

Absence of Protein Kinase C in Nuclei of EL4 Mouse Thymoma Cells¹

David E. Jensen² and Julianne J. Sando³

Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908

ABSTRACT

Since evidence indicates that phorbol ester-induced production of interleukin 2 requires transcription, we investigated the possibility that the phorbol ester receptor acts directly in the nuclei of EL4 thymoma cells. Using a procedure that minimized plasma membrane contamination (as measured by 5'-nucleotidase activity) and maintained the integrity of the double nuclear membrane, we were unable to detect specific binding of [³H]phorbol 12,13-dibutyrate in nuclei of unstimulated cells. Treatment of cells with phorbol 12,13-dibutyrate (100 nM, 37°C) for up to 6 h did not cause appearance of phorbol ester binding capacity in nuclei ($4 \pm 8\%$ of homogenate value; 5'-nucleotidase activity = $10 \pm 3\%$) despite translocation of 40% of the cytosolic binding capacity to the plasma membrane fraction. The failure to detect nuclear binding capacity in treated cells was not due to occupation of nuclear sites with unlabeled ligand; effective exchange binding was demonstrated by recovery of total homogenate binding capacity in treated cells of $82 \pm 13\%$ of that in untreated cells. Treatment of isolated nuclei with DNase to liberate DNA binding proteins also failed to reveal any nuclear phorbol ester binding capacity. Assay of nuclei for protein kinase C enzymatic activity gave similar negative results. These data argue strongly against a direct action of the intact phorbol ester receptor (or the phorbol ester binding fragment) in the transcriptional activation of interleukin 2 in EL4 cells but cannot rule out the possibility of a role for the catalytic fragment.

INTRODUCTION

Treatment of EL4 thymoma cells with PEs⁴ causes inhibition of growth, adherence of the cells to substrate, and production of a number of lymphokines including IL2 (1, 2). Several types of evidence indicate that IL2 production requires RNA and protein synthesis. First, IL2 is not detectable until 6 h following stimulation of the producing cells, and maximal activity is not reached until 24-48 h (3). Second, production has been inhibited by both mRNA and protein synthesis inhibitors (4). Third, mRNA has been isolated from PE-stimulated EL4 cells (3) and lectin- (5) or lectin- and PE-stimulated (6) human lymphocytes and translated *in vitro* into biologically active IL2. More recently, human (7) and mouse (8) IL2 mRNA has been detected using complementary DNA probes. Finally, the hypothesis that the increased mRNA is a result of increased transcription, rather than stabilization, is strongly supported by nuclear "run-on" experiments showing detection of newly synthesized IL2 mRNA in isolated nuclei from stimulated human Jurkat cells (9) as well as by recent findings in our laboratory that no IL2 mRNA can be detected in unstimulated EL4 cells even in the presence of protein synthesis inhibitors (10). Collectively, these data indicate that a mechanism must exist for transducing the PE signal to the nucleus.

All available evidence indicates that the receptor for PEs is the Ca²⁺- and phospholipid-dependent protein kinase, PK-C.

PEs replace the diacylglycerol requirement for activating PK-C (11), diacylglycerols compete with PEs for binding to the PE receptor (12), and both PE binding and PK-C activities have been shown to coelute during purification to apparent homogeneity (13, 14). We have identified PE receptors in intact EL4 cells (2) as well as in cytosolic and crude membrane fractions (15), and we demonstrated that the PE binding exhibits the same requirements for phospholipid as does the kinase activity (15). Treatment of EL4 cells with PE causes loss of PK-C activity from the cytosol (16) concomitant with translocation of PE binding capacity from cytosol to crude membrane (17). Although several potential substrates for PK-C have been identified in cytosol of EL4 cells (18), mechanisms for transducing the signal to the nucleus remain unknown.

One hypothesis for this signal transduction is that PK-C may exist in or translocate to the nucleus (or nuclear membrane). Many of the membrane preparations in which PK-C has been found were crude and thus included all of the organelles [mitochondria, endoplasmic reticulum, plasma membrane, nuclei (*e.g.*, 15, 19)]. Therefore, localization to a specific membrane within the cell could not be determined. Perrella *et al.* have reported that [³H]PMA binding capacity exists in the nuclei of mouse epidermal (20) and liver cells (21). However, several problems in interpreting or generalizing from these data exist: a) PMA rather than the less lipophilic ligand, PDB, was used. PMA exhibits very high nonspecific binding (19). b) The binding was of relatively low affinity for this ligand ($K_d = 2-5$ nM). c) The percentage of the total PMA binding capacity of the cells present in nuclei (9% of the homogenate value) was similar to the amount of plasma membrane contamination present in the nuclear fraction (7% of the homogenate value). d) Detergent was used in the isolation of the nuclei and it has been reported that detergents cause the solubilization of the outer nuclear membrane (22). Thus, if PK-C were binding to this membrane, it would not have been detected.

We have isolated nuclei by a technique that maintains the integrity of the nuclear membranes and have reexamined them for the presence of PE receptors. Our results fail to demonstrate PDB binding capacity or PK-C activity in nuclei from control of PE-treated cells.

MATERIALS AND METHODS

Materials

PDB, PMA, AMP, AMPCP, phosphatidylserine, diolein, DNase I, and Hoechst Dye 33258 were purchased from Sigma Chemical Co. Forskolin was from Calbiochem-Behring Corp. [³H]PDB (15.8 Ci/mmol in ethanol) was purchased from New England Nuclear, [α -³²P]-ATP and [γ -³²P]ATP (75-130 Ci/mol) were obtained from the University of Virginia Diabetes Research and Training Center core lab, and [8-¹⁴C] 5'-AMP (57 mCi/mmol) was from ICN (Irvine, CA). EL4 cells were grown in RPMI 1640 medium with 5% heat-inactivated fetal calf serum (Gibco Laboratories) as previously described (2).

Isolation of Nuclei and Other Subcellular Fractions

Method 1: Cell Lysis in Isosmotic Sucrose. Cells were isolated and resuspended at a concentration of 2×10^7 /ml in Buffer 1 [0.32 M sucrose, 3 mM CaCl₂, 2 mM Mg(CH₃COOH)₂, 0.1 mM EDTA, 2 mM

Received 12/31/86; revised 4/16/87; accepted 4/22/87.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by NIH Grant GM31184.

² Supported by NIH Grant 5T32 GM07055-12.

³ To whom requests for reprints should be addressed. Recipient of American Cancer Society Grant FRA-306.

⁴ The abbreviations used are: PE, phorbol ester; PK-C, protein kinase C; PDB, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; IL2, interleukin 2; AMPCP, α,β -methylene adenosine 5'-diphosphate; NP-40, Nonidet P-40; PE-R, phorbol ester receptor.

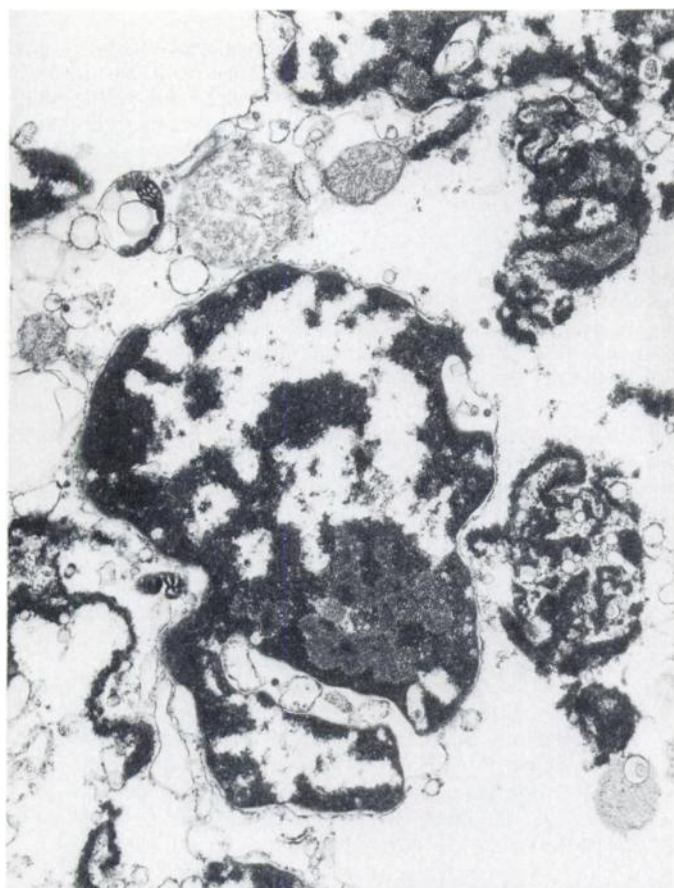


Fig. 1. Electron micrograph of nuclei isolated from untreated EL4 cells. Nuclei were isolated by nitrogen cavitation/sucrose density centrifugation as described in "Materials and Methods". A small aliquot was recentrifuged ($600 \times g$ for 10 min) and the pellet was then treated as described by Purdy-Ramos and Forbes (36). $\times 10,000$.

isolated by N_2 cavitation/sucrose gradient centrifugation. The central nucleus has both inner and outer membranes. Several mitochondria are also present, some expanded to maximum size. No combination of procedures gave nuclei that were free of contamination by mitochondria (as determined by the presence of cytochrome *c* oxidase).

To determine whether any PE receptor (PE-R and by inference PK-C) was present in nuclei of EL4 cells, the binding of [3H]PDB to the nuclei was investigated. Fig. 2A shows that all of the binding capacity in the homogenate could be accounted for by the binding present in the cytosol and plasma membrane. No PE binding could be detected in the nuclei.

Since it is possible that PE-R/PK-C exists in the nucleus only after treatment of cells with PE, the binding of [3H]PDB to the nuclei of PE-treated cells was investigated. This experiment required that there be effective exchange binding between any unlabeled PE remaining in the cells after treatment and the [3H]PDB in the binding assay. When cells were incubated with PMA, effective exchange for the less potent and less lipophilic [3H]PDB was not obtained (data not shown). Treatment of cells with PDB allowed for adequate exchange binding as demonstrated by a recovery of binding in the PDB-treated cells of $82 \pm 13\%$ of the homogenate binding of untreated cells.

As shown in Fig. 2B, specific PDB binding capacity in nuclei of cells treated with PDB (100 nM for 30 min) was 4% of the homogenate value. However, this binding could be easily accounted for by the plasma membrane contamination (10%) as shown by the 5'-nucleotidase activity in the nuclear fraction.

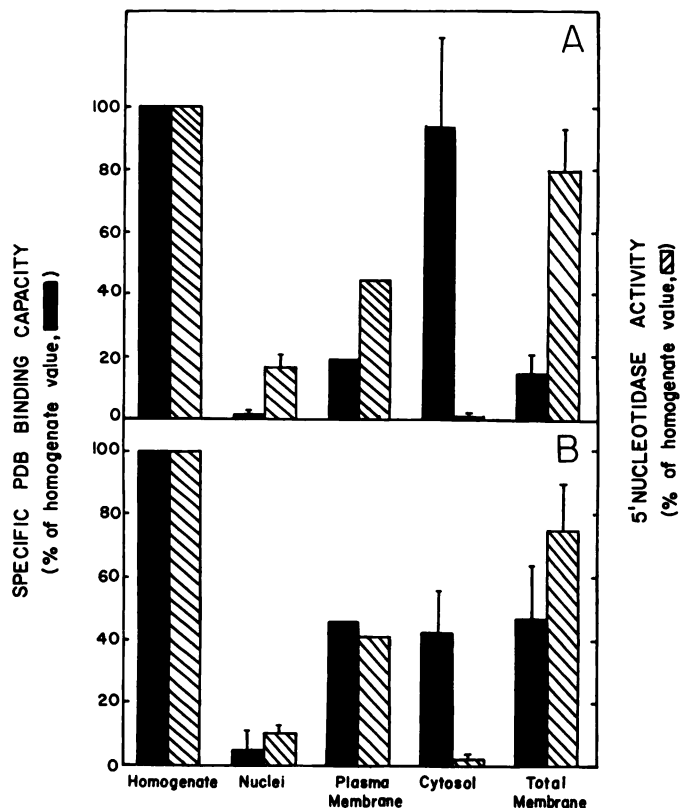


Fig. 2. Specific [3H]PDB binding capacity in fractions of control (A) and PDB-treated (B) EL4 cells. Cells were treated with 100 nM PDB (treated) or 0.01% ethanol (control) for 30 min. Cellular fractions were generated and assayed for PE binding and plasma membrane contamination as described in "Materials and Methods." Percentage of homogenate value, mean from three (5'-nucleotidase) or six (binding) experiments, each with triplicate determinations. Control homogenate: 100% binding = $5,610 \pm 520$ cpm/ μg DNA; 100% 5'-nucleotidase = $10,880 \pm 990$ cpm/ μg DNA. Treated homogenate: 100% Binding = $4,620 \pm 600$ cpm/ μg DNA; 100% 5'-nucleotidase = $11,290 \pm 1,310$ cpm/ μg DNA.

That the cells did respond to PE treatment is clear from the translocation of about 40% of the cytosolic PE binding capacity to the total membrane fraction. The total membrane binding could be completely accounted for by the binding in the plasma membrane fraction (again arguing against a contribution by the nuclei) and the total homogenate binding could be accounted for by the sum of binding in the appropriate fractions.

The whole homogenate 5'-nucleotidase activity could similarly be accounted for by the sum of cytosolic and total membrane activities, but not by the sum of cytosolic, nuclear, and plasma membrane activities. We have observed that whereas the PE binding capacity is stable on ice for up to 20 h, the 5'-nucleotidase activity rapidly decreases after homogenization of the cells such that more than 50% is lost after 8 h and activity is completely absent by 24 h (data not shown). Thus, the failure to recover all of the 5'-nucleotidase activity in the plasma membrane is probably due to the extra 1–1.5 h required to isolate the plasma membrane fraction after isolation of the nuclei. Since the total membrane could be obtained much more rapidly and its PE binding could be attributed to the plasma membrane, we used this fraction routinely.

Since IL2 mRNA is not detectable in EL4 cells until at least 3 h after PE stimulation (10), it is possible that longer times of PE treatment were needed to cause translocation of PE-R/PK-C to the nucleus. Fig. 3A shows the amount of [3H]PDB binding capacity detected in homogenate, cytosol, total membrane, and nuclei after treatment of EL4 cells with 100 nM PDB for 0, 0.5, 3, and 6 h. Fig. 3B shows the amount of plasma membrane

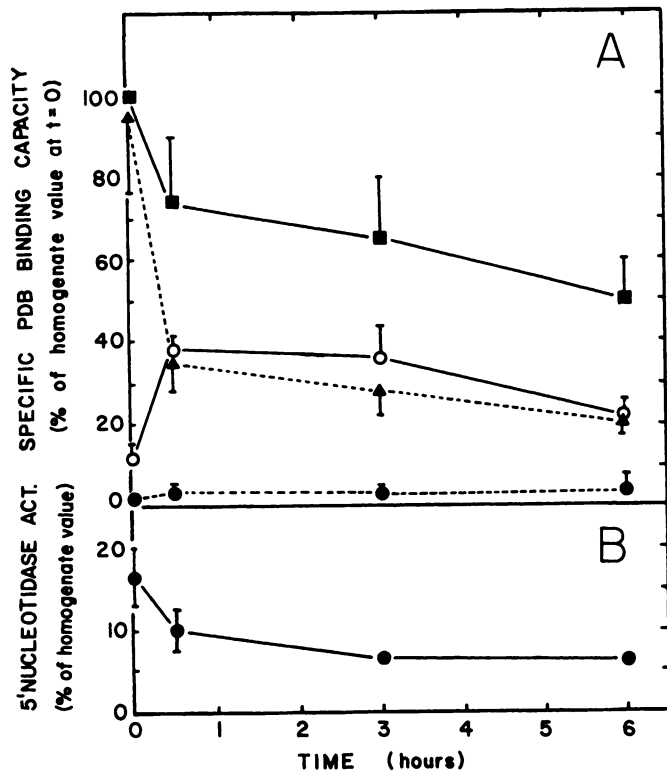


Fig. 3. A, time course for the effect of PDB treatment on subcellular localization of [³H]PDB binding capacity. Cells were treated for the indicated times with 100 nM PDB, cell fractions were generated, and binding capacity was assayed as described in "Materials and Methods." Points, average of the two experiments; values are given as percentages of the time zero homogenate value (100% = 3354 ± 514 cpm/μg of DNA). ■, homogenate; ▲, cytosol; ○, total membrane; ●, nuclei.

B, plasma membrane contamination of the nuclear fraction as a function of time of PDB treatment. The nuclei in Fig. 3A were assayed for 5'-nucleotidase activity. Percentage of activity relative to the homogenate value of each time point is shown. Points, means of triplicate determinations from two experiments (0 and 0.5 h) or one experiment (3 and 6 h); Bars, SE.

Table 1 Effect of DNase treatment on the detection of [³H]PDB binding capacity in subcellular fractions

Samples of each fraction from treated (100 nM PDB for 30 min) and control (0.01% ethanol for 30 min) cells were incubated on ice with 950 units/ml DNase I for 60 min. Aliquots of these samples were then used directly in the binding assay as described in "Materials and Methods." Values are given as the mean ± SE of triplicate determinations in one experiment. Similar results were observed in two other experiments.

Condition	Specific [³ H]PDB binding capacity (cpm/μg DNA ± SE)			Total membrane
	Homogenate	Nuclei	Cytosol	
Control				
-DNase	3020 ± 230	50 ± 5	2670 ± 210	440 ± 60
+DNase	1100 ± 100	50 ± 20	1130 ± 50	100 ± 70
PDB-treated				
-DNase	2540 ± 160	90 ± 50	1380 ± 60	1170 ± 80
+DNase	830 ± 100	80 ± 10	660 ± 40	480 ± 50

contamination (5'-nucleotidase activity) present in the nuclear fraction isolated at each time point. At all times of treatment, the amount of binding in the nuclei was less than the amount of contamination by plasma membrane. Although PEs cause growth inhibition of EL4 cells, there was no increase in cell death over the 6-h time period (data not shown), thus a decrease in cell viability could not have masked an increase in binding activity in the nuclei with time.

Perrella *et al.* (20) reported that treatment of nuclei with DNase I (950 units/ml) increased the amount of [³H]PMA binding capacity released from nuclei. Since it was possible that

Table 2 PK-C activity in subcellular fractions of PDB-treated and untreated EL4 cells

Cells were treated with 100 nM PDB for 30 min and the nuclei and cytosol were then generated as stated in "Materials and Methods." Cytosol values are normalized to amount of DNA in the homogenate from which they were derived (±SE). Results are from a representative experiment.

	PK-C activity (cpm/μg of DNA) ^a		Plasma membrane contamination ^b	
	Cytosol	Nuclei	Cytosol	Nuclei
Control	6760 ± 1660	11 ± 370	4 ± 0.6%	25 ± 3%
Treated	3830 ± 1170	0	3 ± 0.3%	8 ± 0.6%

^a PK-C activity represents the difference in activity with lipids versus without lipids. Phospholipid-independent activity (5500 ± 1120 cpm/μg DNA in cytosol) did not differ between control and PDB-treated samples.

^b Given as percentage of 5'-nucleotidase activity in homogenate. Control, 100% = 10,940 cpm/μg DNA; treated, 100% = 14,430 cpm/μg DNA.

our failure to detect nuclear PE binding capacity in EL4 cells was due to binding of PK-C to DNA, we treated the isolated nuclei with 950 units/ml of DNase I. As shown in Table 1, this treatment did not allow detection of any masked PE binding sites and actually decreased the overall binding capacity.

It is possible that the PE-R assay is not as sensitive as the PK-C enzymatic assay and that the enzyme assay might reveal activity in the nucleus. To test this possibility, PK-C activity was determined in nuclei of EL4 cells. Table 2 shows that no PK-C activity could be found in the nuclei before or after treatment of whole cells with PDB. Cytosolic PK-C activities are given for comparison.

DISCUSSION

Available evidence indicates that the PE-induced production of IL2 in EL4 cells requires initiation of transcription (3-10). A mechanism must therefore exist for transmitting the PE signal to the nucleus. One hypothesis for how this might happen is that PE-R/PK-C may exist in nuclei or translocate to nuclei upon treatment of cells with PE. Our data provide several pieces of evidence against this hypothesis.

(a) Using a homogenization and nuclei isolation procedure that minimized the amount of plasma membrane contamination but maintained the integrity of the double nuclear membrane (Fig. 1), we could detect no [³H]PDB binding capacity in the nuclei of control cells (Fig. 2A). The fact that these nuclear preparations contained most of the mitochondrial cytochrome c oxidase activity of the cells also argues against the presence of PE-R in mitochondria.

(b) Treatment of cells with 100 nM PDB for 30 min did not cause the appearance of [³H]PDB binding capacity in the nuclei, despite the clear translocation of binding capacity from cytosol to plasma membrane fractions (Fig. 2B). The conclusion that no PE-R translocated to nuclei is further supported by the fact that all of the homogenate binding capacity could be accounted for by the sum of the binding capacity in the cytosolic and plasma membrane fractions (Fig. 2B).

(c) Incubation of the cells in the presence of PDB for up to 6 h failed to reveal any [³H]PDB binding capacity in the nuclear fraction that could not be accounted for by the amount of plasma membrane contamination (Fig. 3).

(d) Treatment of the isolated nuclei with DNase (950 units/ml) also did not cause any increase in [³H]PDB binding in the nuclear fraction that could not be accounted for by the amount of plasma membrane contamination (Table 1).

(e) In control and treated (100 nM PDB for 30 min) cells, no PK-C enzymatic activity could be detected in the nuclear fraction (Table 2). Use of the detergent NP-40 to solubilize the

nuclei prior to the assay should not have affected the kinase activity since its final concentration in the assay was 0.004%, an amount shown, by its addition to the cytosolic fraction, not to interfere with the assay.

Although Perrella *et al.* (20) found [³H]PMA binding in the nuclei of mouse epidermal cells, the amount of binding (9% relative to the homogenate value) was very close to the amount of plasma membrane contamination (7%). These data are in accordance with our results. Nishizuka (29) has also mentioned that PK-C is absent or poorly represented in the nuclei of various cells as determined by immunocytochemistry with monoclonal antibody. The recent finding in our laboratory (10) that protein synthesis is required for production of IL2 mRNA also argues against an action of PE-R/PK-C immediately at IL2 transcription. Taken together, these results provide strong support for the conclusion that the PE signaling process does not involve a direct translocation of intact PE-R/PK-C to the nucleus.

Several investigations indicate that the proteolytically derived PE binding fragment is very stable as compared with the similarly generated catalytic fragment (30–32). Our results also rule out involvement of this PE binding fragment in the nucleus, since the calcium and phospholipid requirements for PE binding to the fragment are similar to those of the intact enzyme (30–32). However, our use of PE binding and phospholipid-dependent kinase activity as methods of detection of PK-C precludes us from determining whether the proteolytically derived catalytic fragment of PK-C is involved in the PE signal. Once generated, it is possible that this phospholipid-independent kinase, lacking PE binding capacity, translocates to the nucleus and has an action there. Availability of a monoclonal antibody specific for this kinase might allow detection of this fragment. However, since the catalytic fragment is so similar in sequence to other kinases (33–35), it may be difficult to obtain such a specific monoclonal antibody.

In conclusion, these results favor a mechanism involving an intermediate factor, perhaps a PK-C substrate, in transduction of the PE signal to the nucleus; however, a role involving the catalytic fragment of PK-C cannot be ruled out.

ACKNOWLEDGMENTS

We would like to thank Hannah Anderson for her help with the nitrogen cavitation methodology and the cell culture, Steve Summers for performing the adenylate cyclase assays, and Susan Purdy-Ramos for performing the electron microscopy.

REFERENCES

- Farrar, J. J., Fuller-Farrar, J., Simon, P. L., Hilfiker, M. L., Stadler, B. M., and Farrar, W. L. Thymoma production of T-cell growth factor (interleukin 2). *J. Immunol.*, **125**: 2555–2558, 1980.
- Sando, J. J., Hilfiker, M. L., Piacentini, M. J., and Laufer, T. M. Identification of phorbol ester receptors in T-cell growth factor-producing and -nonproducing EL4 thymoma cells. *Cancer Res.*, **42**: 1676–1680, 1982.
- Bleackley, R. C., Caplan, B., Havele, C., Ritzel, R. G., Mosmann, T. R., Farrar, J. J., and Paetkau, V. Translation of lymphocyte mRNA into biologically active interleukin 2 in oocytes. *J. Immunol.*, **127**: 2432–2435, 1981.
- Wang, Y., Walker, C., Stadler, B. M., and De Weck, A. L. Transcription and translation dependent induction of interleukin 2 (IL-2) and IL-2 receptors. *Immunol. Lett.*, **8**: 227–231, 1984.
- Efrat, S., Pilo, S., and Kaempfer, R. Kinetics of induction and molecular size of mRNA encoding human interleukin 2 and γ -interferon. *Nature (Lond.)*, **297**: 236–239, 1982.
- Hinuma, S., Onda, H., Naruo, K., Ichimori, Y., Koyama, M., and Tsukamoto, K. Translation of interleukin 2 mRNA from human peripheral blood leukocytes in *Xenopus* oocytes. *Biochem. Biophys. Res. Commun.*, **109**: 363–369, 1982.
- Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R., and Hamuro, J. Structure and expression of a cloned cDNA for human interleukin 2. *Nature (Lond.)*, **302**: 305–310, 1983.
- Kashima, N., Nishi-Takaoka, C., Fujita, T., Taki, S., Yamada, G., Hamuro, J., and Taniguchi, T. Unique structure of murine interleukin 2 as deduced from cloned cDNAs. *Nature (Lond.)*, **313**: 402–404, 1985.
- Kronke, M., Leonard, W. J., Depper, J. M., Arya, S. K., Wong-Staal, F., Gallo, R. C., Waldmann, T. A., and Greene, W. C. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA*, **81**: 5214–5218, 1984.
- Harrison, J. R., Lynch, K. R., and Sando, J. J. Phorbol esters induce interleukin 2 mRNA in sensitive but not in resistant EL4 cells. *J. Biol. Chem.*, **262**: 234–238, 1987.
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.*, **257**: 7847–7851, 1982.
- Sharkey, N. A., Leach, K. L., and Blumberg, P. M. Competitive inhibition by diacylglycerol of specific phorbol ester binding. *Proc. Natl. Acad. Sci. USA*, **81**: 607–610, 1984.
- Parker, P. J., Stabel, S., and Waterfield, M. D. Purification to homogeneity of protein kinase C from bovine brain—identity with the phorbol ester receptor. *EMBO J.*, **3**: 953–959, 1984.
- Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., and Nishizuka, Y. Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. Biol. Chem.*, **258**: 11442–11445, 1983.
- Sando, J. J., and Young, M. C. Identification of high-affinity phorbol ester receptor in cytosol of EL4 thymoma cells: requirement for calcium, magnesium, and phospholipids. *Proc. Natl. Acad. Sci. USA*, **80**: 2642–2646, 1983.
- Kraft, A. S., Anderson, W. B., Cooper, H. L., and Sando, J. J. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J. Biol. Chem.*, **257**: 13193–13196, 1982.
- Sando, J. J., Ben-Ari, E., Young, M. C., Mitchell, M. E., Summers, S. T., and Nichols, A. P. *In vitro* and *in vivo* evidence for the similarity between phorbol ester receptors and protein kinase C. *Fed. Proc.*, **42**: 2880, 2250, 1983.
- Kramer, C. M., and Sando, J. J. Substrates for protein kinase C in cytosol of EL4 mouse thymoma cells. *Cancer Res.*, **46**: 3040–3045, 1986.
- Driedger, P. E., and Blumberg, P. M. Specific binding of phorbol ester tumor promoters. *Proc. Natl. Acad. Sci. USA*, **77**: 567–571, 1980.
- Perrella, F. W., Ashendel, C. L., and Boutwell, R. K. Specific high-affinity binding of the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate to isolated nuclei and nuclear macromolecules in mouse epidermis. *Cancer Res.*, **42**: 3496–3501, 1982.
- Perrella, F. W., Bussell, P. A., and Boutwell, R. K. Diversity of nuclear phorbol ester tumor promoter receptors in mouse liver: evidence for two classes of binding sites. *Biochem. Biophys. Res. Commun.*, **108**: 1722–1727, 1982.
- Spelsberg, T. C., Knowler, J. T., and Moses, H. L. Specific methods for the isolation of nuclei from chick oviduct. *Methods Enzymol.*, **31**: 263–279, 1974.
- Gentry, M. K., and Olsson, R. A. A simple, specific, radioisotopic assay for 5' nucleotidase. *Anal. Biochem.*, **64**: 624–627, 1975.
- Gear, A. R. L. Some features of mitochondria and fluffy layer in regenerating rat liver. *Biochem. J.*, **95**: 118–137, 1965.
- Weikel, C. S., Sando, J. J., and Guerrant, R. L. Stimulation of porcine jejunal ion secretion *in vivo* by protein kinase C activators. *J. Clin. Invest.*, **76**: 2430–2435, 1985.
- Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, **72**: 248–254, 1976.
- Summers, S., and Cronin, M. Phorbol esters enhance basal and stimulated adenylate cyclase activity in a pituitary cell line. *Biochem. Biophys. Res. Commun.*, **135**: 276–281, 1986.
- Labarca, C., and Paigen, K. A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.*, **102**: 344–352, 1980.
- Nishizuka, Y. Studies and perspectives of protein kinase C. *Science (Wash. DC)*, **233**: 305–312, 1986.
- Hoshijima, M., Kikuchi, A., Tanimoto, T., Kaibuchi, K., and Takai, Y. Formation of a phorbol ester-binding fragment from protein kinase C by proteolytic digestion. *Cancer Res.*, **46**: 3000–3004, 1986.
- Huang, K. P., and Huang, F. L. Immunochemical characterization of rat brain protein kinase C. *J. Biol. Chem.*, **261**: 14781–14787, 1986.
- Lee, M., and Bell, R. M. The lipid binding, regulatory domain of protein kinase C. *J. Biol. Chem.*, **261**: 14867–14870, 1986.
- Knopf, J. L., Lee, M., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M., and Bell, R. M. Cloning and expression of multiple protein kinase C cDNAs. *Cell*, **46**: 491–502, 1986.
- Ono, Y., Kurokawa, T., Kawahara, K., Nishimura, O., Marumoto, R., Igarashi, K., Sugino, Y., Kikkawa, U., Ogita, K., and Nishizuka, Y. Cloning of rat brain protein kinase C complementary DNA. *FEBS Lett.*, **203**: 111–115, 1986.
- Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., and Ullrich, A. The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science (Wash DC)*, **233**: 853–859, 1986.
- Purdy-Ramos, S. I., and Forbes, M. S. The preparation of cultured cells of vascular origin for transmission electron microscopy. *J. Electron Microsc.*, **1**: 271–277, 1984.