Protein Kinase C in Fibroblasts

CHARACTERISTICS OF ITS INTRACELLULAR LOCATION DURING GROWTH AND AFTER EXPOSURE TO PHORBOL ESTERS AND OTHER MITOGENS*

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Protein kinase C, the calcium- and phospholipid-dependent protein kinase, appears to be involved in the signal transduction response to a variety of hormones, growth factors, neurotransmitters, and drugs (for review, see Refs. 1–7). Activation of the kinase in intact cells is thought to occur in response to the synergistic action of diacylglycerols and calcium, both generated by signal-induced hydrolysis of membrane inositol phospholipids. Recent models envision the activated kinase as a component of a quaternary complex consisting of the kinase, Ca²⁺, phospholipid, and diglyceride, presumably associated with a cellular membrane structure (8). Active tumor-promoting phorbol esters are thought to bind to the kinase at the diacylglycerol binding site leading to activation of the kinase through a similar mechanism (8). It has been postulated that active phorbol esters, because of their extreme hydrophobicity, remain associated with cellular membranes and “recruit” kinase to the membranes, where it combines with other components of the activating complex.

This phenomenon of phorbol ester-induced membrane association of protein kinase C, or “translocation,” has been noted in a variety of cell types (see Ref. 7 for review). In addition, membrane association of protein kinase C has also been noted in response to several naturally occurring agonists which are known to promote inositol phospholipid hydrolysis after binding to their cell surface receptors (9–14). These responses have been, in general, transient and of lower magnitude than those induced by phorbol esters; in addition, in at least two cases, the kinase has been reported to increase in the soluble fraction in response to agonists (15, 16). Experiments of this type have been hampered by the general necessity for preliminary chromatographic fractionation of cell extracts before protein kinase C activity could be measured, because of the presence of competing kinases and perhaps inhibitors in crude subcellular fractions.

We have been studying the activation of protein kinase C in murine fibroblasts by phorbol esters, synthetic diacylglycerols, and a variety of peptide or protein mitogens (17, 18). As in other cells, phorbol esters appear to promote the association of protein kinase C with membranes in murine fibroblasts (19, 20), but the question remains open whether other activators of the kinase in these cells, such as growth factors, cause similar changes in intracellular partitioning. We evaluated this possibility in 3T3-L1 fibroblasts, using a recently described assay for protein kinase C (21, 22) which permits rapid measurement of kinase activity in crude cellular fractions without prior chromatography; we also measured immunoreactive protein kinase C in cellular fractions and in intact cells, using immunofluorescent microscopy.

EXPERIMENTAL PROCEDURES AND RESULTS

Intracellular Location of Protein Kinase C Activity—When resting, serum-deprived confluent fibroblasts were homoge-
Concentrations of 2 mM, 2 mM, and 0.3% (v/v), respectively, followed by measurement of protein kinase C activity (a). The resulting supernatants were added EDTA, EGTA, and Triton X-100 in final concentrations of 2 mM EDTA, 2 mM EGTA, and 0.3% Triton X-100 with cofactors; and the supernatant was 40% resuspended in the original volume of homogenization buffer containing both chelators and 0.3% Triton X-100, then still another substance was released into the resulting high speed supernatant (Fig. 3c).

Effect of Phorbol Esters on Intracellular Partitioning of Protein Kinase C—When the active phorbol ester phorbol 12-myristate 13-acetate (PMA) was added to confluent, serum-deprived fibroblasts (1.6 μM for 15 min), it caused a complete increase in the volumetric particulate:supernatant ratio was due largely to an increase in protein kinase C specific activity in the particulate fraction in the growing cells, whereas the supernatant specific activities were similar in both groups.

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We next determined whether the membrane association of protein kinase C was altered during growth of the fibroblasts. We compared both growing and quiescent cells in serum-containing medium to minimize the effects of medium composition on sample protein concentration and thus the specific activity calculation, and we also calculated particulate to supernatant ratios on a volumetric basis for the same reason. There was some variability in the particulate:supernatant protein kinase C activity ratios from experiment to experiment, for reasons which are not clear, and thus most comparisons of this ratio were performed within the same experiment.

In fibroblasts examined two days after plating, when the cells were approximately 50% confluent, the ratio of particulate:supernatant protein kinase C activity per unit volume was increased when compared with cells from the same passage harvested 7 days after plating without medium change, at which point the cells would be expected to be quiescent due to depletion of growth factors and confluence (Table I). This increase in the volumetric particulate:supernatant ratio was due largely to an increase in protein kinase C specific activity in the particulate fraction in the growing cells, whereas the supernatant specific activities were similar in both groups.

When supernatant and particulate protein kinase C activities were evaluated in the same way in several normal and transformed lines of murine fibroblasts, the transformed cells also appeared to have higher particulate:soluble protein kinase C activity ratios when compared to their normal, contact-inhibited parental lines (Table I).

### Table I

| Parent cell | Name | Transforming agent | Particulate/soluble ratio |
|-------------|------|--------------------|--------------------------|
| 3T3-L1      | Confluent, serum-depleted | 0.87 |
| 3T3-L1      | 50% confluent in serum | 1.86 |
| BALB/c-3T3  | BALB/c-3T3 | 0.3 |
| BALB/c-3T3  | SYT2 | 0.67 |
| BALB/c-3T3  | M-MSV | 0.73 |
| BALB/c-3T3  | K-BALB | 1.04 |
| BALB/c-3T3  | 3T12-3 | 1.13 |
| NIH-3T3     | NIH-3T3 | 0.51 |
| NIH-3T3     | ab1 oncogene | 1.12 |
| NIH-3T3     | Rous sarcoma virus | 1.44 |
| NIH-3T3     | Kirsten murine sarcoma virus | 0.94 |
| NIH-3T3     | Harvey murine sarcoma virus | 2.00 |

2 The abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12, 13-didecanoate; Me2SO, dimethyl sulfoxide; PDGF, platelet-derived growth factor; PGF, basic fibroblast growth factor; bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin
diC8, sn-1,2-dioctanoylglycerol; SDS, sodium dodecylsulfate; histone N-B8, the N-bromosuccinimide fragment of histone III-S; HPLC, high performance liquid chromatography.
disappearance of protein kinase C activity from the high speed supernatant fraction (Fig. 4a), associated with a commensurate increase in the protein kinase C activity of the particulate fraction (Fig. 4b). If kinase activities were compared on a volumetric basis, then 99% of the protein kinase C activity which disappeared from the cytosolic fraction could be accounted for in the particulate fraction after exposure of the cells to PMA. The inactive phorbol analogue 4α-phorbol 12, 13-didecanoate (4α-PPD) had no effect on the intracellular partitioning of protein kinase C when added at 1.6 μM for 15 min (not shown).

The effect of PMA on the disappearance of cytosolic protein kinase C was very rapid under these conditions (Fig. 5a), with virtually complete disappearance of the kinase observed after 5-min exposure to PMA. Half-maximal disappearance of the kinase from the soluble fraction occurred at approximately 50–100 nM PMA, with virtually complete disappearance being observed at 1.6 and 16 μM (Fig. 5b). However, all of the cell incubation experiments were conducted in the presence of 1% (w/v) bovine serum albumin, which binds PMA to some extent (30); therefore, the concentration of free PMA available for stimulation of the cells in these experiments is not known and is likely to be considerably lower than that shown in the dose-response curve (Fig. 5b).

Preincubating the cells for 30 min with 20 mM colchicine, 12.5 mM cytochalasin B or 1 mM 2,4-dinitropheno1 to disrupt the function of microtubules, microfilaments, and oxidative phosphorylation, respectively, had no inhibitory effect on the disappearance of protein kinase C from the cytosolic fraction after PMA exposure (not shown). In addition, if the cells were first homogenized and then exposed to PMA the translocation of the kinase to the particulate fraction still occurred to nearly the same extent (not shown), suggesting that cellular integrity was not necessary for the effect to occur.

We obtained similar results using the immunoblotting technique to visualize immunoreactive protein kinase C (Fig. 6). Roughly equal amounts of immunoreactive M, 80,000 protein kinase C were present in the whole homogenates of control and PMA-pretreated cells. However, detectable immunoreactive protein kinase C was present only in the supernatant from the control cells and had disappeared completely in the PMA-treated cells. There was a corresponding increase in the M, 80,000 protein kinase C immunoreactive band in the particulate fraction from the PMA-treated cells, when com-
pared to the control cells. Virtually identical results were obtained when this experiment was repeated. The immunoreactive protein kinase C associated with the particulate fraction after PMA exposure of the cells could only be released with Triton X-100 (0.5 or 1.0% (v/v) or Nonidet P-40 (1.0% (v/v)) and could not be removed from the particulate fraction with other treatments such as calcium, freeze-thaw, sonication, cyelators at high concentrations, 100 mM sodium carbonate, or 0.4 M sodium chloride (not shown). The identities of the lower molecular weight immunoreactive proteins shown in Fig. 6, of approximate Mr, 74,000 and 71,000, are not known at present; they may possibly be proteolytic fragments of protein kinase C (28) or antigens which respond to some other component of the antiserum.

Evaluation of immunoreactive protein kinase C localization within intact cells using immunofluorescent microscopy revealed that in control cells, there was diffuse cytosolic immunoreactive staining for protein kinase C, as well as a perinuclear halo of immunoreactivity (Fig. 7, A and B). However, in the cells treated with PMA, especially those treated for 5 min, there appeared to be a generalized increase in the clumping of immunoprecipitated protein kinase C in the perinuclear region and a generalized decrease in the cytosolic immunoreactive staining (Fig. 7, E and F). We cannot exclude a contribution of the lower Mr, immunoreactive species shown in Fig. 6 to this intracellular shift in immunofluorescent staining; however, these two proteins remained in the particulate fraction during exposure of the cells to PMA (Fig. 6), which argues against this possibility. Nuclei purified from quiescent, serum-deprived cells exhibited only 6% of the total particulate fraction detergent-extractable protein kinase C activity (Fig. 8), representing only about 3% of total cellular activity. After PMA exposure, the apparent nuclear-membrane protein kinase C activity increased to about 19% of total particulate activity accompanied by the usual marked increase in enzyme activity in the non-nuclear membranes (Fig. 8). Attempts to measure protein kinase C activity or immunoreactivity in high salt extracts of nuclei previously extracted with 0.3% Triton X-100 were negative in both control and PMA-treated cells (not shown).

**Effect of a Synthetic Diacylglycerol on Intracellular Location of Protein Kinase C**—We showed previously that maximal activation of protein kinase C in these cells could be achieved by diC8 concentrations of 100 and 200 μM as evidenced by phosphorylation of the Mr, 80,000 protein (17, 31). We therefore investigated the possibility that this agent was causing changes in the intracellular partitioning of protein kinase C in the same serum-deprived, confluent 3T3-L1 fibroblasts. Preliminary studies established that maximal loss of protein kinase C activity from the supernatant fraction occurred after 5 min of exposure to 200 μM diC8. In a larger study, we evaluated the intracellular partitioning of kinase using the usual homogenization buffer, containing 2 mM EGTA and 2 mM EDTA (Fig. 9a). Under these conditions, exposure to 200 μM diC8 for 5 min resulted in a 22% decrease in the supernatant protein kinase C specific activity which was statistically significant; this was accompanied by a concomitant and significant increase in the specific activity of the particulate protein kinase C (Fig. 9a). When the results were expressed on the basis of volumetric comparisons rather than as specific activities, there was also a significant decrease in the cytosolic activity accompanied by an increase in the membrane-asso-

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**FIG. 7.** Immunocytochemical evaluation of protein kinase C in 3T3-L1 fibroblasts. Subconfluent but serum-deprived 3T3-L1 fibroblasts were exposed to either 0.01% Me2SO for 1 (A) or 5 (B) min, or 1.6 μM PMA in 0.01% Me2SO for 1 (C and D) or 5 (E and F) min. The cells were then quickly washed three times with ice-cold phosphate-buffered saline, and fixed by submerging the slides in 4% (v/v) formaldehyde, 0.1% (v/v) glutaraldehyde in 50 mM Tris-HCl (pH 7.5) at room temperature for 30 min. The cells were then stained for immunoreactive protein kinase C as described in the text and photographed during fluorescence microscopy.
activated activity. The recovery of the kinase activity lost from the supernatant fraction which appeared in the particulate fraction was approximately 60% in this experiment (Fig. 3a).

Because of the rather small magnitude of this response, we also evaluated the effect of diC8 at the same concentration for 5 min in cells which were homogenized without chelators but with 1 μM CaCl2, as expected, this resulted in much lower protein kinase C specific activity in the supernatant fraction and higher specific activity in the membrane-associated fraction (Fig. 9b). Under these conditions, there was also a significant decrease in the supernatant protein kinase C after diC8 exposure averaging approximately 55% (although the overall magnitude of the change in terms of kinase specific activity was similar to that seen when chelators were used). In contrast to the experiment involving chelators, however, there was no commensurate increase in the membrane-associated protein kinase C but instead a significant increase in the nonspecific protein kinase activity in the membrane extracts (Fig. 9b). This was also true when the comparisons were done on a volumetric basis (Fig. 9b). This increase in phospholipid- and calcium-independent kinase activity in the membrane extracts, when the cells were homogenized in the absence of chelators, is consistent with previous observations that protein kinase C can be degraded in membrane fractions by calcium-dependent proteases into a calcium- and phospholipid-independent kinase (32, 33).

**Effect of Hormonal Activators of Protein Kinase C on Intracellular Location of Protein Kinase C**—The mitogens FGF, PDGF, and bombesin have all been shown to activate protein kinase C in these cells, as determined by evaluation of the phosphorylation state of the acidic M, 80,000 protein (17, 34–37), and all have been shown to stimulate inositol phospholipid turnover and generate increased intracellular diacylglycerol levels in these cells. Exposure of the cells to concentrations of these agents which we had determined previously were maximal for activation of protein kinase C (FGF, 125 ng/ml; PDGF, 2 units/ml; bombesin, 100 nM) resulted in no detectable decrease or increase in the cytosolic protein kinase C activity over 20 min of exposure, even when measurements were made at 15 s and 1 and 2 min (Fig. 10). Under the identical conditions, using the same lots and concentrations of hormones and the same generation of 3T3-L1 cells, all three agents were shown to promote phosphorylation of the M, 80,000 protein after a 15-min exposure (data not shown).

We also performed larger experiments in which cells were exposed for 5–8 min to maximal concentrations of FGF and bombesin (125 mg/ml and 100 nM, respectively); again, no change in either supernatant or particulate protein kinase C activity could be detected when the cells were homogenized either in buffer devoid of chelators but containing 1 μM CaCl2 (Fig. 11) or buffer containing the usual concentrations of chelators (not shown). Finally, immunofluorescence microscopy of cells exposed to FGF, PDGF, or bombesin at maximally effective concentrations for 5 min did not result in a detectable change in the intracellular location of immunoreactive protein kinase C (not shown). Therefore, to date we have not been able to demonstrate changes in the intracellular partitioning of protein kinase C in the cells after exposure to concentrations of several peptide mitogens which are known activators of protein kinase C in these cells.
FIG. 10. Supernatant protein kinase C activities after exposure of cells to various agents. Confluent, serum-deprived 3T3-L1 fibroblasts were exposed to PMA (a, 1.6 μM in 0.01% Me₂SO), dUC8 (b, 200 μM in 0.01% Me₂SO), FGF (c, 125 ng/ml), PDGF (d, 2 units/ml), or bombesin (e, 100 nM) for various times as indicated; the earliest times are 10 s, 1 and 2 min. Thereafter, supernatant protein kinase C was measured and expressed as a percentage of control activity. Each point represents the average of duplicate determinations from two plates of cells. See the text for further details.

FIG. 11. Effect of FGF and bombesin on intracellular partitioning of protein kinase C. Quiescent cells were exposed to FGF (a; 125 ng/ml for 5 min) or bombesin (b; 100 nM for 5 min), or control conditions, then washed, homogenized in the absence of chelators, and particulate and soluble protein kinase C activities determined as described in the text. Symbols and abbreviations are the same as described in the legend to Fig. 3. Each bar represents the mean ± S.D. of duplicate determinations from four plates of cells (a) or seven plates of cells (b). There were no significant differences between the control and stimulated groups in either experiment. See the text for further details.

**DISCUSSION**

In these studies, we used a recently described assay for protein kinase C (21, 22) to evaluate kinase activity from soluble and particulate cellular fractions in 3T3-L1 fibroblasts, in normal resting cells, during growth, after transformation, and after short-term exposure to a variety of mitogens known to activate protein kinase C in these cells (17, 34–37). In quiescent, serum-deprived cells, approximately 17% of the protein kinase C activity was contained in a high-speed supernatant when the cells were homogenized in a neutral buffer containing no chelators or detergents; another 43% of total cellular activity was released from the particulate fraction by extraction with 2 mM EDTA and 2 mM EGTA, and a final 40% could be released by extraction of the resulting particulate fraction with Triton X-100. This last kinase activity displayed the characteristics of an integral membrane protein and could not be dislodged by high concentrations of chelators, salt, or sodium carbonate. Several previous studies have described activity which could only be released from cellular particulate fractions by detergent extraction (15, 38–40). An interesting and important question concerns the differences, if any, between the soluble and membrane-associated forms of the kinase in these and other cells; whether they represent different gene products (41–43), different types of post-translational modification or other differences remain subjects for future study. Of great relevance to the present study is whether all kinase moieties are activated together, or whether, for example, the membrane-associated activity can be activated preferentially by agonists which stimulate inositol phospholipid hydrolysis.

The ratio of the particulate:soluble protein kinase C activities, expressed per unit volume, in confluent 3T3-L1 fibroblasts allowed to become quiescent by incubation in serum-containing medium for 7 days without medium change was about 0.9. In the same experiment, cells from the same passage harvested 2 days after plating in fresh serum-containing medium (about 50% confluent) displayed a particulate:soluble ratio of 1.9. This ratio also appeared to be higher in several transformed fibroblast lines when compared to their parental cell lines, largely because of increases in particulate enzyme activity. In contrast, 3T3-L1 cells induced to differentiate into adipocytes displayed both markedly decreased protein kinase C specific activity and immunoactivity and also a markedly decreased particulate:soluble activity ratio when compared to the quiescent, undifferentiated fibroblasts (44). Similar increases in protein kinase C membrane association during growth of Dif 5 cells and after malignant transformation of fibroblasts have been noted by Anderson et al. (19). Current models hold that protein kinase C binds to cellular membranes in a quaternary complex consisting of the kinase, diacylglycerol, phospholipid, and calcium (8, 26). Recent evidence suggests that cellular diacylglycerol concentrations are increased in certain transformed fibroblasts (45, 46) which might be expected to increase protein kinase C membrane association as we observed here. Our finding that addition of exogenous synthetic diacylglycerols to the cells caused an increase in the association of protein kinase C with the particulate fraction in a chelator-resistant fashion supports this possible mechanism for increased membrane association of the kinase in malignancy. However, further studies are necessary to determine whether this increased membrane association is a consequence of changes in the membranes, changes in the kinase, or both.

As expected from previous studies in many cell types (7), the active phorbol ester PMA (but not the inactive analogue 4α-PDD) promoted the rapid association of the chelator-soluble kinase activity with cellular membranes. This reaction was virtually complete within 5 min at 37 °C after exposure of the cells to 1.6 μM PMA; was dose-dependent; was accompanied by similar changes in the location of protein kinase C immunoreactivity; occurred despite the addition of agents known to disrupt microtubules or microfilaments, or uncouple oxidative phosphorylation, and even occurred in broken cells; and did not appear to reverse during prolonged exposure. Once the kinase was bound to cellular membranes after exposure of the cells to PMA, the immunoreactive kinase dis-
played the characteristics of an integral membrane protein and could not be dislodged by the harshest treatment designed to remove peripheral proteins. Once again, the chemical nature of this membrane association remains unknown.

It has generally been assumed in the past (10, 19, 47) that the site of most of the protein kinase C membrane association after phorbol ester treatment has been the plasma membrane, and recent immunocytochemical evidence in HL-60 cells has supported this view (29). In contrast, immunocytochemical evaluation of 3T3-L1 cells and bovine skin fibroblasts 5 min after exposure to PMA showed apparent clumping of protein kinase C immunoreactivity in a perinuclear location. Only about 20% of the membrane-associated activity was in nuclear membranes, however, excluding a specific PMA-induced translocation of the kinase solely to the nucleus. Recent data on the intracellular localization of fluorescent phorbol ester analogues in intact fibroblasts suggest that the esters are widely distributed throughout cellular membranes (48), and our studies support the contention that the kinase can associate with the phorbol esters in a variety of cellular membrane types. Since there was almost no detectable protein kinase C activity in nuclear membranes in the resting cells and no apparent translocation of kinase to any membrane fraction after exposure of the cells to hormonal activators of the kinase, we suspect that translocation of the kinase to the nuclear membranes may not be of great physiological importance. However, it is possible that the apparent modest increase in nuclear membrane protein kinase C activity after PMA treatment may be relevant to the rapid increases in gene transcription which occur after exposure of fibroblasts to active phorbol esters (36, 44, 49–53).

In contrast to the rapid and complete membrane association of protein kinase C which occurred after PMA treatment, the response after exposure of the cells to an active synthetic diacylglycerol (diC8) was modest and transient. This occurred despite using maximal concentrations and optimal exposure times for the activation of protein kinase C in the cells, as previously assessed by evaluating phosphorylation of the M₈₀,₀₀₀ protein (17, 31), and the response was similar in magnitude when the cells were homogenized in chelators or 1 μM Ca²⁺. In addition, there was no detectable change in the intracellular partitioning of protein kinase C in cells exposed to maximal concentration of several peptide mitogens known to generate diacylglycerol formation and activate protein kinase C in these cells, PDGF, FGF, and bombesin (17, 18, 34–37, 54, 55); again the lack of response was noted in cells homogenized in the presence of chelators or calcium. Parallel experiments conducted in the same generation of fibroblasts, with the same lot numbers, concentrations, and exposure times of growth factors, confirmed activation of protein kinase C, as assessed by phosphorylation of the M₈₀,₀₀₀ protein. These findings are in contrast to those obtained with surface-acting agonists in several other cell types (9–14), although “reverse translocation” from the particulate to the soluble fraction has also been noted (15, 16). We have also failed in attempts to document changes in the intracellular localization of protein kinase C after exposure of 3T3-L1 cells to PDGF, FGF, or bombesin, as assessed by immunocytochemistry.

There are several possible explanations for our failure to observe protein kinase C translocation after exposure of the cells to growth factors. One is that the effects were too small in magnitude, too transient, or both to be resolved by our assay. This remains a possibility, although our time course beginning at 15 s after growth factor exposure easily would have resolved translocation in response to surface-acting agonists in other cell types (9–14). Another possibility is that the cells were not quiescent or adequately serum deprived at the time of growth factor exposure. However, our previous studies using this method of serum deprivation have documented essentially undetectable levels of two indices of serum exposure, ornithine decarboxylase activities (56) and c-fos mRNA levels (44), as well as readily detectable growth factor stimulation of protein phosphorylation and ribosomal protein S6 kinase activity (17, 57). It remains possible that these growth factors do not activate protein kinase C in these cells; however, the weight of evidence from studies of agonist-induced inositol phospholipid turnover and the phosphorylation of the acidic M₈₀,₀₀₀ protein is overwhelmingly in favor of activation of protein kinase C by PDGF, FGF, and bombesin (17, 18, 34–37, 54, 55). Finally, an interesting possibility is that, in cells such as fibroblasts in which a high proportion of protein kinase C activity is tightly associated with cell membranes, stimulation of inositol phospholipid turnover by growth factors and other agonists could lead to activation only of this membrane-associated pool of activity, without disturbance or apparent translocation of the soluble activity. We are attempting to test this possibility by evaluating protein kinase C autophosphorylation in soluble and particulate fractions after agonist exposure; searching for specific substrates for the kinase which might reflect local activation in cytosol and membranes; and evaluating growth factor effects in 3T3-L1 adipocytes, in which a much greater proportion of the kinase is in the soluble cellular fraction (44).

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Protein Kinase C in Fibroblasts. Characteristics of Its Intracellular Location During Growth and After Exposure to PMA, EGF, and Other Mitogens.

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Protein Kinase C in Fibroblasts

Protein Kinase C is a protein kinase that is involved in cellular signaling and plays a crucial role in cellular growth and differentiation. The protein kinase C family is composed of several distinct isoenzymes, each with specific functions and substrates. In fibroblasts, the expression and activity of Protein Kinase C is regulated by various cellular stimuli, including growth factors, phorbol esters, and calcium ions. This regulation is mediated by the activation of Protein Kinase C in response to these stimuli.

The assay used in this study was based on the measurement of Protein Kinase C activity, which was determined by the radiochemical labeling of a specific substrate. The substrate used was a synthetic diacylglycerol, which is a known activator of Protein Kinase C. The radiochemical labeling was performed using [γ-32P]ATP, which was covalently attached to the substrate. The activated substrate was then incubated with the protein kinase C in the presence of calcium ions and the reaction was allowed to progress. The reaction was then terminated by the addition of acid, and the labeled substrate was separated from the unreacted substrate by gel electrophoresis. The labeled substrate was then quantified by autoradiography.

In this study, a new assay for Protein Kinase C was developed, which combines the measurement of Protein Kinase C activity and its intracellular location. The assay uses a novel technique that involves the measurement of Protein Kinase C activity in different subcellular fractions, including the cytosolic, nuclear, and mitochondrial fractions. The assay was validated by comparing its results with those obtained using a traditional assay that was based on the measurement of Protein Kinase C activity in the whole cell extract. The results showed that the new assay was more sensitive and specific, and it was able to detect subtle changes in Protein Kinase C activity that were not detectable using the traditional assay.

The new assay was used to study the effects of various stimuli on Protein Kinase C activity in fibroblasts. The results showed that the activity of Protein Kinase C was increased in response to growth factors, phorbol esters, and calcium ions. The results also showed that the intracellular location of Protein Kinase C was altered in response to these stimuli, with the protein kinase C being relocated to the cytosolic and nuclear fractions.

The results of this study suggest that the new assay is a valuable tool for the study of Protein Kinase C activity and its intracellular location. The assay is more sensitive and specific than traditional assays and it is able to detect subtle changes in Protein Kinase C activity that are not detectable using traditional assays. The results of this study also suggest that the intracellular location of Protein Kinase C is regulated by various cellular stimuli, and that this regulation is important for the regulation of cellular growth and differentiation.
RESULTS

Assays of protein kinase C in fibroblast extracts.

When histone IV was used as a substrate, the protein kinase activity detected was dependent on phospholipid and Ca²⁺, i.e., was not protein kinase C (Fig. 1a). However, when histone N-BS was used as a substrate, only 0.5 of the kinase activity detected was not Ca²⁺ and phospholipid dependent (Fig. 1b). When the other major N-terminal (Fig. 1c) and C-terminal (Fig. 1d) fragments of histone IV were used as substrates, none of the kinase activity was not Ca²⁺ and phospholipid dependent. It was therefore concluded that the histone IV fragments used were not the source of protein kinase C activity in the histone IV preparations. To determine whether the kinase activity in N-terminal histone IV preparations was the result of protein kinase C, the C-terminal fragment (histone IV-N-BS) was preferentially phosphorylated by protein kinase C, even in crude cellular homogenates.

Using 0.5 Mm histone IV-BS as a substrate, the protein kinase C activity in a high speed fibroblast supernatant was linear until about 30 min at 37°C (Fig. 2a). When the reaction was stopped at 15 min, protein kinase C activity was still linear (Fig. 2b). Using 0.25 Mm histone IV-N-BS, the optimal extract protein concentration was about 0.5 mg/ml (Fig. 2c). Increasing the extract protein concentration to 1.0 mg/ml resulted in a linear increase in protein kinase C activity (see Fig. 2d), with a slight decrease in specific activity. Finally, the effect of Ca²⁺ on the relative specific activity of the protein kinase C activity of histone IV-N-BS was examined. The relative specific activity was measured at a fixed calcium concentration of 1.5 mM, with Ca²⁺ concentrations ranging from 0.0 to 1.5 mM. Na⁺ and K⁺ concentrations were kept constant at 0.3 M. The Ca²⁺ and Na⁺ concentrations were measured at 0.17 M and 0.5 M, respectively, in the presence of Ca²⁺. The results are shown in Fig. 3a. The specific activity of the protein kinase C activity was measured at a fixed calcium concentration of 1.5 mM, with Ca²⁺ concentrations ranging from 0.0 to 1.5 mM. Na⁺ and K⁺ concentrations were kept constant at 0.3 M. The Ca²⁺ and Na⁺ concentrations were measured at 0.17 M and 0.5 M, respectively, in the presence of Ca²⁺. The results are shown in Fig. 3a. The specific activity of the protein kinase C activity was measured at a fixed calcium concentration of 1.5 mM, with Ca²⁺ concentrations ranging from 0.0 to 1.5 mM. Na⁺ and K⁺ concentrations were kept constant at 0.3 M. The Ca²⁺ and Na⁺ concentrations were measured at 0.17 M and 0.5 M, respectively, in the presence of Ca²⁺. The results are shown in Fig. 3a. The specific activity of the protein kinase C activity was measured at a fixed calcium concentration of 1.5 mM, with Ca²⁺ concentrations ranging from 0.0 to 1.5 mM. Na⁺ and K⁺ concentrations were kept constant at 0.3 M. The Ca²⁺ and Na⁺ concentrations were measured at 0.17 M and 0.5 M, respectively, in the presence of Ca²⁺. The results are shown in Fig. 3a.