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Calcium–Phospholipid Enhanced Protein Phosphorylation in Human Placenta (42353)

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Abstract. Calcium-activated, phospholipid-dependent protein phosphorylation has not been studied in placenta. Human placental cytosol was subjected to an endogenous protein phosphorylation assay using [γ-^{32}P]ATP in the presence of calcium and phosphatidylserine. Protein phosphorylation was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. When compared to basal levels, calcium (10^{-6} *M*) in combination with phosphatidylserine (50 μg/ml) significantly enhanced ($P < 0.01$) ^{32}P incorporation into phosphoproteins having mol wt 47,000, 43,000, and 37,000. Half-maximal ^{32}P incorporation was observed with 3.5×10^{-7} *M* Ca^{2+} in the presence of phosphatidylserine (50 μg/ml). The effect of phosphatidylserine was biphasic. In the presence of Ca 10^{-6} *M*, ^{32}P incorporation increased to a maximum at 70 μg/ml of phosphatidylserine. The increase was suppressed at 150 μg/ml. Tetracaine caused a dose-dependent inhibition of calcium-activated, phospholipid-dependent enhancement of the three phosphoproteins. Calcium in the absence of phospholipid enhanced the phosphorylation of a protein of 98,000 mol wt. Phosphatidylserine suppressed this enhancement. Calmodulin (10^{-6} *M*) had no detectable effect upon phosphorylation beyond that of calcium alone, but the calmodulin inhibitor R-24571 specificlly inhibited the calcium-stimulated 98,000 mol wt phosphoprotein. Calcium-activated, phospholipid-dependent phosphoproteins are present in human placental cytosol; whether calcium-activated, calmodulin-dependent phosphoproteins also are present remains a question.

Calcium-activated phospholipid-dependent protein kinase (kinase C) was described initially by Takai *et al.* (1, 2) and has since been demonstrated in many phyla (3, 4). This kinase can be activated in intact cells by phorbol esters (5) but has not been definitively connected with hormone-mediated events. Recently, however, many investigators have presented evidence that kinase C may be activated by several hormones which affect phosphatidylinositol metabolism in cell membranes (6, 7) including muscarinic cholinergic agonists, angiotensin II, and epidermal growth factor. Such hormones are thought to activate kinase C by first interacting with receptors on the cell membrane, resulting in rapid changes in phosphatidylinositol metabolism and the release of a second messenger; arachidonate, myoinositol triphosphate, and diacylglycerol have been proposed as second messengers. The second messenger then elevates intracellular calcium levels and participates jointly with calcium in activating kinase C (6, 7). Conformational changes in specific cell proteins resulting from phosphorylation by kinase C would cause the intracellular effects of the hormone that initiated the cascade.

Muscarinic cholinergic receptors (8), epidermal growth factor receptors (9), and angiotensin II receptors (10) have all been described in human placenta. Physiological effects of some of these hormones on the human placenta have also been reported. Cholinergic antagonists inhibit the release of acetylcholine and the transport of AIB by placental villi *in vitro* (11). Also, epidermal growth factor increases hCG secretion in placental villi (12). Because the physiological effects of these hormones in placenta may be mediated through kinase C activation and the resulting phosphorylation of specific cell proteins, it was of interest to examine calcium-activated phospholipid-dependent phosphoproteins in human placenta. In this study we demonstrate that calcium in association with phospholipids enhances the phosphorylation of distinct phosphoproteins in human placenta.

Materials and Methods. *Materials.* [γ-^{32}P]ATP (35 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). Phosphatidylserine, phosphatidylinositol, phosphatidylcholine, spermine, cAMP, and other reagents were purchased from Sigma Chemical Company (St. Louis, Mo.). All other

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reagents were obtained from Fisher Scientific Company. R-24571 was a generous gift of Dr. D. Johnson, Columbus, Ohio.

Tissue preparation. Placentas from uncomplicated, term gestation pregnancies were obtained at delivery and processed immediately. Cotyledons to be used were perfused free of blood with phosphate-buffered saline. Decidual tissue was removed by sharp dissection. The remaining tissue was then washed in iced buffer containing 0.25 *M* sucrose, 1 m*M* EGTA, and 10 m*M* Tris–HCl (pH 7.2). Throughout the remainder of the preparation procedure the tissue was maintained at 4°C. Approximately 3 g of washed tissue was homogenized with a Tekmar Tissuemizer (Tekmar Co., Cincinnati, Ohio) in 30 ml of the sucrose buffer (3 × 10 sec at high setting). The homogenate was poured through four layers of cheesecloth. The filtrate was centrifuged at 5000*g* for 10 min. The pellet was discarded. The supernatant was then centrifuged at 100,000*g* for 60 min and used as the placental cytosol preparation. Placental cytosol was assayed on the day of preparation or after freezing and storage at −70°C. No differences were seen in fresh and frozen preparations under these conditions. Protein concentrations were determined by the method of Lowry *et al.* (13).

Gel electrophoresis and autoradiography. Gel electrophoresis and autoradiography were done by a modification of methods previously reported (14). Phosphorylated products were identified by incubation of placental cytosol with [γ-^{32}P]ATP followed by gel electrophoresis and autoradiography. The reaction mixture (total volume 0.2 ml) consisted of the protein sample (10–20 μg), 5 μM [γ-^{32}P]ATP (5–20 cpm/pmole), 20 m*M* Tris–HCl (pH 7.2), 5 m*M* $MgCl_2$, 1 m*M* EGTA, and 10 m*M* NaF. Calcium, phospholipids, and other potential effector compounds were added as indicated. The amount of $CaCl_2$ required to give specific free Ca^{2+} concentrations was calculated by a computer program according to the method of Sillen and Martell (15). Assays were initiated by addition of [γ-^{32}P]ATP and incubated for 6 min at 30°C in a water bath. The reaction was terminated by the addition of 200 μl of ice-cold 12% trichloroacetic acid (TCA). The protein was then precipitated by centrifugation in a Beckman B microfuge for 3 min and the pellet washed twice with 350 μl H_2O. The pellet was dissolved in a solution (60 μl) containing 2% SDS, 10% glycerol, 5% B-mercaptoethanol, and 0.001% bromophenol blue and boiled for 5 min. Samples were then loaded on an SDS–polyacrylamide gel (4.3% stacking, 9.4% running) for electrophoresis according to the method of Laemmli (16). The molecular weight standards used included myosin (205,000), β-galactosidase (116,000), phosphorylase b (97,400), BSA (66,000), ovalbumin (45,000), and carbonic anhydrase (29,000). The gels were fixed overnight in 50% methanol and 7.5% acetic acid, stained with Coomassie blue, and destained. Gels were dried on Whatman Grade 3 filter paper, autoradiographed on Kodak XR film, and developed for the desired length of time (24 hr–2 weeks) to demonstrate the phosphorylated proteins. The autoradiographs were then scanned at 550 nm by a Beckman Du-8 spectrophotometer with a slab gel scanner attachment.

Phosphate bond analysis. In order to demonstrate that phosphate was incorporated into proteins as well as to establish the type of phosphate bond formed, the following experiments were performed: Protein phosphorylation was performed exactly as described in the preceding paragraph except that two tubes each containing 50 μg of cytsol protein in total incubation volumes of 2 ml were used. One contained 10^{-6} *M* calcium with 50 μg of phosphatidylserine and the other contained neither. After protein precipitation with a comparable amount of 12% TCA, the 2.0-ml samples were each divided into seven 200-μl aliquots (a–g) and treated as follows: (a) The first aliquot was centrifuged and the pellet extracted three times with acetone (350 μl). The resulting pellet was then placed in a 60-μl solution (Laemmli) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromphenol blue and boiled for 5 min. (b) The second tube was treated identically as (a) except that the pellet was extracted three times with chloroform/methanol (2/1) immediately following the acetone step. (c) The third tube was treated as (a) except that following the initial centrifugation and prior to the acetone extraction, the pellet was resuspended in ice-cold 0.5 *M* NaOH for 5 min and then precipitated in 5% TCA (350 μl). (d) The fourth was treated exactly as (c) except that the NaOH solution was boiled for 1 of the 5 min. (e) The fifth was treated as (a) except that following the 12%

TCA addition, the solution was boiled for 1 min prior to centrifugation. Both (f) and (g) were treated as (a) except that following addition of the Laemmli solution they were exposed to Pronase (0.01 mg) and ribonuclease A (0.01 mg), respectively, for 10 min at 37°C. All of the boiled samples were then loaded on gels and processed exactly as in the preceding paragraph.

Results. Phosphatidylserine (50 μg/ml) and calcium (10^{-6} *M*) activated protein phophorylation of several endogenous proteins in human placental cytosol. Phosphoproteins of 37,00, 43,000, and 47,000 mol wt consistently demonstrated calcium-activated, phospholipid-dependent phosphorylation in eight different placental preparations (Fig. 1). Densitometer scans of autoradiographs of SDS–polyacrylamide gels of placental cytosol in the presence or absence of phosphatidylserine (50 μg/ml) and calcium (10^{-6} *M*) demonstrated

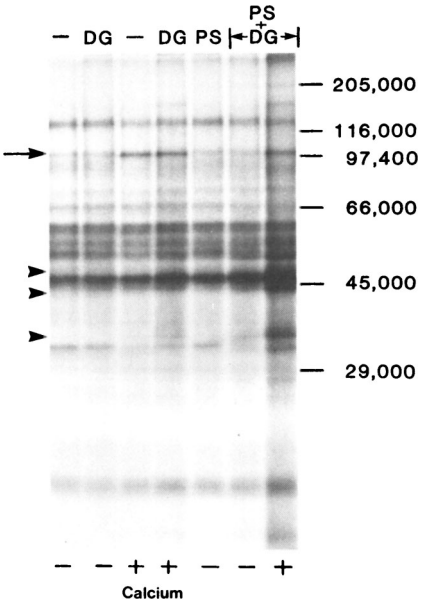

FIG. 1. Autoradiograph of phosphorylated proteins in placental cytosol. Placental cytosol was incubated as described under Methods with no added effector, or calcium (10^{-6} *M*), phosphatidylserine (50 μg/ml), and/or diacylglycerol (1 μg/ml) in the combinations indicated. The arrowheads (to the left) indicate proteins differentially phosphorylated by calcium and phosphatidylserine. The arrows indicates the 98,000 mol wt phosphoprotein activated by calcium alone. The positions of the marker proteins are indicated at the right. The results shown are representative of gels from placental cytosol of eight placentas (n = 8).

TABLE I. ^{32}P INCORPORATED INTO CALCIUM-ACTIVATED, PHOSPHOLIPID-DEPENDENT PHOSPHOPROTEINS

Phosphoprotein mol wt	Density (arbitrary units)	
	$+Ca^{2+}$/PS	$-Ca^{2+}$/PS
47,000	3.8 ± 1.7*	1.8 ± 0.83
43,000	3.1 ± 1.9*	1.9 ± 0.89
37,000	0.78 ± 0.01*	0.03 ± 0.04

Note. Values are means ± SD. Phosphorylation and gel electrophoresis of placental cytosol were performed as described under Methods. The densitometer readings (arbitrary units) of the 47,000, 43,000, and 37,000 mol wt phosphoproteins in the presence and absence of 10^{-6} *M* calcium and 50 μg/ml phosphatidylserine were determined (n = 8).

*$P < 0.01$ by paired Student t test.

significant differences ($P < 0.01$) in each of these activated phosphoproteins (Table I). In many preparations phosphoproteins of 205,000, 52,000, and 28,000 mol wt also appeared to incorporate additional ^{32}P in the presence of phosphatidylserine (50 μg/ml) and calcium (10^{-6} *M*). However, augmented phosphorylation of these proteins was not as pronounced and/or consistent with that observed for the 37,000, 43,000, and 47,000 mol wt proteins.

The nature of the phosphate bond was studied by exposure of the phosphorylated cytosol to hot and cold TCA, hot and cold 0.5 *N* NaOH, Pronase, ribonuclease A, acetone, and chloroform/methanol (see Methods). As illustrated in Table II, none of these treatments had major effects on the incorporation of ^{32}P into calcium-activated, phopholipid-dependent phosphorylated proteins except hot NaOH and Pronase. Treatment with hot NaOH or Pronase removed the proteins from the gel as well as the ^{32}P label. The results of these experiments indicate that the ^{32}P associated with the three proteins whose phosphorylation is stimulated by calcium and phosphatidylserine was incorporated into protein rather than lipid or nucleic acid. In addition the stability of these phosphorylated compounds in base at low temperatures, and in trichloroacetic acid at elevated temperatures, indicates that the bonds are of the phosphoester rather than acylphosphate type (17).

In the presence of 50 μg/ml phosphotidylserine, Ca^{2+} concentrations above 10^{-7} *M* re-

TABLE II. PHOSPHATE BOND ANALYSIS EXPERIMENTS

Treatment	Arbitrary density units	
	$+Ca^{2+}$/PS	$-Ca^{2+}$/PS
1. Control	1.000	0.347 ± 0.012
2. Acetone	0.990 ± 0.007	0.357 ± 0.010
3. Chloroform/ methanol	0.935 ± 0.013	0.334 ± 0.012
4. NaOH (cold)	0.850 ± 0.005	0.291 ± 0.016
5. NaOH (boiling)	—	—
6. TCA (boiling)	0.802 ± 0.014	0.266 ± 0.025
7. Pronase	—	—
8. Ribonuclease A	1.023 ± 0.017	0.328 ± 0.009

Note. Values are means ± SE. Phosphorylation of placental cytosol was performed with the after treatment listed in the table as described under Methods. The control sample was without any after treatments. For each treatment the densitometer scanner reading of the 37,000 mol wt calcium-activated, phospholipid-dependent phosphoprotein is given. The data have been normalized to the control in the presence of activator (n = 4).

sulted in a dose-dependent stimulation of phosphorylation of the 37,000, 43,000, and 47,000 mol wt proteins (Fig. 2a). Half-maximal enhancement was observed with 1×10^{-6} M calcium (Fig. 2b). Phosphatidylserine in the absence of calcium did not effect protein phosphorylation. In the presence of 10^{-6} M Ca^{2+}, phosphatidylserine induced a dose-dependent increase in the phosphorylation of the three specific calcium-activated, phospholipid-dependent proteins (Fig. 3a). Maximum phosphorylation was attained with 70 μg/ml of phosphatidylserine and higher levels resulted in a decrease in phosphorylation (Fig. 3b). Phosphorylation was suppressed at 250 μg/ml of phosphatidylserine. Two other membrane phospholipids, phosphatidylcholine and phosphatidylinositol, showed respectively very little and approximately equal effects as phosphatidylserine as a coeffector with calcium (data not shown). Tetracaine, an anesthetic agent known to inhibit the calcium-

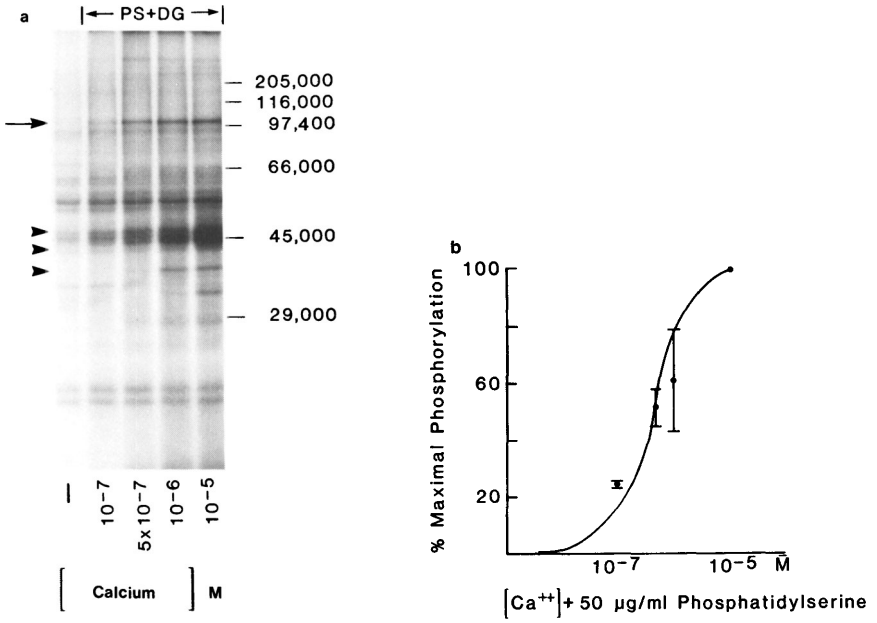

FIG. 2. Calcium dose-dependent enhancement of phosphorylation of calcium-activated, phospholipid-dependent phosphoproteins. (a) Representative autoradiograph demonstrating the effect of increasing concentrations of calcium (10^{-7} to 10^{-5} M) in the presence of phosphatidylserine (50 μg/ml) upon the phosphorylation of placental cytosol. The major calcium-activated, phospholipid-dependent phosphoproteins are indicated by arrowheads; the 98,000 mol wt phosphoprotein activated by calcium alone is indicated by the arrow (left). Molecular weight standards are indicated to the right. (b) Densitometer readings (percentage of maximum phosphorylation) of the 37,000 mol wt calcium-activated, phospholipid-dependent protein (vertical axis) are plotted against the calcium concentration (M). These results show the means and SE of three experiments.

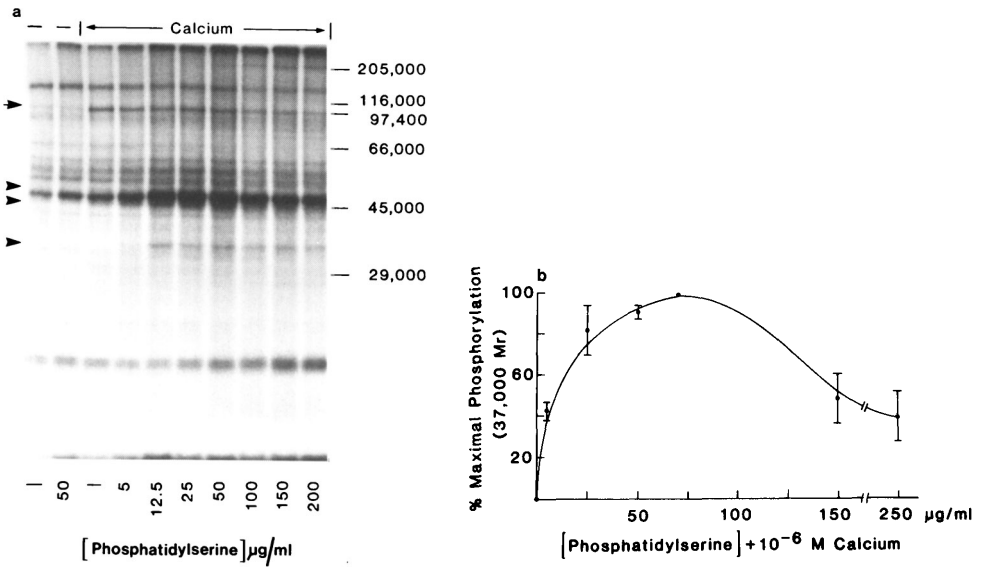

FIG. 3. Phosphatidylserine dose-dependent enhancement of phosphorylation. (a) Representative autoradiograph demonstrating the effect of increasing concentrations of phosphatidylserine (5 to 200 μg/ml) in the presence of calcium (10^{-6} *M*) upon the phosphorylation of placental cytosol. The major calcium-activated, phospholipid-dependent phosphoproteins are indicated by arrowheads; the 98,000 mol wt phosphoprotein activated by calcium alone is indicated by the arrow (left). Molecular weight standards are indicated to the right. (b) Densitometer readings (percentage of maximum phosphorylation) of the 37,000 mol wt calcium-activated, phospholipid-dependent protein (vertical axis) are plotted against the calcium concentration (*M*). These results show the means and SE of three experiments.

activated, phospholipid-dependent kinases (18), inhibited the phosphatidylserine-induced phosphorylation in a dose-dependent manner. Half-maximal inhibition of phosphorylation of the 37,000 mol wt calcium-activated, phospholipid-dependent protein occurred with 4×10^{-4} *M* tetracaine (Fig. 4).

In the absence of phospholipid, calcium at concentrations above 10^{-6} *M*, induced dose-dependent increases in the phosphorylation of a major phosphoprotein of 98,000 mol wt. Phosphoproteins of mol wt 90,000, 20,000, and 19,000 are also enhanced by calcium in the absence of phospholipids in many preparations (21). The calcium-induced 98,000 mol wt phosphoprotein is half-maximally enhanced at approximately $3.7 \pm 0.6 \times 10^{-7}$ *M* Ca^{2+} (Fig. 5). In the presence of 70 μg/ml of phosphatidylserine, phosphorylation of the 98,000 mol wt phosphoprotein was suppressed (Fig. 3). The other, more variably observed Ca^{2+}-enhanced phosphoproteins (90,000, 20,000, and 19,000 mol wt) also appear to be suppressed by phosphotidylserine (data not shown). In many tissues a calcium-activated, calmodulin-dependent kinase has been associated with major physiological processes (19). Stimulation of phosphorylation by these substances was therefore investigated. Calmodulin

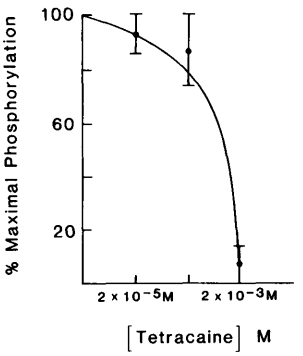

FIG. 4. Inhibition of the 37,000 mol wt calcium-activated, phospholipid-dependent phosphorylation by tetracaine. Densitometer readings (percentage of maximum phosphorylation) demonstrating the effect of increasing concentrations of tetracaine (2×10^{-5} to 2×10^{-3} *M*) upon phosphorylation of proteins by calcium (10^{-6} *M*) and phosphatidylserine (50 μg/ml). These results show the means and SE of five experiments.

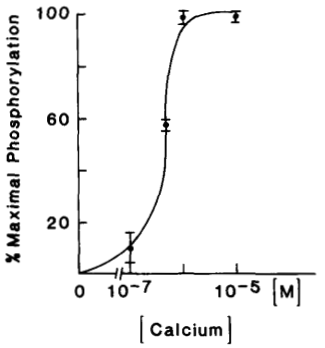

FIG. 5. Calcium dose-dependent enhancement of phosphorylation of the 98,000 mol wt protein. The percentage of maximum phosphorylation of the 98,000 mol wt protein as determined by densitometer scanning of autoradiographs (vertical axis) is shown versus concentration (molar) of added calcium. The results show the means and SE of three experiments.

in concentrations of 10^{-10} to 10^{-6} *M* had no detectable effect upon Ca^{2+}-enhanced protein phosphorylation in placental cytosol (data not shown). However, the calmodulin inhibitor, R-24571 (Calmidazolium) specifically inhibited calcium enhanced phosphorylation of the 98,000 mol wt phosphoprotein. This effect was half-maximal at 5×10^{-6} *M* Calmidazolium (Fig. 6). This inhibition could not be overcome with additional calmodulin. Calmidazolium did not inhibit calcium-activated, phospholipid-dependent phosphorylation in placental cytosol.

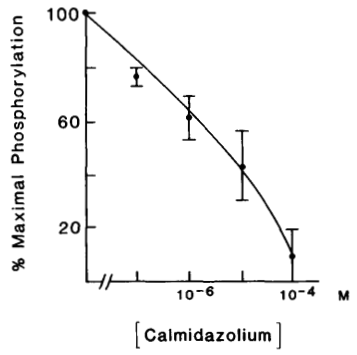

FIG. 6. Inhibition of calcium-activated phosphorylation by Calmidazolium. Densitometer readings (percentage of maximum phosphorylation) demonstrating the effect of increasing concentrations of Calmidazolium (10^{-7} to 10^{-4} *M*) upon phosphorylation of the 98,000 mol wt phosphoprotein by calcium (10^{-6} *M*). These results show the means and SE of five experiments.

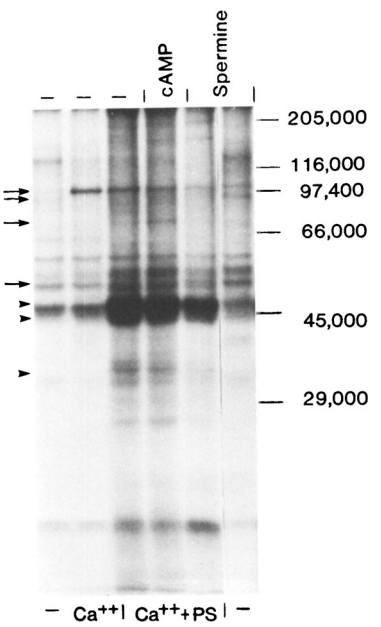

FIG. 7. The effect of cAMP and spermine upon calcium-activated, phospholipid-dependent phosphorylation. Autoradiograph demonstrating the results of combinations of activating agents. The wells contain cAMP (10^{-6} *M*), spermine (10^{-3} *M*), and/or calcium (10^{-6} *M*), as indicated. The arrowheads indicate the calcium-activated, phospholipid-dependent phosphoproteins. The arrows indicate phosphoproteins activated by other agents. Molecular weight standards are indicated to the right. These results are representative of three experiments ($n = 3$).

The effects of cAMP and spermine, other known effectors of phosphorylation in human placenta, were tested for possible interactions with calcium-activated phospholipid-dependent phosphorylation (14, 20, 21). Proteins activated by cAMP and spermine were distinct from those activated by calcium and phosphotidylserine. Spermine (1 m*M*) partially inhibited the calcium-activated, phospholipid-dependent phosphorylation (Fig. 7).

Discussion. The central finding of this study is that calcium in conjunction with phospholipid enhances the phosphorylation of a distinct group of proteins. This raises the possibility that hormones which modulate the metabolism of cell membrane phospholipids with the intracellular release of phospholipid components may exert physiological effects by the activation of placental kinase C and the subsequent phosphorylation of specific proteins.

An extensive list of hormones has been postulated to act through kinase C in this manner,

including insulin, α-adrenergic agonists, several growth factors, angiotensin II, vasopressin, and muscarinic cholinergic agents (4, 5). Receptors for many of these hormones have been described in the human placenta. Placental insulin receptors have been localized on the microvillus brush border of the placental trophoblast, facing the maternal circulation (22, 23). Although a small effect of insulin upon placental glycogen synthase and phosphorylase has been reported by a few workers (24), this finding has been disputed by others (25). In addition there have been reports of failure of insulin to influence uptake or transport of amino acids or glucose in the placenta (26, 27). The importance of insulin in the placenta thus remains questionable. Epidermal growth factor receptors have also been localized in the microvillus brush border (9). *In vitro,* EGF has been shown to increase hCG production in placental explants (12). In identical experiments, phorbol esters which are known to stimulate kinase C in some tissues also stimulated hCG production (12). Angiotensin II receptors have been described in placental membrane fractions but the physiological role of this hormone in placenta remains unknown (10). Although it has been known for almost 40 years that the placenta produces large amounts of acetylcholine, it remains uncertain whether this neurotransmitter acts upon the placenta to mediate placental function. Sastry *et al.* (11) have reported that muscarinic cholinergic blockers inhibit AIB transport in the placenta. Pharmacologic doses of these blockers are required to obtain these effects, however, and attempts by two groups to demonstrate specific, CNS-like muscarinic cholinergic binding sites (QNB binding) have failed (28). Therefore, although the number of hormones which could potentially exert effects through the placental kinase C with resulting phosphorylation of calcium-activated, phospholipid-dependent phosphoproteins is large, the physiological importance of many of these hormones in the placenta is still unknown.

In addition to calcium-activated, phospholipid-dependent enhancement of phosphorylation, calcium-activated calmodulin-dependent phosphorylation has been shown to be of great importance in many tissues (19). Major known effects of the latter include modulation of the activity of the enzymes phosphorylase kinase and myosin light chain kinase, both of which have been described in the human placenta (29, 30). Large amounts of calmodulin have been reported in the human placenta (31). An attempt was therefore made to identify calcium-activated, calmodulin-dependent phosphoproteins and compare them with the calcium-activated, phospholipid-dependent phosphoproteins. Calmodulin addition to placental cytosol resulted in no detectable change in phosphorylation from that observed in the presence of calcium alone. To determine whether saturating concentrations of calmodulin in placenta might prevent a detectable response to calmodulin addition, inhibitors of calmodulin were included along with calcium in some assays. The calmodulin inhibitor Calmidazolium (R-24571) inhibited the 98,000 mol wt protein enhanced by calcium alone. This inhibition, however, could not be overcome by calmodulin. Whether the 98,000 mol wt phosphoprotein and others which are enhanced by calcium alone in placental cytosol are, in fact, enhanced by calcium and endogenous calmodulin therefore still remains uncertain.

In previous reports we demonstrated that cAMP and spermine each enhance distinct sets of phosphoproteins in human placenta (14, 21). Further, we reported that spermine inhibits cAMP-dependent phosphorylation in the placenta (14). The calcium-activated, phospholipid-dependent phosphoproteins are distinct from those enhanced by either cAMP or spermine. Spermine, however, at 10^{-3} *M* inhibited the calcium-activated, phospholipid-dependent phosphoproteins (Fig. 7).

Specific human placental phosphoproteins are activated by calcium in combination with phospholipids. The significance of this finding must remain preliminary until the proteins are shown to be phosphorylated under *in vivo* conditions and the identity of the proteins is known. However, receptors for several hormones which are postulated to exert physiological effects through the activation of kinase C exist in the human placenta. Therefore, these hormones may exert effects upon the placenta by the phosphorylation of these phosphoproteins.

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