Activation of protein kinase in the bovine corpus luteum by phospholipid and Ca^{2+}

John S. DAVIS* and Martin R. CLARK

The Endocrine Laboratory, Department of Obstetrics and Gynecology and Department of Biochemistry, University of Miami School of Medicine, Miami, FL 33101, U.S.A.

(Received 7 April 1983/Accepted 13 May 1983)

A new species of protein kinase has been identified in cytosol preparations from bovine corpora lutea. Enzyme activity required the simultaneous presence of Ca^{2+} and phospholipid, and was also enhanced by glyceryl dioleate. Phosphatidylserine was the most effective phospholipid for stimulating histone phosphorylation. Other phospholipids capable of supporting enzymic activity were, in order of decreasing activity, phosphatidylinositol, phosphatidic acid, cardiolipin and phosphatidylglycerol. Several other phospholipids tested were ineffective. A cyclic AMP-dependent protein kinase was also present in the luteal cytosol. This enzyme activity was eliminated by protein kinase inhibitor without affecting the Ca^{2+} - and phospholipid-stimulated activity. Lysine-rich histone (IIIS) was a much better substrate than type-IIA histone for Ca^{2+} - and phospholipid-dependent phosphorylation. Ca^{2+} and phospholipid also enhanced phosphorylation of endogenous luteal cytosol protein. Calmodulin, alone or in the presence of Ca^{2+} , was unable to increase phosphorylation. Trifluoperazine inhibited protein kinase activity stimulated by Ca^{2+} and phospholipid. These data suggest that a phospholipid-sensitive, Ca^{2+} -dependent protein kinase may provide an important link between hormonally-induced changes in phospholipid metabolism and corpus-luteum function.

Although many of the effects of LH in the corpus luteum are thought to be mediated by cyclic AMP (Marsh, 1976), the exact mechanism of action of LH is unknown. It is presumed that the LH-induced events in ovarian tissues mediated by cyclic AMP occur via cyclic AMP-dependent protein kinases. Fluxes in cellular Ca^{2+} may also have a mediatory role in the action of LH in ovarian tissues (Higuchi *et al.*, 1976; Sawyer *et al.*, 1979; Veldhuis & Klase, 1982). Like cyclic AMP, Ca^{2+} may regulate cellular function through phosphorylation of endogenous substrate proteins (Means, 1981; Flockhart & Corbin, 1982). In addition, hormone-induced alterations in phospholipid metabolism may regulate a variety of cellular processes (Fourcans, 1974; Michell, 1975), including steroidogenesis (for

review, see Farese, 1983). Recently, this laboratory has reported that LH induces rapid alterations in phosphatidylinositol metabolism and that these events are temporally- and dose-related to LH-induced steroidogenesis in the bovine corpus luteum (Davis *et al.*, 1981). However, the effects of LH on phospholipid metabolism could not be duplicated by exogenous cyclic nucleotides, suggesting that this action of LH occurs either before or independent of LH-induced cyclic AMP accumulation.

Recent reports (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981) describe the widespread occurrence of a new species of cyclic nucleotide-independent protein kinase that is selectively activated by the simultaneous presence of Ca^{2+} and phospholipid. The enzyme shows a marked preference for phosphatidylinositol and PtdSer (Takai *et al.*, 1979*a*). In addition to these phospholipids, diacylglycerol sharply increases the affinity of this protein kinase for both PtdSer and Ca^{2+} and thereby renders the enzyme active at micromolar Ca^{2+} concentrations (Takai *et al.*, 1979*b*; Kishimoto *et al.*, 1980; Kuo *et al.*, 1980; Nishizuka & Takai, 1981). The unique

Abbreviations used: PtdSer, phosphatidylserine; PKi, beef heart protein kinase inhibitor; LH, lutropin ('luteinizing hormone').

* To whom requests for reprints should be sent at: Research Service, James A. Haley Veterans Hospital, 13000 North 30th Street, Tampa, FL 33612, U.S.A.

characteristics of the enzyme, taken together with the close correlation between phosphatidylinositol turnover and diacylglycerol formation (Michell, 1975), led Nishizuka and co-workers to propose that activation of this protein kinase may be related to receptor-mediated phosphatidylinositol turnover (Takai *et al.*, 1979b; Kishimoto *et al.*, 1980; Nishizuka & Takai, 1981). Although a direct demonstration of the specific regulation of this protein kinase in any system has not been reported, it has been suggested that the phospholipid-sensitive Ca^{2+} -dependent protein kinase is activated in platelets upon stimulation by thrombin (Nishizuka & Takai, 1981; Sano *et al.*, 1983). Supporting this hypothesis are the observations that (1) thrombin-induced breakdown of phosphatidylinositol to form diacylglycerol accompanies the phosphorylation of a 40000-mol. wt. protein, (2) addition of purified protein kinase to crude platelet homogenates enhances the phosphorylation of this protein and (3) the phosphorylated sites of the 40000-mol. wt. protein appear to be similar in both purified cell-free systems and intact platelets. These observations on a protein kinase that is selectively activated by Ca^{2+} and phospholipid and our evidence for the regulation of phosphatidylinositol metabolism in the bovine corpus luteum provided the impetus for the following studies describing a phospholipid-sensitive, Ca^{2+} -dependent protein kinase in the bovine corpus luteum.

Materials and methods

Materials

Histones, phospholipids, cyclic AMP, cyclic GMP, bovine brain calmodulin (4300 units/mg) and beef heart protein kinase inhibitor (PKi) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (triethylammonium salt) was obtained from New England Nuclear and was diluted with unlabelled ATP as required.

Ovaries from cows, 2–6 months pregnant, were obtained at slaughter and transported to the laboratory in 0.154 M-NaCl at 0°C. The corpora lutea were dissected free from adjacent ovarian tissue, weighed and slices (0.5 mm) were prepared using a Stadie-Riggs hand microtome. Luteal slices were homogenized in 5 vol. of a buffer containing 20 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 5 mM-EGTA, 0.25 M-sucrose and 50 mM-2-mercaptoethanol (Minakuchi *et al.*, 1981). The homogenate was centrifuged for 10 min at 800 g and the pellet discarded. Bovine luteal cytosol was obtained from the supernatant after an additional centrifugation for 60 min at 100000 g and was used immediately or stored frozen at -80°C. All procedures were performed at 0–4°C.

Assay for protein kinase activity

The luteal cytosol fraction was assayed for protein kinase activity by measuring its ability to transfer ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to histone. All reactions were carried out in plastic tubes. The standard reaction mixture (0.25 ml) contained 5 μmol of Tris/HCl, pH 7.5, 1.25 μmol of magnesium acetate, 5 nmol of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1.5×10^6 c.p.m./nmol), 50 μg of lysine-rich histone (IIIS), 100 μl of luteal cytosol and various combinations of glyceryl 1,2-dioleate (0.2 μg), phospholipid (5 μg) and CaCl_2 (125 nmol in excess of the cytosol EDTA and EGTA concentrations). Lipids were suspended in 20 mM-Tris/HCl, pH 7.5, by sonication for 3 min at 0°C (Takai *et al.*, 1979a,b). Enzyme activity was determined to be linear with time (0–4 min) and proportional to the concentration of luteal cytosol enzyme preparation (50–200 μg of protein) at 30°C. All subsequent determinations of protein kinase activity were conducted with 70–100 μg of cytosol protein and were terminated after 3 min at 30°C. Cyclic AMP-dependent protein kinase activity was assayed under similar conditions except that 500 pmol of cyclic AMP and various concentrations of the heat-stable beef heart PKi were added. Cyclic GMP-dependent protein kinase activity was assayed in the presence of 500 pmol of cyclic GMP. Calmodulin-sensitive phosphorylation was assayed with calmodulin (2 μg) in the presence and in the absence of Ca^{2+} . The reaction was terminated by addition of 2.0 ml of ice-cold 25% trichloroacetic acid and acid-precipitable materials were collected on Millipore (0.45 μm ; HAWP) membrane filters (Takai *et al.*, 1979a,b). After washing with three 2 ml portions of trichloroacetic acid, the filters were dried and counted for radioactivity in scintillation fluid. The c.p.m. of ^{32}P were converted into pmol of ^{32}P incorporated into histone from the specific radioactivity of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the assay mixture and the protein kinase activity expressed as pmol of ^{32}P incorporated/min per mg of cytosol protein. Experimental values are corrected for background activity (less than 25% of the activity observed in the absence of any treatments). Background activities observed in the absence of enzyme or in the presence of a portion of boiled enzyme were similar. Data are expressed as means $\pm$ S.E.M. of triplicate determinations or as means after expression as a percentage of enzymic activity. Individual values generally varied less than 5% from the mean.

Cytosol protein was measured by the Coomassie Brilliant Blue G-250 binding method with bovine γ -globulin as standard (Bradford, 1976).

Results and discussion

The effects on protein kinase activity of Ca^{2+} , PtdSer and unsaturated diacylglycerol (glyceryl

Table 1. *Activation of lipid-sensitive Ca²⁺-dependent protein kinase from bovine corpora lutea*

Bovine luteal cytosol was prepared and lipid-sensitive Ca²⁺-dependent protein kinase activity was determined as described in the Materials and methods section. CaCl₂ (500 μM), PtdSer (20 μg/ml) and glyceryl dioleate (0.8 μg/ml) were added alone or in various combinations. Protein kinase activity is expressed as a percentage of the activity observed in samples treated with Ca²⁺, PtdSer and glyceryl dioleate after subtraction of the activity observed in the absence of treatments. The means of triplicate samples are shown.

Glyceryl dioleate	PtdSer	Ca ²⁺	Enzymic activity (%)
+	+	+	100
—	+	+	66
+	+	—	51
+	—	+	—11
—	—	+	0
—	+	—	15
+	—	—	—2
—	—	—	0

Table 2. *Activities of purified phospholipids on lipid-sensitive Ca²⁺-dependent protein kinase from bovine corpora lutea*

The effects of purified phospholipids (20 μg/ml) on protein kinase activity were determined in the presence of CaCl₂ (500 μM) and glyceryl dioleate (0.8 μg/ml). Enzymic activity is expressed as a percentage of the activity observed in samples treated with PtdSer after subtraction of the activity observed in the absence of phospholipid. Data are the means of triplicate samples.

Phospholipid	Enzymic activity (%)
Phosphatidylserine	100
Phosphatidylinositol	76
Phosphatidic acid	75
Cardiolipin	65
Phosphatidylglycerol	37
Phosphatidylethanolamine	1
Sphingomyelin	—12
Phosphatidylcholine	—14

ase from other tissues required both Ca²⁺ and phospholipid for enzyme activation (Kishimoto *et al.*, 1980; Ogawa *et al.*, 1981; Wise *et al.*, 1982a,b). Furthermore, unsaturated diacylglycerol stimulates the activity of this protein kinase (Nishizuka & Takai, 1981; Ogawa *et al.*, 1981; Wise *et al.*, 1982b). Addition of glyceryl dioleate to luteal cytosol enhanced enzyme activity in samples containing PtdSer alone, and in samples containing both Ca²⁺ and PtdSer (Table 1). The activation of the enzyme in the presence of glyceryl dioleate and phospholipid may reflect the presence of low levels of free Ca²⁺ in the assay system and the ability of glyceryl dioleate to reduce the *K_a* for Ca²⁺ and thereby enhance enzyme activity (Kishimoto *et al.*, 1980; Ogawa *et al.*, 1981; Wise *et al.*, 1982b). Because of its unique stimulatory effects on this protein kinase, Nishizuka & Takai (1981) have proposed that diacylglycerol formed as a result of receptor-mediated phosphatidylinositol turnover serves as a second messenger in the activation of this phospholipid-sensitive, Ca²⁺-dependent protein kinase (Takai *et al.*, 1979b; Nishizuka & Takai, 1981).

Various purified phospholipids were tested for their ability to activate this protein kinase (Table 2). PtdSer appeared to be the most effective phospholipid for stimulating enzyme activity in luteal cytosol preparations. Other phospholipids capable of stimulating enzyme activity were, in order of decreasing activity, phosphatidylinositol, phosphatidic acid, cardiolipin and phosphatidylglycerol. Phosphatidylethanolamine was ineffective and phosphatidylcholine and sphingomyelin were slightly inhibitory. PtdSer was also reported to be the most effective phospholipid for activating this protein kinase in preparations from rat brain (Nishizuka & Takai, 1981), beef heart (Wise *et al.*, 1982b) and human lymphocytes (Ogawa *et al.*, 1981). The relative abilities of some of the less effective phospholipids to support protein kinase activity are different in various enzyme preparations (Table 2; Takai *et al.*, 1979a; Ogawa *et al.*, 1981; Wise *et al.*, 1982b). At present it is not clear why these differences exist, but it seems likely that dissimilar tissues may have selective phospholipid requirements.

To determine whether or not the protein kinase activity observed in the presence of Ca²⁺ plus lipid (PtdSer and glyceryl dioleate) was merely a reflection of the activation of the luteal cyclic AMP-dependent protein kinase, experiments were conducted in the presence of 2 μM-cyclic AMP and increasing doses of the heat-stable PKi. Fig. 1 shows that cyclic AMP markedly increased protein kinase activity under control conditions (absence of Ca²⁺ plus lipid). However, the effects of cyclic AMP and Ca²⁺ plus lipid were not additive. Instead, the level of phosphorylation observed with the combination of

dioleate) either alone or in combination were examined in cytosol extracts from bovine corpora lutea (Table 1). When tested alone, these treatments had no or very little effect on protein kinase activity. In addition, no stimulatory effects were observed in the presence of both Ca²⁺ and glyceryl dioleate. However, when PtdSer was added in the presence of Ca²⁺, a marked stimulatory effect on enzyme activity was observed. Similarly, purified preparations of lipid-sensitive Ca²⁺-dependent protein kin-

Fig. 1. Effects of cyclic AMP and PKi on control and lipid-sensitive, Ca^{2+} -dependent protein kinase activity. Protein kinase activity was determined in the absence (control, open columns) or in the presence of Ca^{2+} + lipid [CaCl_2 (500 μM), PtdSer (20 $\mu\text{g}/\text{ml}$) and glyceryl dioleate (0.8 $\mu\text{g}/\text{ml}$); hatched columns]. In addition, the effects of cyclic AMP (2 μM) on control and Ca^{2+} -plus-lipid-dependent protein kinase activity were determined in the presence of increasing doses of PKi. Assay conditions are described in the Materials and methods section. The bars represent $\pm$ S.E.M.

cyclic AMP and Ca^{2+} plus lipid was similar to the level produced by Ca^{2+} plus lipid alone. This lack of additivity may be explained by the observations of Kuo *et al.* (1970) and Yamashita & Field (1972), who reported that Ca^{2+} inhibits cyclic AMP-dependent protein kinase activity in a variety of bovine tissues. In addition, Endo & Hidaka (1981) reported that PtdSer and phosphatidylinositol interfere with cyclic AMP-dependent protein kinase activity, presumably by directly interacting with substrate proteins. The contribution of cyclic AMP-dependent protein kinase was also evaluated by using a specific cyclic AMP-dependent protein kinase inhibitor. Addition of increasing doses of PKi (0–30 units) to samples containing cyclic AMP resulted in dose-dependent reductions in cyclic AMP-dependent protein kinase activity (Fig. 1). When increasing doses of PKi were added in the presence of cyclic AMP and Ca^{2+} plus lipid, enzyme activities were similar to those observed in the presence of Ca^{2+} plus lipid alone. Furthermore, the addition of PKi alone had no effect on enzyme activity in control or Ca^{2+} -plus-lipid-treated samples (Fig. 2).

Fig. 2. Lack of effect of PKi on lipid-sensitive Ca^{2+} -dependent protein kinase activity.

Protein kinase activity was determined in the absence (control; open columns) or in the presence of Ca^{2+} + lipid [CaCl_2 (500 μM), PtdSer (20 $\mu\text{g}/\text{ml}$) and glyceryl dioleate (0.8 $\mu\text{g}/\text{ml}$); hatched columns] with increasing doses of PKi. The bars represent $\pm$ S.E.M.

Table 3. Effect of protein kinase inhibitor on lipid-sensitive Ca^{2+} -dependent and cyclic AMP-dependent protein kinase activities

Protein kinase activity in bovine luteal cytosol was assayed under conditions described in the Materials and methods section. Cyclic AMP (2 μM) or CaCl_2 (500 μM), PtdSer (20 $\mu\text{g}/\text{ml}$) and glyceryl dioleate (0.8 $\mu\text{g}/\text{ml}$) were tested for their ability to stimulate phosphorylation of histones IIA and IIIS (50 μg each). Enzymic activity is also expressed as a ratio of the activities observed with histones IIIS and IIA. In some samples, a submaximal dose (5 inhibitory units) of PKi was added. The means of triplicate samples are shown. Numbers in parentheses represent the average percentages of the activities observed in the absence of PKi.

Treatment	Protein kinase activity (pmol/min per mg of protein)		
	Histone IIA	Histone IIIS	Ratio IIIS/IIA
Ca^{2+} and lipid	55.8	97.0	1.74
Ca^{2+} and lipid + PKi	53.7 (96)	94.1 (97)	1.75
Cyclic AMP	190	109	0.57
Cyclic AMP + PKi	137 (72)	88 (81)	0.64

Table 3 shows the relative abilities of cyclic AMP and Ca^{2+} plus lipid to stimulate the phosphorylation of histones IIA and IIIS. Lysine-rich histone (IIIS) was a much better substrate for the lipid-sensitive

Table 4. *Bovine luteal protein kinase activity in the presence of PtdSer, calmodulin and trifluoperazine*
The effects of PtdSer (20 µg/ml) and calmodulin (4300 units/ml) were assayed in the presence of CaCl₂ (500 µM). Control groups were treated with CaCl₂ alone. Enzyme activity in the absence of CaCl₂ was 48 ± 0.7 pmol/min per mg of protein. Some samples were also treated with the indicated concentrations of trifluoperazine.

Additions	Trifluoperazine (µM)	...	Protein kinase activity (pmol/min per mg)		
			0	10	50
None			47 ± 0.9	35 ± 0.1	42 ± 2.6
PtdSer			93 ± 0.4	59 ± 3.1	52 ± 3.2
Calmodulin			44 ± 0.3	41 ± 0.2	39 ± 1.8

Fig. 3. *Phosphorylation of endogenous luteal cytosol proteins by lipid-sensitive Ca²⁺-dependent protein kinase*
Protein kinase activity was determined in the absence of exogenous phosphate acceptor proteins. Increasing concentrations of luteal cytosol protein were incubated for 3 min at 30°C in the absence of Ca²⁺ (control; —) or in the presence of Ca²⁺ + lipid [CaCl₂ (500 µM), PtdSer (20 µg/ml) and glyceryl dioleate (0.8 µg/ml); ----].

Ca²⁺-dependent enzyme, whereas histone IIA was much better for cyclic AMP-dependent phosphorylation, which agrees with previous observations by others (Takai *et al.*, 1979a; Ogawa *et al.*, 1981; Wise *et al.*, 1982a). A submaximal dose of PKi had no effect on the lipid- and Ca²⁺-dependent phosphorylation of either histone. In cyclic AMP-treated samples, PKi reduced the phosphorylation of histones IIIS and IIA by a similar extent (80%) in proportion to the activity observed in the absence of PKi. Cyclic GMP had no effect on enzyme activity in the presence of histone IIIS (results not shown).

Ca²⁺-dependent protein kinases that require calmodulin as a cofactor have been described (Means, 1981; Flockhart & Corbin, 1982). Maizels &

Jungman (1982) have recently reported that cytosol isolated from immature rat ovaries contains a Ca²⁺-dependent protein kinase that is sensitive to chlorpromazine. These results led to the suggestion that calmodulin may have a role in the regulation of Ca²⁺-dependent phosphorylation in the rat ovary. The possibility that calmodulin was somehow involved in the activation of this enzyme was considered, but this seemed unlikely since enzyme activity was not directly stimulated by Ca²⁺ (Tables 1 and 4). The addition of exogenous calmodulin (2 µg) also had no effect on histone phosphorylation in the presence of added Ca²⁺ (Table 4). Table 4 also shows the inhibitory effect of trifluoperazine on histone phosphorylation. Although phenothiazines such as chlorpromazine and trifluoperazine have been reported to be selective probes for calmodulin-sensitive enzymes (Means, 1981), the present paper and other studies (Mori *et al.*, 1980; Schatzman *et al.*, 1981; Wise *et al.*, 1982a) indicate that these drugs inhibit lipid-sensitive Ca²⁺-dependent protein kinases as well. Phenothiazines may exert their inhibitory effects by interacting at hydrophobic regions on the enzyme (Wise *et al.*, 1982a) or by directly interacting with phospholipid (Mori *et al.*, 1980; Schatzman *et al.*, 1981). Furthermore, endogenous substrate proteins for the lipid-sensitive and calmodulin-sensitive Ca²⁺-dependent protein kinases appear to be different in heart and brain tissue from the rat and guinea-pig (Wrenn *et al.*, 1980; Katoh *et al.*, 1981).

Lipid-sensitive, Ca²⁺-dependent protein kinases are capable of phosphorylating, in addition to histones (Takai *et al.*, 1979a; Ogawa *et al.*, 1981; Wise *et al.*, 1982a), phosphorylase kinase (Nishizuka *et al.*, 1978; Minakuchi *et al.*, 1981), glycogen synthase (Nishizuka *et al.*, 1978; Minakuchi *et al.*, 1981), myelin basic protein (Minakuchi *et al.*, 1981; Wise *et al.*, 1982a), troponin (Wise *et al.*, 1982a) and a variety of endogenous proteins in cytosol and particulate fractions of rat, guinea-pig, bovine and human tissues (Kuo *et al.*, 1980; Nishizuka & Takai, 1981; Wrenn *et al.*, 1981). The results of an experiment conducted to demonstrate the phosphorylation of endogenous luteal cytosol protein are shown in Fig. 3. When Ca²⁺, PtdSer and glyceryl dioleate were added to preparations of luteal cytosol in the absence of exogenous phosphate acceptor proteins, the incorporation of radioactive phosphate into cytosol proteins was increased. The increases in protein kinase activity were proportional to the amount of cytosol protein assayed. These results suggest that bovine luteal cytosol contains a substrate protein(s) that may be phosphorylated by activation of a lipid-sensitive Ca²⁺-dependent protein kinase.

In conclusion, these results demonstrate for the first time the presence of a cyclic nucleotide-inde-

pendent phospholipid-sensitive Ca^{2+} -dependent protein kinase in the corpus luteum. Enzyme preparations from the whole ovaries of pregnant mare serum gonadotropin-primed immature rats (Davis & Clark, 1982a) and mature rats (Kuo *et al.*, 1980) have also been found to have a similar activity. Whether or not this new ovarian protein kinase is hormonally regulated is unknown. These observations on ovarian lipid-sensitive Ca^{2+} -dependent protein kinase, and evidence for the hormonal regulation of ovarian phosphatidylinositol metabolism and presumably diacylglycerol levels (Davis *et al.*, 1981, 1983; Davis & Clark, 1982b; Naor & Yavin, 1982) raise intriguing new possibilities for the regulation of ovarian function.

This work was supported in part by NIH Grant HD-03142. We thank Mrs. Adalgisa Rojo for her excellent technical assistance and Ms. Gloria Urrea for help in the preparation of the manuscript. We also thank William J. LeMaire for his advice and support in this research.

References

- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Davis, J. S. & Clark, M. R. (1982a) *Proc. Annu. Meet. Endocrine Soc.* 63rd 339 (abstr.)
- Davis, J. S. & Clark, M. R. (1982b) *Biol. Reprod.* **2** (Suppl. 1), 41 (abstr.)
- Davis, J. S., Farese, R. V. & Marsh, J. M. (1981) *Endocrinology* **109**, 469–475
- Davis, J. S., Farese, R. V. & Clark, M. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2049–2053
- Endo, T. & Hidaka, H. (1981) *Arch. Biochem. Biophys.* **211**, 108–112
- Farese, R. V. (1983) *Endocrine Rev.* **4**, 78–95
- Flockhart, D. A. & Corbin, J. D. (1982) *CRC Crit. Rev. Biochem.* 133–186
- Fourcans, B. (1974) *Adv. Lipid Res.* **12**, 147–226
- Higuchi, T., Kaneko, A., Abel, J. M. & Niswender, G. D. (1976) *Endocrinology* **99**, 1023–1032
- Katoh, N., Wrenn, R. W., Wise, B. C., Shoji, M. & Kuo, J. F. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4813–4817
- Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276
- Kuo, J. F., Krueger, B. K., Sane, J. R. & Greengard, P. (1970) *Biochim. Biophys. Acta* **212**, 79–87
- Kuo, J. F., Anderson, R. G., Wise, B. C., Mackerlova, L., Solomonsson, I., Brackett, N. L., Katoh, N., Shoji, M. & Wrenn, R. W. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7039–7043
- Maizels, E. T. & Jungman, R. A. (1982) *Biochem. Biophys. Res. Commun.* **107**, 32–37
- Marsh, J. M. (1976) *Biol. Reprod.* **14**, 30–53
- Means, A. R. (1981) *Rec. Prog. Horm. Res.* **37**, 333–362
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Minakuchi, R., Takai, Y., Yu, B. & Nishizuka, Y. (1981) *J. Biochem. (Tokyo)* **89**, 1651–1654
- Mori, T., Takai, Y., Minakuchi, R., Yu, B. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 8378–8380
- Naor, Z. & Yavin, E. (1982) *Endocrinology* **111**, 1615–1619
- Nishizuka, Y. & Takai, Y. (1981) in *Protein Phosphorylation* (Rosen, O.M. & Krebs, E. G., eds.), pp. 237–249, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Nishizuka, Y., Takai, Y., Kishimoto, A., Hashimoto, E., Inoue, M., Yamamoto, M., Criss, W. E. & Kuroda, Y. (1978) *Adv. Cyclic Nucleotide Res.* **9**, 209–220
- Ogawa, Y., Takai, T., Kawahara, Y., Kimura, S. & Nishizuka, Y. (1981) *J. Immunol.* **127**, 1375–1379
- Sano, K., Takai, Y., Yamanishi, J. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 2010–2013
- Sawyer, H. R., Abel, J. H., McCellan, M. C., Schmitz, M. & Niswender, G. D. (1979) *Endocrinology* **104**, 467–486
- Schatzman, R. C., Wise, B. C. & Kuo, J. F. (1981) *Biochem. Biophys. Res. Commun.* **98**, 669–676
- Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. & Nishizuka, Y. (1979a) *J. Biol. Chem.* **254**, 3692–3695
- Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. & Nishizuka, Y. (1979b) *Biochem. Biophys. Res. Commun.* **91**, 1218–1224
- Veldhuis, J. D. & Klase, P. A. (1982) *Biochem. J.* **202**, 381–389
- Wise, B. C., Glass, D., Chau, C.-H. J., Raynor, R. L., Katoh, N., Schatzman, R. C., Turner, R. S., Kibler, R. F. & Kuo, J. F. (1982a) *J. Biol. Chem.* **257**, 8489–8495
- Wise, B. C., Raynor, R. L. & Kuo, J. F. (1982b) *J. Biol. Chem.* **257**, 8481–8488
- Wrenn, R. W., Katoh, N., Wise, B. C. & Kuo, J. F. (1980) *J. Biol. Chem.* **255**, 12042–12046
- Wrenn, R. W., Katoh, N. & Kuo, J. F. (1981) *Biochim. Biophys. Acta* **676**, 266–269
- Yamashita, K. & Field, J. B. (1972) *Metab. Clin. Exp.* **21**, 150–158