]	925 ± 132[d]	87 ± 10[e]	1288 ± 138[g]
IFP	247 ± 42[b]	960 ± 154[d]	97 ± 12[e]	1304 ± 161[g]

*Mammary gland explants from virgin mice were either processed immediately (time zero) or were cultured in the above hormones for 3 days. After an epithelial cell-enriched fraction was isolated, the DNA linker proteins were labeled with ADP-ribose by the endogenous enzyme and extracted and separated by two-dimensional polyacrylamide gel electrophoresis. Labeled spots were excised, dissolved in hydrogen peroxide, and counted. All data were expressed as the mean ± 1 SEM for three experiments; within each column all values with the same superscript do not differ significantly from each other, whereas those with different superscripts do ($P < .05$).

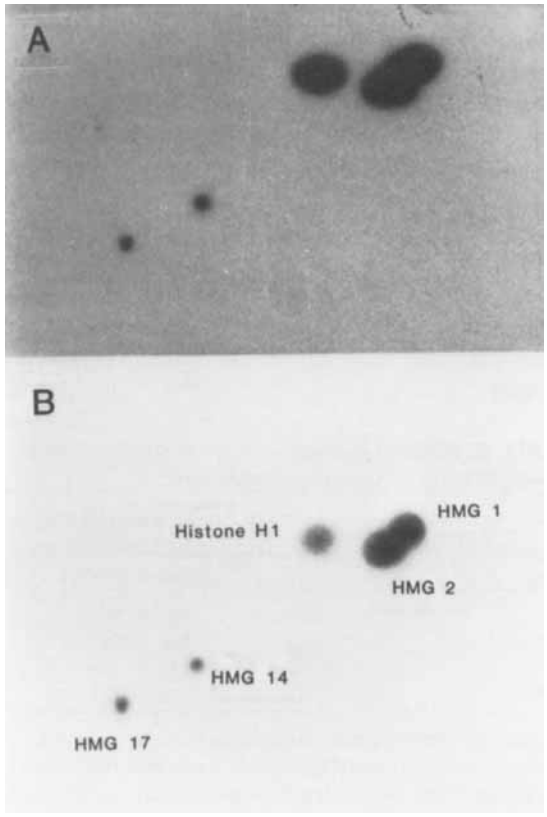

Fig. 3. Radioautogram of a two-dimensional gel of perchloric acid extracted nuclear proteins labeled with [^{32}P]NAD^{+}. Mammary gland explants from virgin mice were cultured for 3 days in either IF (A) or IFP (B) and then processed as described in the text.

Since the degree of ADP-ribosylation of nuclear proteins is a balance between synthesis and degradation, and since other investigators have suggested that the degradation of these polymers is both important and hormonally modulated [36], poly(ADP-ribose) degradation was also studied in the mammary gland. The ability of mammary epithelium from intact mice to degrade poly(ADP-ribose) remains constant through midpregnancy (Table IV). However, this activity begins to increase in late pregnancy and peaks during lactation.

In culture, the poly(ADP-ribose) degradatory activity doubles in the presence of I (Table V); the additional presence of F and P has no further effect. Since the PADPR-glycohydrolase has been reported to be inhibited by cAMP [23,37] and since I is known to lower cAMP levels in other tissues [38] and can elevate cAMP phosphodiesterase activity in mammary acini [39], I may be stimulating the glycohydrolase by reducing the tissue concentration of cAMP. The data in Table VI demonstrate that, in the presence of I, the cAMP level in mammary epithelium does decline to 30% of its original value; again, the presence of F and P has no further effect.

TABLE IV. Poly(ADP-ribose) Degradation in Mammary Epithelial Cells From Mice in Different Reproductive States*

Experimental group	Poly(ADP-ribose) degradation (nmol/10^7 cells)
Virgin	0.53 ± 0.03[a]
Early pregnant	0.52 ± 0.09[a]
Midpregnant	0.53 ± 0.07[a]
Late pregnant	0.86 ± 0.12[b]
Lactating	1.80 ± 0.28[c]

*Enzyme activity was determined in an epithelial cell-enriched fraction prepared from fresh mammary glands. All data are expressed as the mean ± 1 SEM for three experiments; all values with the same superscript do not differ significantly from each other, whereas those with different superscripts do ($P < .05$).

TABLE V. Poly(ADP-ribose) Degradation in Cultured Mammary Epithelium*

Experimental group	Poly(ADP-ribose) degradation (nmol/μg protein · min)
Time zero	2.20 ± 0.10[a]
No hormone	2.38 ± 0.07[a]
Insulin	5.67 ± 0.15[b]
Insulin, cortisol, and prolactin	5.45 ± 0.12[b]
Insulin and cholera toxin	2.90 ± 0.47[c]

*Mammary gland explants from virgin mice were either processed immediately (time zero) or were cultured in the above compounds for 3 days; cholera toxin was present at a concentration of 10 ng/ml. Poly(ADP-ribose) degradation was then measured in an epithelial cell-enriched fraction. All data are expressed as the mean ± 1 SEM for three experiments; all values with the same superscript do not differ significantly from each other, whereas those with different superscripts do ($P < .005$).

TABLE VI. cAMP Concentrations in Cultured Mammary Epithelium†

Culture conditions	cAMP concentration (pmol/mg epithelial protein)
Time zero	8.3 ± 1.0
No hormone	7.8 ± 0.6
Insulin	2.5 ± 0.4*
Insulin, cortisol, and prolactin	2.6 ± 0.7*

†Mammary gland explants from virgin mice were either processed immediately (time zero) or were cultured in the above hormones for 3 days. An epithelial cell-enriched fraction was then isolated and assayed for cAMP by radioimmunoassay. All data are expressed as the mean ± 1 SEM for three experiments.
*$P < .005$ vs time zero.

Furthermore, the insulin response is suppressed, if the cAMP concentration is stimulated by cholera toxin (Table V), which activates the adenylyl cyclase. Identical results are obtained when dibutryl cAMP (0.1 mM) is substituted for the cholera toxin (data not shown).

Finally, it should be noted that 3-MBA has no effect on poly(ADP-ribose) degradation, whereas cholera toxin has no effect on PADPR-synthetase activity. These data, combined with the differences in hormonal control and time courses, clearly demonstrate the distinctive nature of the synthesis and breakdown of this polymer.

DISCUSSION

These data support a reciprocal relationship between ADP-ribosylation and mammary gland differentiation both *in vivo* and *in vitro*. However, one cannot infer a causal relationship since (1) pharmacological inhibition of the PADPR-synthetase will enhance the effects of hormones but cannot replace them, and (2) P is capable of suppressing enzyme activity in the presence of only I, but an IP system will not support mammary differentiation [15]. It may be more accurate to describe the reciprocal relationship as being between ADP-ribosylation and a differentiative potential.

This reciprocal relationship and the hormonal inhibition of ADP-riboslyation were unexpected findings, since this process has been associated with differentiation in several other systems [19,40] and can be induced by sex steroids [12,14,33]. Nonetheless, there are three reports of hormonal inhibition of this process. Shimoyama et al [41] reported that F injected *in ovo* inhibited ADP-ribosylation in chick embryo livers. However, the dose was 100 μg, which is 2.5 times the teratogenic dose in these animals [42]; therefore, their results may reflect nonspecific toxicity. The induction of teratocarcinoma cell differentiation by retinoic acid is also accompanied by decreased ADP-ribosylation [43]. Finally, Tanuma et al [36] studied mouse mammary tumor virus (MMTV) production in a mouse mammary tumor cell line, and they reported that F reduced the ADP-ribosylation of nuclear proteins and induced MMTV RNA. These events were temporally related, and inhibition of the PADPR-synthetase by 3-aminobenzamide alone also induced MMTV RNA. The similarities between the tumor system and the mammary explant system are noteworthy, as (1) both systems use cells derived from mammary tissue, (2) in both systems, the primary hormones of differentiation inhibit ADP-ribosylation, and (3) enzyme inhibitors either induce or augment the induction of mammary gland specific markers. However, there is also a significant difference: the primary substrates for this enzyme in the tumor cells are HMG 14/17, whereas histone Hl and HMG 1/2 are the major targets in explants. A difference in assay technique probably accounts for this disparity [44].

In addition to establishing this reciprocal relationship in normal mammary epithelium, the data herein clarify the individual functions of several of the differentiation-inducing hormones in this system. The decrease in ADP-ribosylation seen with mammary differentiation appears to be the result of two processes, each of which is under separate hormonal control: P inhibits the PADPR-synthetase activity, whereas I stimulates the PADPR-glycohydrolase activity (Fig. 4). The latter effect may occur via an insulin-mediated decrease in the cAMP level.

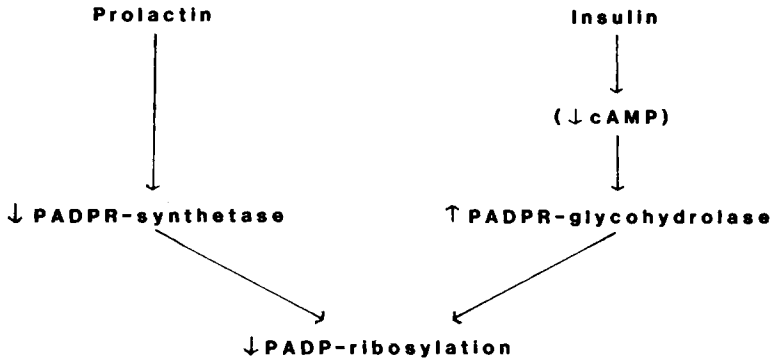

Fig. 4. A hypothetical scheme for the hormonal regulation of poly(ADP-ribosyl)ation in mouse mammary epithelium.

cAMP is known to be an important negative regulator of mammary gland differentiation: *in vivo*, levels decline abruptly at the initiation of lactation [45–47], and *in vitro*, this nucleotide inhibits biochemical differentiation in mouse mammary explants [48]. In addition, Oka et al [49] have reported that cAMP concentrations in these explants are reduced below 50%, when the tissue is cultured in IFP, and Aitchison et al [39] demonstrated that I can stimulate cAMP phosphodiesterase in mammary acini. The data in this study show that I alone is responsible for both the fall in the cAMP concentration and the increase in the PADPR-glycohydrolase activity, suggesting that the mechanism of action of cAMP in the mammary gland may be related to a stimulation of ADP-ribosylation within the nucleus. The data also suggest that I may be a primary regulatory of cAMP levels in mammary explants. However, it should be noted that, although I is known to reduce cAMP levels in several tissues [38], it is still not clear if this reduction actually mediates any of the actions of I. Nonetheless, the experiment with cholera toxin does lend additional support to a causal relationship.

Although P has usually been considered to be the most important hormone responsible for mammary gland differentiation, I and F have also been shown to be corequisites at the level of casein gene expression [50–53]. The results described herein would suggest that at least part of the function of I may be to alter the chromatin proteins in a manner that complements the action of P.

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