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Possible Involvement of the Phospholipases in the Mitogenic Actions of Prolactin (PRL) on Nb_2 Node Lymphoma Cells[1] (42529)

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Abstract. The possible role of the phospholipase enzymes in the prolactin stimulation of mitogenesis in Nb_2 node lymphoma cells was investigated. Two phospholipase inhibitors including quinacrine and *α-para*-dibromoacetophenone (BPB) were employed. Quinacrine at concentrations of 1–5 μM attenuated the magnitude of the PRL stimulation of cell division; at concentrations of 10 μM and above quinacrine abolished the PRL response. BPB at concentrations of 1–10 μM also inhibited the mitogenic effect of PRL in a concentration response fashion. The polyunsaturated fatty acid arachidonic acid partially reversed the inhibitory effects of these drugs. In further studies, exogenously added phospholipase C at concentrations of 5–50 ng/ml was found to potentiate the mitogenic effect of prolactin when prolactin was employed at a concentration that evoked a half-maximal response. By itself, however, phospholipase C had no effect on the rate of cell division. Phospholipase A_2 either by itself or in the presence of prolactin was without effect.

Prolactin has receptors on a variety of cell types and is known to elicit a number of different mitogenic and differentiative responses. In the mammary gland, prolactin stimulates both milk product formation and cell division (1). The molecular events involved in the actions of prolactin following its receptor interactions are not completely understood. Our laboratory has been investigating the possible role of the phospholipases, specifically phospholipases A_2 and C, during the initial receptor-generated events. Several years ago our laboratory reported that quinacrine, an inhibitor of phospholipase activity, abolishes the effect of prolactin on the stimulation of the rate of [^{3}H]thymidine incorporation into DNA in cultured mouse mammary tissues (2). In order to perform more definitive studies on the possible role of the phospholipases in the action of prolactin on mitogenesis, experiments were carried out employing the Nb_2 node lymphoma cell line (3, 4); mitogenesis in these cells is specifically stimulated by prolactin and other lactogenic hormones (5, 6).

Materials and Methods. The Nb_2 node lymphoma cells employed in these studies were a gift from Dr. C. T. Beer of the Cancer Control Agency of British Columbia (Vancouver, British Columbia, Canada). Ovine prolactin (NIH-P-S-16) was provided by the NIAMDD. Other materials used in these studies were purchased from the following sources: fetal calf serum and horse serum from Hyclone (Logan, UT); penicillin and streptomycin from Eli Lilly Inc. (Indianapolis, IN); phospholipase C (Bacillas Cereus, sp act 133 units/mg) from Calbiochem (La Jolla, CA); arachidonic acid, phospholipase A_2 (sp act 940 units/mg), and quinacrine from Sigma Chemical Co. (St. Louis, MO); *α-para*-dibromoacetophenone from Aldrich Chemical Co. (Milwaukee, WI).

The Nb_2 node lymphoma cells were maintained as suspension cultures in 25-cm^2 culture flasks containing "growth media" (Fisher's medium supplemented with 10% fetal calf serum (FCS); 10% horse serum (HS); 1×10^{-4} M 2-mercaptoethanol; 50,000 IU/liter penicillin; 50,000 μg/liter streptomycin). The flasks were flushed with a 95% air–5% CO_2 gas mixture and incubated at 37°C. Media were changed every 72 hr. Twenty-four hours before beginning an assay the cells were collected by centrifugation at 300g in "stationary media" (same components as growth media except that the FCS is deleted). In the absence of FCS the Nb_2 cells only divide if prolactin (or another lactogenic hormone) is added to the culture medium.

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After a 24-hr culture period in the absence of FCS, the cells were again harvested by slow-speed centrifugation and resuspended in stationary media. One-milliliter aliquots (approximately 1×10^5 cells/ml) were transferred to the wells of sterile multiwell tissue culture plates (24 wells/plate). The cells were then cultured for an additional 72 hr in the presence of various concentrations of phospholipases and/or phospholipase inhibitors and/or prolactin. The plates were incubated in a humidified incubator at 37°C in a constantly maintained environment of 95% air–5% CO_2. After 72 hr the cells were transferred to vials containing 9 ml hematall isotonic diluting fluid and counted on a Coulter Model ZM counter. The data in the tables represent the means of at least four replicates and statistical comparisons were made using an analysis of variance and Tukey's *hsd* procedure or paired *t* test (7).

Results. Table I shows the results of a study in which cells were cultured with various concentrations of PLC in the presence and absence of prolactin. Two prolactin concentrations were tested: an intermediate concentration of 200 pg/ml and a maximum stimulatory concentration of 20 ng/ml. PLC by itself or in the presence of the maximum prolactin concentration had no effect on cell numbers. However, in the presence of an intermediate concentration of prolactin, PLC at 5, 10, and 50 ng/ml potentiated by 10–12% prolactin's stimulatory effect on cell division. Phospholipase A_2 tested under the same experimental protocol as employed in the previous experiment (Table II) exhibited no stimulatory effect on cell division in either the presence or the absence of prolactin. At higher concentrations, however, PLA_2 caused the demise of the cells. This decrease in cell number by high concentrations of PLA_2 could be circumvented if exogenous PLC was added to the media at the same time as the PLA_2 (data not presented).

As shown in Table III the phospholipase inhibitor quinacrine (8) interferes with the

TABLE I. EFFECT OF PHOSPHOLIPASE C (PLC) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

PLC concentration (ng/ml)	Number of cells × 10^{-3}		
	No prolactin	+ Prolactin (200 pg/ml)	+ Prolactin (20 ng/ml)
0 (control)	217 ± 3.4[b]	515 ± 5.8	1127 ± 10
1.0	203 ± 3.2	550 ± 7.0	1162 ± 17
5.0	200 ± 2.0	569 ± 11*	1169 ± 17
10	215 ± 2.6	578 ± 15*	1147 ± 14
50	207 ± 6.6	579 ± 10*	1139 ± 12
100	195 ± 6.4	551 ± 12	1108 ± 6.4

[a] Nb_2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or PLC at the concentrations indicated in the table. Cell numbers were then determined.

[b] Numbers represent the mean ± SE of four observations.

* Represents results significantly ($P < 0.01$) greater than controls heading the same column.

TABLE II. EFFECT OF PHOSPHOLIPASE A_2 (PLA_2) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

PLA_2 concentration (pg/ml)	Number of cells × 10^{-3}		
	No prolactin	+ Prolactin (200 pg/ml)	+ Prolactin (20 ng/ml)
0 (controls)	291 ± 5.0[b]	675 ± 10	1024 ± 29
25	284 ± 4.4	683 ± 14	1030 ± 19
50	287 ± 3.0	688 ± 2.0	1063 ± 12
100	287 ± 4.0	713 ± 21	1058 ± 21
200	232 ± 7.8*	667 ± 9.6	1027 ± 21
500	17.6 ± 1.6*	20.8 ± 4.8*	128 ± 51*

[a] Nb_2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or PLA_2 at the concentrations indicated in the table. Cell numbers were then determined.

[b] Numbers represent the mean ± SE of four observations.

* Represents significantly ($P < 0.01$) less than controls heading the same column.

TABLE III. EFFECT OF QUINACRINE ON PROLACTIN STIMULATION OF CELL DIVISION[a]

Quinacrine concentration (μM)	Number of cells × 10^{-3}		
	No prolactin	+ Prolactin (200 pg/ml)	+ Prolactin (20 ng/ml)
0 (control)	243 ± 8.4[b]	600 ± 22	1518 ± 58
1.0	246 ± 8.8	518 ± 18**	1472 ± 66
2.0	230 ± 19	466 ± 10*	1344 ± 46**
5.0	200 ± 10*	296 ± 7.6*	868 ± 9.2*
10.0	184 ± 2.0*	190 ± 4.8*	216 ± 4.4*

[a] Nb_2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or quinacrine at the concentrations indicated in the table. Cell numbers were then determined.

[b] Numbers represent the mean ± SE of four observations.

* Represents results significantly ($P < 0.01$) less than controls heading the same column.

** $P < 0.05$.

prolactin stimulation of cell division in the Nb_2 node cells. Quinacrine at concentrations of 1–5 μM attenuated the response to the intermediate and high concentrations of prolactin, while 10 μM quinacrine abolished these responses. A second phospholipase inhibitor employed in these studies is α-*p*-dibromoacetophenone (BPB) (9). At concentrations between 1 and 10 μM, this drug attenuated the mitogenic effect of both intermediate and maximum stimulatory concentrations of prolactin (Table IV).

In order to establish specificity for the actions of the phospholipase inhibitors on the Nb_2 node lymphoma cells, products of the actions of these enzymes were added to the cultured cells. In studies not presented several diacylglycerides including dicaprin, diolein, and 1-oleoyl-2-acetyl-*rac*-glycerol (OAG) were added to the media of the Nb_2 node cells. At concentrations from 1 ng/ml to 100 μg/ml these agents had no effect on mitogenesis, in either the presence or the absence of prolactin. In contrast arachidonic acid was found to have effects on mitogenesis in the Nb_2 node lymphoma cells. The data in Table V show that arachidonic acid at concentrations from 0.5 to 5 μg/ml potentiated by 10–13% the effect of an intermediate concentration of prolactin. No stimulating effects of arachidonic acid were observed with arachidonic acid by itself or in the presence of a maximum stimulatory concentration of prolactin.

TABLE IV. EFFECT OF α-*p*-DIBROMOACETOPHENONE (BPB) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

BPB concentration (μM)	Number of cells $\times 10^{-3}$		
	No prolactin	+ Prolactin (200 pg/ml)	+ Prolactin (20 ng/ml)
0 (control)	86 ± 1.2[b]	500 ± 17	1514 ± 46
0.1	93 ± 6.2	460 ± 20	1446 ± 42
1.0	66 ± 1.6*	393 ± 23*	1254 ± 68**
5.0	57 ± 1.8*	256 ± 11*	799 ± 41*
10	50 ± 2.2*	172 ± 10*	183 ± 16*

[a] Nb_2 node lymphoma cells (75,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or BPB at the concentrations indicated in the table. Cell numbers were then determined.

[b] Numbers represent the mean ± SE of four observations.

* Represents results significantly ($P < 0.01$) less than controls heading the same column.

** $P < 0.05$.

TABLE V. EFFECT OF ARACHIDONIC ACID (AA) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

AA concentration (μg/ml)	Number of cells $\times 10^{-3}$		
	No prolactin	+ Prolactin (200 pg/ml)	+ Prolactin (20 ng/ml)
0 (control)	168 ± 5.0[b]	631 ± 12	1087 ± 18
0.5	172 ± 4.4	669 ± 22	1088 ± 21
1.0	178 ± 2.2	694 ± 12*	1018 ± 9.8
2.5	163 ± 2.6	714 ± 12*	1057 ± 26
5.0	186 ± 4.2	716 ± 26*	900 ± 9.6
10	157 ± 6.9	621 ± 10	864 ± 14

[a] Nb_2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or AA at the concentrations indicated in the table. Cell numbers were then determined.

[b] Numbers represent the mean ± SE of four observations.

* Represents results significantly ($P < 0.01$) greater than controls heading the same column.

In Table VI, 5 μg/ml arachidonic acid is shown to potentiate the mitogenic effect of an intermediate concentration of prolactin. In addition, arachidonic acid is shown to more than double the cell number (115% increase) when both quinacrine and prolactin are present in the culture medium. Arachidonic acid also prevents the decreased cell number which occurs in the presence of 5 μM quinacrine when prolactin is not present. Similarly, the effect of arachidonic acid in the presence of BPB and/or prolactin is described in Table VII. Arachidonic acid potentiates the effect of the intermediate concentration of prolactin by 26%. Arachidonic acid also potentiated the prolactin response in the presence of 5 μM BPB by 92%. However, arachidonic acid did not prevent the decrease in cell number in the presence of 5 μM BPB by itself.

The effect of phospholipase C at various concentrations in combination with prolactin (200 pg/ml) and/or arachidonic acid (5 μg/ml) is presented in Table VIII. The control values obtained when no PLC was added is shown in the top row; a 12% increase in cell number is observed when arachidonic acid is added with prolactin as described earlier. However, this arachidonic acid effect is not observed when phospholipase C is present. There is no significant difference between column 2 (PLC and prolactin) and column 3 (PLC, prolactin, and arachidonic acid).

TABLE VI. EFFECT OF QUINACRINE IN COMBINATION WITH ARACHIDONIC ACID (AA) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

Quinacrine concentration	Number of cells × 10^{-3}			
	No prolactin		+ Prolactin (200 pg/ml)	
	No AA	+ AA (5 μg/ml)	No AA	+ AA (5 μg/ml)
0 (control)	234 ± 9.4[b]	248 ± 28	608 ± 9.4	910 ± 10*
5 μM	159 ± 4.0*	226 ± 5.1*,**	285 ± 3.4*	614 ± 6.3*,**

[a] Nb^2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or AA + inhibitor at the concentrations indicated in the table. Cell numbers were then determined.
[b] Numbers represent the mean ± SE of four observations.
* Represents results significantly ($P < 0.01$) less than controls not receiving quinacrine.
** Represents results significantly ($P < 0.01$) greater than cells not receiving arachidonic acid.

Discussion. In order to determine if phospholipase C may be involved in the prolactin stimulation of mitogenesis in Nb_2 node lymphoma cells, two types of experiments were carried out. First, cells were cultured with exogenously added phospholipase C to determine whether by itself PLC would stimulate cell division or whether PLC would potentiate the stimulatory effect of prolactin. Second, we employed two phospholipase inhibitors to determine whether they would attenuate or abolish prolactin's stimulatory effect on mitogenesis.

The data from Table I show that the presence of PLC potentiates prolactin's (intermediate concentration) stimulatory action on Nb_2 node cell division. Since PLC catalyzes the release of diglycerides from phospholipids and diglycerides are known to activate protein kinase C, we would propose that the phospholipase C potentiation of the prolactin response may occur by the generation of diglycerides and the activation of protein kinase C. In accord with this conclusion is the observation by Gertler *et al.* (10) that the phorbol ester TPA also potentiates the mitogenic action of prolactin on cell division in the Nb_2 node lymphoma cells. TPA is known to stimulate protein kinase C activity like the diglycerides. We have subsequently confirmed the results of the TPA studies of Gertler *et al.* (unpublished).

PLC cleaves the phosphate-containing group from the number 3 position of the glycerol moiety of phospholipids. In order to establish specificity for the PLC effect on cell division we tested another phospholipase, PLA_2, which cleaves the 2-acyl free fatty acid from phospholipids; PLA_2 had no effect on the division of the Nb_2 node cells, either by itself or in the presence of prolactin.

As was stated in the introduction, quinacrine (one of the two phospholipase inhibitors tested) has already been shown to abolish the

TABLE VII. EFFECT OF α-*p*-DIBROMOACETOPHENONE (BPB) IN COMBINATION WITH ARACHIDONIC ACID (AA) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

BPB concentration	Number of cells × 10^{-3}			
	No prolactin		+ Prolactin (200 pg/ml)	
	No AA	+ AA (5 μg/ml)	No AA	+ AA (5 μg/ml)
0 (control)	86.2 ± 1.2[b]	97.2 ± 2.4	558 ± 25	704 ± 19**
5 μM	66.8 ± 1.6*	68.4 ± 3.8*	265 ± 49*	509 ± 40*,**

[a] Nb_2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or AA + inhibitor at the concentrations indicated in the table. Cell numbers were then determined.
[b] Numbers represent the mean ± SE of four observations.
* Represents results significantly ($P < 0.01$) less than controls not receiving BPB.
** Represents results significantly ($P < 0.01$) greater than cells not receiving BPB.

TABLE VIII. EFFECT OF PHOSPHOLIPASE C (PLC) IN COMBINATION WITH ARACHIDONIC ACID (AA) ON PROLACTIN STIMULATION OF CELL DIVISION[a]

PLC concentration (ng/ml)	Number of cells × 10^{-3}		
	No prolactin	+ Prolactin (200 pg/ml)	+ Prolactin and AA (200 pg/ml and 5 μg/ml)
0 (control)	244 ± 8.2[b]	614 ± 15	690 ± 12**
5.0	251 ± 2.2	769 ± 33*	843 ± 5.4*
50	312 ± 10	829 ± 37*	824 ± 45*
500	207 ± 8.6	815 ± 49*	769 ± 54
5,000	198 ± 4.6	637 ± 37	611 ± 37
50,000	57 ± 2.4	109 ± 10	111 ± 11

[a] Nb^2 node lymphoma cells (100,000 per well initially) were cultured for 72 hr in the presence of prolactin and/or PLC at the concentrations indicated in the table. Cell numbers were then determined.

[b] Numbers represent the mean ± SE of four observations.

* Represents results significantly ($P < 0.01$) greater than controls heading the same column.

** Represents results significantly ($P < 0.01$) different between cells not receiving arachidonic acid and those cells that did receive arachidonic acid.

prolactin stimulation of the [^{3}H]thymidine incorporation into DNA in mouse mammary gland explants (2). As presented in the present study, two phospholipase inhibitors including quinacrine and BPB also abolish the prolactin stimulation of cell division in the Nb_2 node lymphoma cells. We therefore conclude from this study that ongoing phospholipase activity is essential for prolactin to express mitogenic actions in the Nb_2 node lymphoma cells as well as in the mammary gland.

In order to establish the specificity of action of quinacrine and BPB we attempted to reverse the inhibition of these drugs by adding the products of phospholipase C's actions on the cultured cells. Various diglycerides including dicaprin, dioleate, and OAG did not reverse the inhibitory effects of the drugs. Somewhat surprisingly, however, the polyunsaturated fatty acid, arachidonic acid, may have partially reversed the inhibitory effects of these drugs. Arachidonic acid by itself or in the presence of a maximum stimulatory concentration of prolactin had no effect on the role of cell division. Arachidonic acid, like phospholipase C, however, potentiates the prolactin stimulation of mitogenesis when prolactin is employed at intermediate concentrations. Whether arachidonic acid reversed the quinacrine and/or BPB inhibition of the prolactin response is therefore not entirely substantiated by these data.

If arachidonic acid and phospholipase C are functioning via a common metabolic pathway in potentiating the prolactin stimulation of cell division, then their responses would be predictively nonadditive when tested in concert. Table VIII confirmed that arachidonic acid did not enhance the phospholipase C potentiation above that elicited with phospholipase C alone. Precisely how arachidonic acid has its action in this system is not known. It apparently does not function via the formation of icosanoid products since in studies not presented very high concentrations of indomethacin (50 μg/ml) were found not to affect the mitogenic effect of prolactin in these cells. It is possible that arachidonic acid functions via a stimulation of protein kinase C since polyunsaturated fatty acids have been shown in several reports to stimulate PKC (11).

In summary, we have shown that phospholipase C and arachidonic acid enhance the mitogenic effect of prolactin on the Nb_2 node lymphoma cell line. In addition, two phospholipase inhibitors were shown to abolish the prolactin response. We conclude that the data are compatible with a possible involvement of phospholipase C in the prolactin stimulation of lymphoma cell mitogenesis. Also, the phospholipase and arachidonic acid data are compatible with the possible involvement of protein kinase C.

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