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The Role of Protein Kinase C in Laminin-Mediated Neurite Outgrowth

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Laminin is a potent stimulator of neurite outgrowth in rat pheochromocytoma (PC12) cells. Here, we investigated the role of protein kinase C (PKC) in the mechanism of laminin-mediated neurite outgrowth in PC12 cells. Phorbol ester activators of PKC have been shown to have divergent effects on laminin-mediated neurite outgrowth. Therefore, we tested the effect of the non-phorbol PKC activator, indolactam V. At 1.0 μM indolactam V inhibited laminin-mediated neurite outgrowth by 85%. Further, the PKC inhibitor H7 blocked the inhibitory effect of indolactam V on laminin-mediated neurite outgrowth. Direct measurement of protein kinase C activity in the soluble (cytosolic) and particulate (membrane) fractions of PC12 cells showed that laminin failed to alter protein kinase C activity. These data demonstrate that PKC activation inhibits laminin-mediated neurite outgrowth and that laminin does not activate PKC in PC12 cells.

Laminin is a basement membrane specific extracellular matrix protein which has been shown to promote neurite outgrowth in primary peripheral and central nervous system neurons as well as many neuronal cell lines including rat pheochromocytoma cells (PC12 cells) (1, 2). Three laminin domains capable of stimulating neurite outgrowth have been identified using synthetic peptides (1, 2). These domains have the amino acid sequences RNIAEIIKDI, IKVAV, and LQVQLSIR (1, 3, 4). A 110 kDa protein related to amyloid precursor protein (APP) is known to bind to the laminin IKVAV domain (5), and cranin, a 120 kDa neuronal cell surface receptor for laminin, is known to bind to laminin in the general vicinity of the IKVAV sequence although the precise binding site for this receptor is not yet known (6, 7). In addition to APP and cranin, as many as ten cell surface adhesion receptors within the integrin super gene family have been shown to bind to laminin. However, only $\alpha1\beta1$, $\alpha6\beta1$, and $\alpha3\beta1$ integrin have been associated with neuronal cell interaction with laminin (1, 2). While laminin domains and some of their neuronal cell surface receptors have been identified, the intracellular signaling pathways through which laminin promotes neurite outgrowth remain poorly understood.

Focal adhesion kinase (FAK) is a central component in signal transduction pathways triggered by extracellular matrix proteins and has been shown to be phosphorylated during laminin-mediated neurite outgrowth in human neuroblastoma cells (8–11) and during integrin-mediated adhesion of retinal neurons to laminin (12). Activation of FAK in neuronal cells has been reported to be positively regulated by protein kinase C activity (13), however investigation of the role of protein kinase C in laminin-mediated neurite outgrowth has yielded conflicting data.

Protein kinase C comprises a family of at least eleven protein kinase C isotypes (14) all of which are serine/threonine protein kinases. While some of the protein kinase C isotypes are associated with the plasma membrane, protein kinase C is generally found in the cell cytosol and then translocates to the plasma membrane upon activation (14). Compounds which activate and inhibit protein kinase C are available. For example, phorbol esters such as TPA and PMA are known to activate protein kinase C (14) and indolactam V is a non-phorbol activator of protein kinase C (15). Alternatively, H-7 is an inhibitor of several kinases including protein kinase C (16). In sparate studies, TPA and H-7 have been reported to both inhibit and enhance neurite outgrowth (17, 18). Further, overexpression of protein kinase C has been shown to enhance neurite outgrowth (19) while a decrease in protein kinase C expression and activity has been associated with neurite outgrowth in neuroblastoma cells (20). Therefore the role of protein kinase C in the mechanism of neurite outgrowth is yet to be clarified.

Here we investigate the role of protein kinase C in the signaling mechanism of laminin-mediated neurite outgrowth in PC12 cells. We have avoided the pleiotrophic effects of the phorbol esters and have measured the effect of indolactam V on protein kinase C activa-

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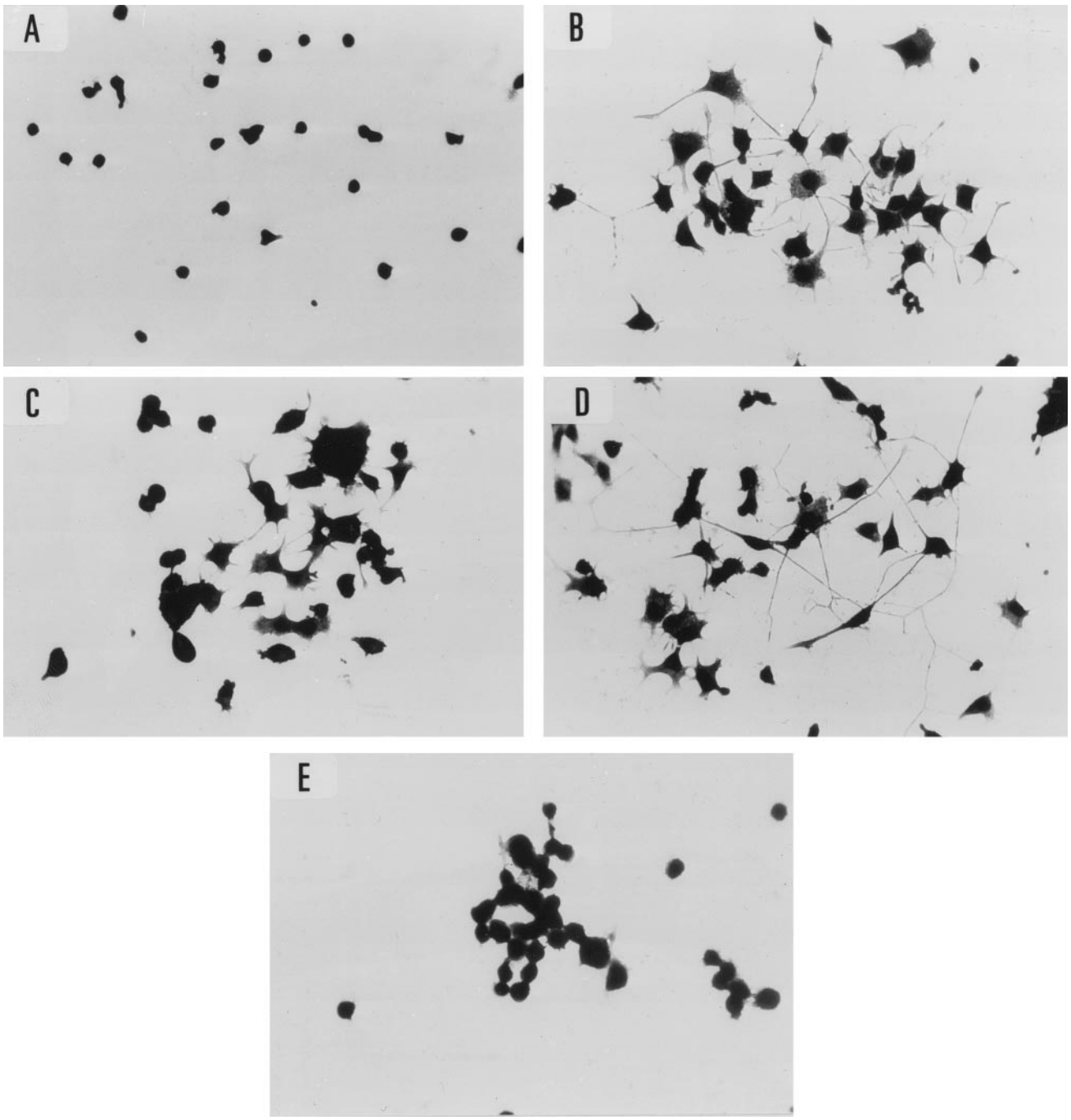

FIG. 1. Morphology of PC12 cells cultured on laminin in the presence of indolactam V, insulin and calyculin A. PC12 cells were cultured for 18 hours in a serum-free medium in the absence of laminin (A) or the presence of laminin (B–E). At the time of seeding on laminin the PC12 cells were untreated (B) or treated with 1.0 μM(−)-indolactam V (C), 10 μg/ml insulin (D), or 1.0 nM calyculin A (E). The cells were fixed and stained and photographed at a 40X magnification.

tion and laminin-mediated neurite outgrowth. We also test the effects of a serine/threonine phosphatase inhibitor, calyculin A (21), on laminin mediated neurite outgrowth. Most importantly, we have measured the effects of laminin on protein kinase C activation in PC12 cells. Our investigation directly measures protein kinase C activity in response to laminin treatment and our data clarify the role of protein kinase C in the mechanism of laminin-mediated neurite outgrowth.

MATERIALS AND METHODS

Materials. Laminin was prepared from the murine Englebreth-Holm-Swarm tumor as previously described (22). The PC12 cells were provided by Gordon Guroff (National Institute of Child Health

and Human Development). Nerve Growth Factor (NGF) was purchased from Boehringer Mannheim (Indianapolis, Indiana). The (−)-indolactam V, (+)-indolactam V, calyculin A (LC Laboratories, Woburn, MA), phorbol didecanoate (PDD) and phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) were dissolved in absolute EtOH and were stored at −80°C. Stocks of these compounds were prepared so that at all concentrations tested, the final concentration of EtOH in the test well was 1.0%. Bovine insulin was (Sigma, St. Louis, MO) dissolved in Dulbecco's modification of Eagle's medium (DMEM) and stored at −20°C.

Cell culture and neurite outgrowth assays. The PC12 cells were grown in Dulbleco's modification of Eagle's medium (DMEM) containing 7.5% fetal bovine serum, 7.5% equine serum, 0.1 units/ml of penicillin G sodium and 0.1 μg/ml streptomycin in a 37°C CO_2 incubator. All neurite outgrowth assays were performed in triplicate in wells of a 24 wells tissue cluster (Costar, Cambridge, MA). Prior to harvest, the PC12 cells were treated for 24 hours with 100 ng/ml nerve growth factor (NGF). The NGF primed PC12 cells were then collected and washed three times with serum-free DMEM. Cells were then diluted to 2×10^4 cells/ml in a defined medium composed of a 50%/50% mixture of DMEM and HAMS/F12 containing 100ng/ml NGF, 100μg/ml transferrin, 100μM putrescene, 20nM progesterone, and 30nM $NaSeO_3$. To each well, 1×10^4 cells were plated and 10 μg of laminin and any other compound to be tested was added immediately after cell seeding. The cells were then incubated for 18 hours at 37°C in a CO_2 incubator and then fixed and stained using DIF QIK (Baxter Scientific Products, McGraw Park, IL). Cells were considered to be positive for neurite outgrowth if they had neurites which were two times the cell diameter in length or longer. A total of 150 cells were assessed by examining 50 cells in each of triplicate wells and the number of cells positive for neurites was determined. The number of the cells extending neurites in wells which did not receive treatment with any of the kinase or phosphatase modulator were considered to be the maximal response to laminin and was given a value of 100%. The control values used to establish 100% neurite outgrowth were determined with 1% EtOH added to control for solvent effects. Neurite outgrowth is presented as percent of control which was determined using the following formula:

$$\frac{\%\ \text{N.O. in treatment group} \times 100}{\%\ \text{N.O. in control (solvent) group}} = \text{N.O. as \% of Control}$$

where N.O. is neurite outgrowth and the % N.O. was determined by the following formula:

$$\frac{\text{Number of cells with neurites} \times 100}{\text{Total number of cells viewed}} = \%\ \text{N.O.}$$

Cell viability. The viability of the cells with drug treatment was determined by trypan blue exclusion.To determine cell viability cells were treated in the same fashion described for the neurite outgrowth assays above. After the 18 hour period of exposure to laminin and the test compounds the cells were harvested using 250 μl trypsin-EDTA for 10 minutes and transferred to 1.5 ml microfuge tubes. The trypsin-EDTA was immediately neutralized by adding 250 μl of culture medium containing 10% serum. A volume of 10μl of the cells was then mixed with 10 μl of 10% trypan and viewed using a hemocytometer under 20× magnification. The number of cells excluding trypan blue was divided by the total number of cells in the same area and multiplied by 100 to determine the percent of viable cells for each of the triplicate wells. The viability of untreated cells was used as the control and was defined as the control and 100%. The control cell viability was 97% ± 5%. When a compound was observed to increase neurite outgrowth, cell viability in the presence of this compound was not determined.

Protein kinase C measurement. Protein kinase C (PKC) activity was determined in PC12 cells that were cultured in the absence and presence of laminin and (−)-indolactam V using the Biotrak TM RPN77 protein kinase C kit from Amersham. Cells were primed with NGF and harvestedThe cells were then washed free of serum and resuspended in a defined medium composed of a 50%/50% mixture of DMEM and HAMS/F12 containing 100ng/ml NGF, 100μg/ml transferrin, 100μM putrescene, 20nM progesterone, and 30nM $NaSeO_3$. The cells were then plated in 100 mm diameter tissue culture plates and allowed to attach and re-stabalize overnight at 37°C. The next day, the effect of indolactam V and laminin on PKC activity was determined as follows. For indolactam V, cells were either untreated or treated with various concentrations of (−)-indolactam V for 30 minutes prior to harvest and assay. For laminin, cells were either untreated or treated with 50 μg of laminin for 1.5, 15, 45, 60, 90, or 1080 minutes prior to cell harvest and assay for protein kinase C activity. To measure protein kinase C activity, 2×10^6 cells from each treatment were harvested, pelleted, and homogenized in 50 mM Tris/HCL, pH 7.5 containing 0.3% b-mercaptoethanol, 5 mM EDTA, 10mM EGTA and 50μg/ml PMSF (homogenizing buffer). The homogenate was then centrifuged at 100,000 × g for 30 minutes at 4°C. The supernatant (cytosolic fraction) was collected and used for measurement of cytosolic PKC as described below. The pellet (particulate fraction) containing membrane associated PKC was extracted with 200 μl of the homogenizing buffer (50 mM Tris/HCL, pH 7.5, 0.3% b-mercaptoethanol, 5 mM EDTA, 10mM EGTA and 50μg/ml PMSF) containing 0.3% Triton X-100 and incubated for one hour at 4°C. For PKC activity 25 μl of each cell treatment sample and fraction was added separately to 25 μl of the target substrate peptide mixture as described by the manufacturer. Next, 5 μl of 40 μCi/ml [^{32}P]ATP was added to each tube and the reaction mixture was incubated at 37°C for 15 minutes. The reaction was then stopped with 10 ml of stop reagent as described by the manufacturer. The phosphorylated peptide was separated for quantitation of PKC activity by pipetting 35 μl of the reaction mixture to the center of paper discs which were allowed to dry. Next, each disc was washed three times with 10 ml of orthophosphoric acid for 5 minutes each wash. The radioactivity remaining on the paper discs was quantitated in a scintillation counter. Protein kinase C activity was then calculated as the picomoles of phosphate added to the substrate peptide per minute based on the following formula:

$$P = T \times 1000/I \times R$$

where P = pmoles transferred per minute, I = incubation time, R = the specific activity of the magnesium [^{32}P]ATP and T = the sample CPM − blank CPM. Blank CPM was determined by running a reaction with no substrate peptide added to the reaction mixture. The cytosolic and membrane (particulate) PKC activity in the PC12 cells which were not treated with either laminin or (−)-indolcatam V cell cytosol and membranes were considered control values and were considered 100% of the PKC activity under control conditions. The PKC activity in treatment groups was expressed as the percent of the control using the following formula:

$$\frac{\text{Treatment PKC activity} \times 100}{\text{Control PKC activity} = \%\ \text{of Control}} = \text{PKC activity as \% of Control}$$

RESULTS

In order to test the effects of a nonphorbol protein kinase C activator on laminin-mediated neurite outgrowth, we treated PC12 cells with (−)-indolactam V in the presence of laminin. Cells treated with (−)-indolactam V showed a decrease in neurite outgrowth (Fig. 1 and 2A). Trypan blue exclusion showed that the inhibitory doses of indolactam V were not toxic to the

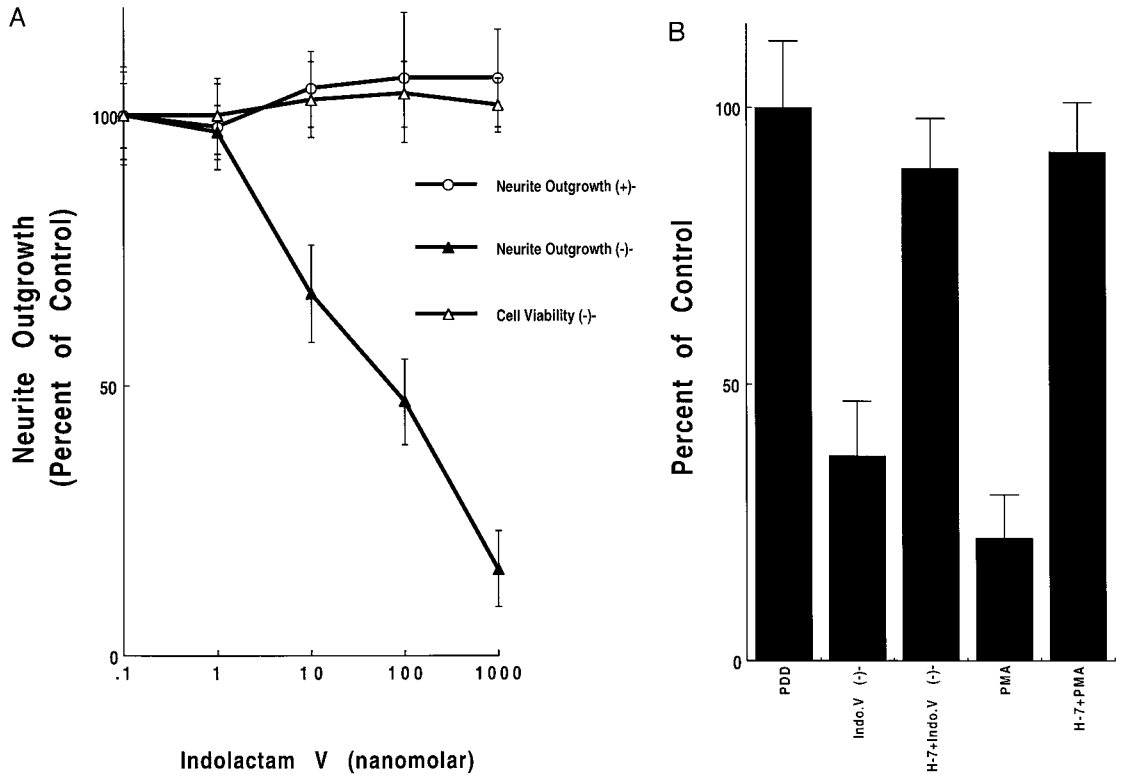

FIG. 2. The effect of idolactam V on laminin-mediated neurite outgrowth. (A). The promoter of protein kinase C, (−)-indolactam V and its inactive stereo isomer control (+)-indolactam V were added to PC12 cell cultures at the time of cell seeding with laminin. The cells were cultured for 18 hours and then fixed and stained and neurite outgrowth was determined as described in the Materials and Methods section. Maximum laminin-mediated neurite outgrowth in control wells was defined as 100%. The percent of cells with neurites in the control wells with the 1% EtOH solvent was 78% ± 5% with the 1% EtOH solvent control. Cell viability in the presence of (−)-indolactam V was determined by trypan exclusion in triplicate parallel wells. Cell viability was always above 90%. Error bars represent the standard error of the mean. (B). Indolactam V-mediated inhibition of neurite outgrowth is reversed by 30 μM H-7. PC12 cells were cultured for 18 hours in the presence of laminin. Maximum laminin-mediated neurite outgrowth in control wells containing 100nM PDD was defined as 100% and was was 85% ± 7. At the time of cell seeding both 1.0 μM (−)-indolactam V and 100 nM PMA were either added alone or with a concomitant addition of 30 μM H-7. After 18 hours the cells were fixed and stained and neurite outgrowth was determined as described in the Materials and Methods section. Treatment groups were tested in triplicate and the error bars represent the standard error of the mean.

PC12 cells (Fig. 2A). Further, the inactive stereoisomer of (−)-indolactam V, (+)-indolactam V, had no effect on laminin mediated neurite outgrowth (Fig. 2A). Further, the inhibitory effect of 1.0 μM (−)-indolactam V on laminin-mediated neurite outgrowth could be blocked by concomitant treatment with the protein kinase C inhibitor, H-7 (Fig. 2B). These data suggest that the protein kinase C activity inhibits laminin-mediated neurite outgrowth.

The phosphatase inhibitor calyculin A was added to PC12 cells cultured in the presence of laminin. Calyculin A inhibited neurite outgrowth by over 50% without demonstrable cellular cytotoxicity (Fig. 1 and 3). Further, insulin significantly enhanced laminin-mediated neurite outgrowth by as much as 235 % (Fig. 1 and 3). These data suggest that protein phosphatase activity is also required in the mechanism of laminin mediated neurite outgrowth.

The effect of laminin on protein kinase C (PKC) activity in PC12 cells was directly measured. Both the cytosolic (soluble fraction) and membrane (particulate fraction) levels of PKC activity were measured in PC12 cells in the presence and absence of (−)-indolactam V (30 minute incubation) and the absence and presence of laminin (1.5, 15, 45, 60, 90, 1080 minutes). The PKC activity in the cytosolic and particulate fractions in untreated PC12 cells were 402 ± 24 and 134 ± 5.0 pmol ^{32}P incorporated/minute/3.5 × 10^4 cells, respectively, and these values were used as controls to establish the 100% PKC activity in cytosolic and membrane fractions in PC12 cells. Laminin treatment for up to 18 hours had no effect on protein kinase C activity and did not effect the subcellular localization of this enzyme (Fig. 4). (−)-indolactam V was used as a positive control and increases in membrane protein kinase C activity was observed after 30 minutes of treatment (Fig. 4). Taken together, these data demonstrate that protein kinase C is not activated in PC12 cells with laminin treatment and that protein kinase C activity is

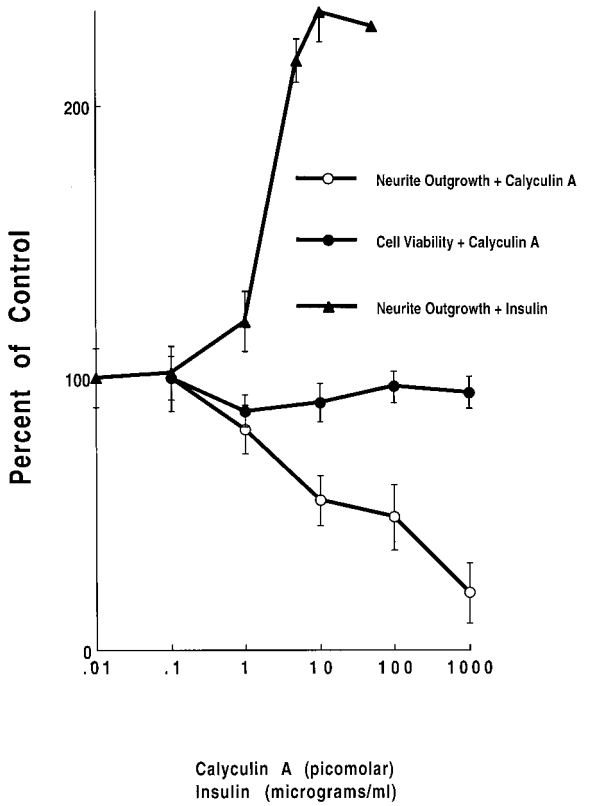

FIG. 3. The effect of calyculin A and insulin on laminin-mediated neurite outgrowth. PC12 cells were cultured for 18 hours in the presence of laminin. At the time of cell seeding, either calyculin A or insulin were added to triplicate wells. Maximum laminin-mediated neurite outgrowth in control wells was defined as 100%. The percent of cells with neurites in the control wells were 85% ± 7 and 69% ± 7% in 1% EtOH and DMEM blank solvent controls, respectively. In the case of calyculin A, cell viability was monitored using trypan blue exclusion and was always above 90%. All measurements are from triplicate wells and the error bars represent the standard error of the mean.

not required in the mechanism of laminin-mediated neurite outgrowth.

DISCUSSION

Investigation of laminin signal transduction pathways in neurons has often provided conflicting results regarding the role of protein kinase C (17, 18). For example, a 24 hour pre-treatment of chick ciliary ganglial cells with phorbol ester was shown to promote laminin-mediated neurite outgrowth (18). Further, overexpression of protein kinase C epsilon has been shown to enhance neurite outgrowth in PC12 cells (19). In contrast, laminin-mediated neurite outgrowth in PC12 and NG108-15 cells was inhibited by phorbol ester treatment (17) and neurite outgrowth in neuroblastoma cells has been associated with decrease protein kinase C expression and activity (20). Further, the PKC inhibitor H-7 has been shown to enhance neurite outgrowth in both the presence and absence of laminin (17, 23, 24). Here we found that the protein kinase C activator, indolactam V, is a potent inhibitor of laminin-mediated neurite outgrowth. This observation is the first to show that a non-phorbol activator of protein kinase C inhibits laminin-mediated neurite outgrowth and helps to clarify the negative role of protein kinase C in neurite outgrowth.

In addition to activators of protein kinase C, the protein phosphatase inhibitor, okadaic acid, has also been shown to inhibit neurite outgrowth (17). Here, we found that the potent protein phosphatase-1 and -2A inhibitor, calyculin A, also inhibits laminin-mediated neurite outgrowth. Conversely, insulin is an activator of protein phosphatases-1 and -2 (25, 26) and stimulates the dephosphorylation of paxillin and focal adhesion kinase (FAK) (27–30). Interestingly, insulin is

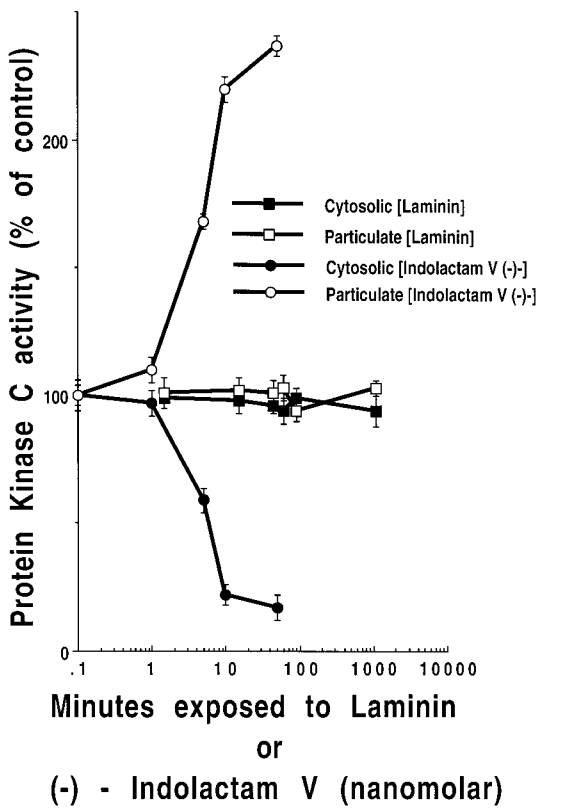

FIG. 4. Protein kinase C activity in PC12 cells treated with indolactam V or laminin. PC12 cells were either treated with various concentrations of indolactam V for 30 minutes prior to assay or treated with laminin (50 μg) for 1.5, 15, 45, 60, 90, 1080 minutes prior to assay. After cell harvest, the cytosol (soluble) and membrane (particulate) fractions were isolated and the protein kinase C activity in these fraction was determined as described in the Materials and Methods section. The PKC activity in cytosolic and membrane fractions of untreated PC12 cells were 402 ± 24 and 134 ± 5.0 pmol 32P incorporated/minute/3.5 × 10^4 cells, respectively, and these values were used as controls to establish the 100% PKC activity in cytosolic and membrane fractions in PC12 cells. Triplicate PKC assays were performed using the fractions from each treatment and the error bars represent the standard error of the mean.

known to enhance neurite outgrowth in several systems in the absence of laminin (31–33) and here we found that insulin also enhances laminin-mediated neurite outgrowth. The observations that insulin both induces neurite outgrowth and dephosphorylates paxillin and FAK are at odds since phosphorylated paxillin and FAK are associated with neurite outgrowth and laminin signal transduction in a variety of cell types including neurons (9, 10, 12, 34). Our observations with calyculin A and insulin support the hypothesis that protein phosphatase-1 and -2A activation is important in the mechanism of laminin mediated neurite outgrowth. However, as noted with the use of modulators of protein kinase C activity, modulators of protein phosphatase activities also provide conflicting data regarding the precise role of phosphorylation systems in the mechanism of laminin-mediated neurite outgrowth.

The disparate effects of phosphorylation system modulators on laminin mediated neurite outgrowth are likely related to the type of neuron studied, the lack of specificity of many of these modulators, the complexity of phosphorylation systems, the binding of laminin to multiple receptors and the activation of multiple signaling pathways. In order to clarify the role of protein kinase C in the mechanism of laminin-mediated neurite outgrowth, we directly measured the the level of soluble and particulate neuronal cell protein kinase C activity in the presence of laminin. We demonstrated that laminin does not activate PC12 cell protein kinase C activity within the time-frame of neurite outgrowth. Therefore, while protein kinase C modulation clearly affects neurite outgrowth, we conclude that the mechanism of laminin-mediated neurite outgrowth does not require protein kinase C activation.

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