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Endogenously Inhibited Protein Kinase C in Transgenic *Drosophila* Embryonic Neuroblasts Down Regulates the Outgrowth of Type I and II Processes of Cultured Mature Neurons

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Abstract Embryonic neurons were cultured from transgenic *Drosophila melanogaster* expressing a highly specific pseudosubstrate inhibitor of protein kinase C (PKC). Flies homozygous for this transgene, which is under the control of the yeast UAS promoter, were crossed to flies homozygous for the yeast heat shock inducible transcription factor GAL 4. Following heat shock, the progeny express the pseudosubstrate inhibitor at high levels. This strategy, which has the advantage of avoiding the non-specific effects of drugs, was used to study the role of PKC in process growth of cultured, differentiating neuroblasts. An external gold particle labeling procedure using a cell surface antigen expressed by mature neurons and processes was used to visualize neuronal processes directly in the scanning electron microscope. We observed that cell cultures expressing a low concentration of the pseudosubstrate inhibitor showed a significant decrease in the number of type I and II processes as compared to control cultures, while the proportions of neuroblasts, ganglion mother cells (GMCs), and mature neurons in the clusters were little affected.

Key words: protein kinase C, *Drosophila melanogaster*, embryonic neurons

INTRODUCTION

Protein kinase C (PKC) is a member of the serine/threonine family of kinases and appears to be involved in a large number of biological functions, including cell growth and proliferation [Nishizuka, 1988], activation or inactivation of channels [Swartz, 1993], and in long-term potentiation observed in the hippocampus [for review, see Bourne and Nicoll, 1993]. Furthermore, PKC isoenzymes have been extensively characterized biochemically [House and Kemp, 1987, for review see Ogita et al., 1991, Pearson and Kemp, 1991] and constitute one of the most ubiquitous second messenger mechanisms in the cell [Berridge, 1993]. The fruit fly, *Drosophila melanogaster*, has far fewer PKC isoforms than mammals, but the three fly PKC genes bear significant (60%) homology to mammalian PKC genes [Shaeffer et al., 1989]. To begin to dissect the multiple functional roles of PKC, we sought to set up a system in which PKC could be inhibited endogenously using molecular engineering techniques.

One of the mechanisms in which PKC has been implicated is neurite extension by developing and regenerating cultured vertebrate neurons [Bixby, 1989], studied by the use of pharmacological inhibitors and activators. In vivo studies of *Drosophila* larvae have shown two types of neuronal processes. Type I processes have large buttons with few branches, and type II processes are thinner, more branched, and beaded [Littleton et al., 1993]. The beads express synaptotagmin in vivo on the muscle of third instar larvae, suggesting that they are functional or maturing synapses. Cultured neurons from early *Drosophila* embryos display the same range of process morphology as well as the normal progression of differentiation from neuroblast to ganglion mother cell (GMC) to neuron [Prokop and Technau, 1991].

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Here, we report the results of a morphological comparison using scanning electron microscopy of neuronal cultures from embryos with normal, inhibited, or defective PKC activity. The technique we used allows for the attachment of myoblasts and neuroblasts to the surface of plastic Petri dishes. Under such conditions, these precursor cells undergo maturation and division, providing an excellent model for studying the process of neuronal differentiation. We have developed a technique to label processes and mature neurons externally using an anti-HRP (horseradish peroxidase) antibody, which recognizes a specific neuronal surface marker [Jan and Jan, 1982], and a second antibody attached to gold particles which can be visualized directly in the scanning electron microscope (SEM).

Transgenes encoding specific inhibitory peptides are an effective approach for studying kinase functions in vivo [Griffith et al., 1993, Wang et al., 1994]. In this study, transgenic flies express a pseudosubstrate inhibitor of PKC derived from fly eye PKC, the amino acid sequence of which is MKNRLRKGAMKRKGLEM [Shaeffer et al., 1989]. The inactive form of a number of different kinases results from the presence of an autoinhibitory domain that occupies the catalytic site. For PKC, this autoinhibitory domain, which has a high affinity for the catalytic site, constitutes the pseudosubstrate with a glycine or alanine (nonphosphorylable) instead of the usual serine or threonine [Kemp et al., 1991, for review]. Using such a pseudosubstrate inhibitor that is endogenously synthesized in neurons and which has a high affinity and specificity for PKC, we have circumvented the major problems associated with drugs (PKC activators or inhibitors) over a long period of time. Such problems include the disappearance of several subspecies of PKC following treatment with TPA, which initiates their degradation and sustained disappearance from the cell [Nishizuka et al., 1988]; the down-regulation of PKC, which complicates the interpretation of any long-term effects such as those on the morphology of processes over a 24- or 48-h period; and the competition of PKC inhibitors such as H7 and staurosporine with ATP for the catalytic site, raising questions about specificity and their possible interference with other ATP-ase molecules over a long period [Suidan et al., 1992]. Another major uncertainty in using drugs is the possibility of these amphoteric molecules becoming "trapped" in the lipid bilayer when they pass through the cell membrane, changing the dynamics of process growth by artifactually modifying membrane fluidity. Finally, in the system reported here, we observed that the reduction of PKC activity is time dependent after heat shock.

MATERIALS AND METHODS

Assay for PKC Activity

Protein kinase C was assayed by measuring the quantity of ^{32}Pi incorporated into a PKC-specific peptide substrate, as has been described elsewhere [Ogita et al., 1991]. The substrate VRKRTLRRL (Sigma) was used at a concentration of 10 μM. The typical reaction mixture contained aliquots of [^{32}P]-ATP (5,000 cpm/pmol), reaction buffer composed of 10 mM MgCl, 20 mM Tris HCl, 2 mM $CaCl_2$, 50 μM ATP, and 10 μl of enzyme extract in 20 mM Tris HCl, 2 mM EGTA, 1 mM EDTA, 50 μM b-mercaptoethanol, 1 μg/ml phenylmethyl sulfonyl-fluoride (PMSF), leupeptin, and aprotinin 1 μg/ml. Diacylglycerol (DAG) and phosphatidylserine (PS) were added to specifically activate PKC at the respective concentrations 0.8 μg/ml and 8 μg/ml. DAG and PS were prepared by combining 50 μl of DAG in chloroform and 50 μl of PS and blowing air over the mixture to evaporate most of the chloroform. The partially dried mixture was then sonicated for 5 min in 500 μl of reaction buffer. After a specified period of incubation at 30°C (usually 10 min), tubes were put on ice and the reaction was stopped by adding 10 μl of glacial acetic acid. Aliquots of 50 μl were spotted on 3 cm × 3 cm square pieces of cation exchanger, P81 chromatography paper. This method makes use of the ability of basic peptides to bind via ionic interaction with phosphocellulose. The spotted papers were washed in phosphoric acid solution (5 ml in 1 L) 3 times for 5 min, dried, and the radioactivity counted in scintillation liquid. CaM kinase activity and cAMP-dependent kinase were assayed with specific peptides, KKRQET [a gift from L. Griffith, Hanson et al., 1989] and KKRASGP [Drain et al., 1991] (Sigma) respectively.

For CaM kinase, head extracts were prepared by homogenization in 25 mM Tris, pH 7.5, 1 mM EGTA, 50 μM 2-mercaptoethanol, 0.1% Triton X-100, 1 μg/ml phenylsulfonylfuoride, 1 μg/ml leupeptin, and centrifuged at 100,000*g* for 10 min. For PKC, head extracts were typically homogenized in extraction buffer (20 mM Tris pH 7.5,1 mM EDTA and 50 mM β-mercaptoethanol). The homogenate was then centrifuged for

a few minutes at 10,000 rpm using a Beckman centrifuge and the pellet resuspended in EGTA extraction buffer (20 mM Tris pH 7.6, 5 mM EGTA) on ice for 1 h. The suspension was then centrifuged at 40,000 rpm for 30 min. The resulting supernatant was enriched in PKC because this enzyme is normally attached to membranes but is released as a soluble protein when calcium ions are removed.

Extraction of the Pseudosubstrate Inhibitor From Adult Flies or Third Instar Larvae

Flies or larvae were ground in 6 M guanidinium chloride using a glass Poter. After centrifugation, the extract was passed through a Sepak column, washed with 1% TFA, and then the peptide was released with 60% acetonitrile, 1% TFA. The eluates were dried up, solubilised in 1% BSA in PBS, and cross-linked with 0.1% glutaraldehyde for 10 min. Then, this material was spotted on nitrocellulose using a vacuum chamber. For the standards, different known concentrations of peptides in 6 M guanidium chloride were treated in the same way. The staining was performed using an antibody against the peptide (gift from Denise Malicke, Zuker Lab) and a second antibody coupled to alkaline phosphatase (substrate BCIP/NBT tablets, Sigma).

Immunoprecipitation of ACh Receptor and PKC Molecule

The reactions were carried out according to the Maniatis manual [Sambrook et al., 1989]. Briefly, flies were set on ice for 30 min, and then the heads cut off and ground in 250 mM sucrose, 20 mM Tris HCl, pH 7.4, PMSF, 1 μg/ml, aprotinin, and leupeptinin 1 μg/ml. After centrifugation for 5 min at 10,000 rpm, the supernatant was discarded and the pellet was solubilized in Triton X-100 at a final concentration of 1% in the above buffer at 4°C. After brief centrifugation, the supernatant was removed and complemented with a second buffer, 150 mM NaCl, 20 mM Tris HCl pH 7.4, 1 mM EDTA, and 1 μg/ml PMSF, with a final concentration of Triton X-100 of 0.1%. Overnight, 5 μl of the antibody anti-AChR (nicotinic) [Lindstrom and Tsardos, 1981] was added at 4°C. Then, a second incubation with Sepharose beads (Sigma) attached to protein A was performed with a slight rocking motion for 1 h at room temperature. After brief centrifugation the pellet was washed twice in the second buffer. Then the pellet was boiled for 1 min in loading buffer and run on an electrophoretic gel. For *in vivo* phosphorylation of Ach-R by PKC, flies were fed with inorganic ^{32}P in sucrose for 48 h. The flies were kept in sealed vials into which radioactivity was injected using a syringe through the caps, and flies were subsequently killed by freezing at −70°C. Membranes were isolated from entire flies [Choi et al., 1991], and treated as described above.

Neuronal Cell Culture

This method was based on P.M. Salvaterra [1987] with substantial modifications.

Egg collection and aging. The different fly genotypes were maintained in plastic bottles capped with yeast paste covered Petri plates, at 25°C in the dark. Eggs were collected on yeast for 1 or 2 h, depending on the critical timing of the experiments. The eggs were allowed to age undisturbed on the plates for 6–7 h at 25°C to make sure that the embryos had proceeded to the early gastrula stage. Embryos were then washed onto a nylon mesh with distilled water.

Extract of embryos in culture. One hundred embryos for each culture were dechorionated by immersion for 1 min in a 1:1 solution of 95% ethanol and commercial Clorox bleach. Cells were mechanically dissociated in Schneider's medium using a Dounce homogenizer. The material was then centrifuged at 2000 rpm for 2 min, the supernatant discarded and the cells resuspended in same medium with gentle pipetting. This material was seeded in Primaria (Fisher) Petri dishes in 2 ml Schneider's medium complemented with heat-treated calf bovine serum (final concentration 17%), and the plates were kept at 25°C and 60% humidity for 24 h. Standarization of the cell culture system in the light microscope prior to the analysis of the various genotypes in the SEM: This standardization was initially performed using w^z control cultures. The number of myotubes was used as an internal control of each culture. Cell cultures were retained for further analysis when the density of cell clusters per dish was approximately the same as that which in control cultures produced the standardized outgrowth of processes. This is an important control because such an effect of cell cluster density on outgrowth of processes is possibly due to the secretion of diffusible factors by the neurons or myotubes. This analysis in the light microscope, which was carried out after fixation in glutaraldehyde and alcohol treatment, was of sufficient resolution to quantify

myotubes and neuronal cell clusters. However, it is not appropriate for quantifying type I and II processes which require the higher resolution of the electron microscope. Therefore, the presence myotubes and the number of cell clusters per culture dish was a constant for each culture that was further analysed in the SEM.

Scanning Electron Microscopy

Cells on plates were incubated with 3% glutaraldehyde in PBS for 2 h, then dehydrated in an ethanol series (from 30% to 100%) for 10 min at each 10% increase in concentration. The specimens were dried by CO_2 critical point drying and coated with carbon and gold/paladium. For the external labeling, cells were incubated in Schneider's medium and bovine serum with the primary antibody rabbit anti-HRP (Cappel, PA) for 4 h, then washed twice and incubated with a gold particle labeled anti-rabbit secondary antibody, which were enhanced by silver for a few minutes (Amersham kit RPN 470 and RPN 490). After 2 washings the cells were fixed with 3% glutaraldehyde in PBS and the drying steps were carried out as described above. The externally labeled specimens were coated with carbon only. Furthermore, prior to EM analysis, the cell density of the fixed specimens can be rechecked in the light microscope. Clusters appear as brown spots and myotubes as clear elongated cells.

Morphological and Statistical Analysis of Each Genotype in the Electron Microscope

Again, this analysis was initially standardized in control cultures, when it was determined that the quantification of 5 cell clusters per culture dish was sufficient to obtain representative averages for each culture dish. Three culture dishes were analysed for each genotype, so that a total of 15 cell clusters were quantified per genotype. Data for each genotype were tested for normal distribution using a Shapiro-Wilk W test. Statistical analysis using Dunnett's method ($P < 0.05$) was performed to compare each genotype to the w^2 control.

P-Element Transformations

The UAS-PKC inhibitor P-element transgene (UAS-PKCi) was constructed by subcloning the PKC inhibitor sequence into the pUAST vector [Brand and Perrimon, 1993]. The corresponding PKC inhibitor amino-acid sequence is MKNRLRKGAMKRKGLEM which is the pseudosubstrate of fly eye PKC [Shaeffer et al., 1991]. Transgenic lines were generated by microinjection of plasmid DNA at a concentration of 500 μg/ml into *w; +/+; P[^2-3] Sb e/Tm6 UBX e* strain embryos using standard procedures [Spradling, 1986]. Two transformed lines, *2A* and *4A*, were used.

Fly Culture and Maintenance

Flies were grown on standard cornmeal-molasses-agar medium at 25°C in a humidified incubator. The chromosome bearing the insert (UAS-PKCi) was determined for each transformant line using dominantly marked balancer chromosomes, which simultaneously allowed us to obtain single insert homozygous lines. Two transformed lines, *4A* or *2A*, both carrying the insert on the 3rd chromosome were used in each assay. Assays were performed using the progeny of the cross between males homozygous for UAS-PKCi and virgin females homozygous for hspGAL4 [Brand and Perrimon, 1993]. All the progeny were, therefore, heterozygous for both UAS-PKCi and hspGAL4, and their genotype may be written *UAS-PKCi/hspGAL4* (which is referred to in the text as *PKCi/GAL4*). Heat-shocking these double heterozygous flies at 37°C stimulates synthesis of GAL4, which in turn binds to the UAS sequence resulting in increased production of the pseudosubstrate inhibitor, PKCi.

Drosophila Stocks

w; P[^2-3] Sb e/TM6 Ubx e. [^2-3] is a modified P element producing transposase, marked with *Stubble* and *ebony* in a white eyed background.

FM7a [Lindsley and Zimm, 1992].

w; Cyo/Gla [Lindsley and Zimm, 1992].

w; y + TM3 Ser/Sb [Lindsley and Zimm, 1992].

w^2—White eyed Canton S strain-control, same genetic background as transformed strains.

P[w⁺, hspGAL4]—GAL4 protein coding sequence under control of heat-shock promoter hsp-70. Homozygous on third chromosome. This strain is the generous gift of Dr. Andrea Brand (Wellcome/CRC Institute, Cambridge, England).

The *turnip* strains were the generous gift of Dr. Tim Tully (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

The series of PKC substrate peptides (derivatives) were a generous gift from Dr. Bruce Kemp (U. Southern Alabama).

PKCi DNA, peptide, and anti-peptide antibodies were the generous gifts of Drs. Dean Smith, Deborah Malicke, and Charles Zuker (UCSD, La Jolla, CA).

Anti-AChR monoclonal antibody MAb 35 [Lindstrom and Tsardos, 1981] was a gift from Dr. Darwin Berg (UCSD, La Jolla, CA).

RESULTS

Comparative Inhibitory IC_{50} of the PKC Pseudosubstrate Inhibitor for cAMP Kinase, Ca^{++}/Calmodulin Dependent Protein Kinase II (CaM Kinase) and PKC

Since little enzymology on fly PKC has been studied, we analyzed the sequence requirement for fly PKC substrates. PKC displays low rates of phosphorylation for peptides with dibasic residues at the N terminal of the phospho-serine. However, the inclusion of C terminal dibasic residues increases the apparent affinity to 3 μM (Table I).

Data plotted in Figure 1A–C shows the comparative efficiency of the pseudosubstrate inhibitor to inhibit different kinases. cAMP kinase shows no affinity (>1 mM). The IC_{50} for CaM kinase is 80 μM, and for PKC is 0.4 μM. These numbers provide evidence for the high specificity of the pseudosubstrate inhibitor for PKC and its slight interference with other competing kinases. We also performed an immunoprecipitation of CaM kinase (Fig. 1D) after heat shock to show the in vivo specificity of the inhibition. No differences in the labelling of the two species (55 and 60 kDa) constituting the enzyme are noticeable between the w^z control and the *PKCi4A/GAL4* genotype. In addition, the 60 kDa species disappears in hsp-ala2 flies (which express an inhibitory peptide of CaM kinase) following heat shock.

TABLE I. Measure of Relative Km for a Series of Derivatives from the Original Peptide G-G-R-L-A-R-A-L-S-V-A-A-G Abbreviated to R3-R6-S9*

Peptide derivative	Relative Km (μM)
R3-R6-R7-S9	40
R3-R6-S9	>100
R5-R6-S9	>100
R3-R5-R7-S9	>100
R5-R6-S9-R12	20
R5-R6-S9-R11-R12	5
R6-S9-R12	20
R3-R6-R7-S9-R11-R12	3

*The derivatives are R substituted by A or vice versa. The relative Km's were determined with head extracts from w^z using approximately the concentration corresponding to half of the Vmax.

In Vivo Expression of Pseudosubstrate Inhibitor

Flies homozygous for a transgene encoding the pseudosubstrate inhibitor, MKNRLRKGAMKRKGLEM, under the control of the yeast UAS (Upstream Activating Sequence) promoter were crossed to flies homozygous for the yeast heat shock inducible transcriptional factor GAL4. Following heat shock, the progeny of such a cross (*PKCi/GAL4*) express the inhibitor at high levels. After a standard heat shock, *PKCi4A/GAL4* flies were allowed to recover for different lengths of time before the pseudosubstrate inhibitor was extracted and analyzed (Fig. 2). Using a rabbit polyclonal antiserum against the peptide, we were able to detect the synthesis of the peptide after cross-linking to BSA in a Western dot blot. Densitometric comparison of a defined quantity of peptide, treated in the same conditions to assess recovery, gave concentrations which spiked around 10 μM at 4 h (Fig. 2A). Furthermore, without heat shock the embryos gave a concentration of about 2 μM, suggesting that a low level of GAL4 (heat shock promotors are known to be leaky) may trigger active peptide synthesis. Although it is difficult to assess accurately the peptide concentrations, we have been able to rule out a possible interference with other major kinases due to the fact that the Ki's show large differences in affinity (Fig. 1).

PKC activities of head extracts, before and after heat shock for *PKCi4A/GAL4* and w^z, were measured (data not shown). A 20% decrease in PKC activity was obtained for PKC inhibited flies, and a slight increase of 10% for the equivalent wild type, w^z. We also performed the assay for cAMP dependent kinase and Ca^{2+}/calmodulin dependent kinase (CaM kinase). No differences were seen after heat shock for both the w^z control and *PKCi4A/GAL4*. The dilution of the peptide (affinity 0.5 μM) plus the competition of the peptide substrate used in the assay may explain these inconclusive results. Due to these limitations, we performed immunoprecipitation of putative targets in vivo (namely, the Ach receptor and PKC itself) to address the efficiency of the system and the results are described in the following two sections.

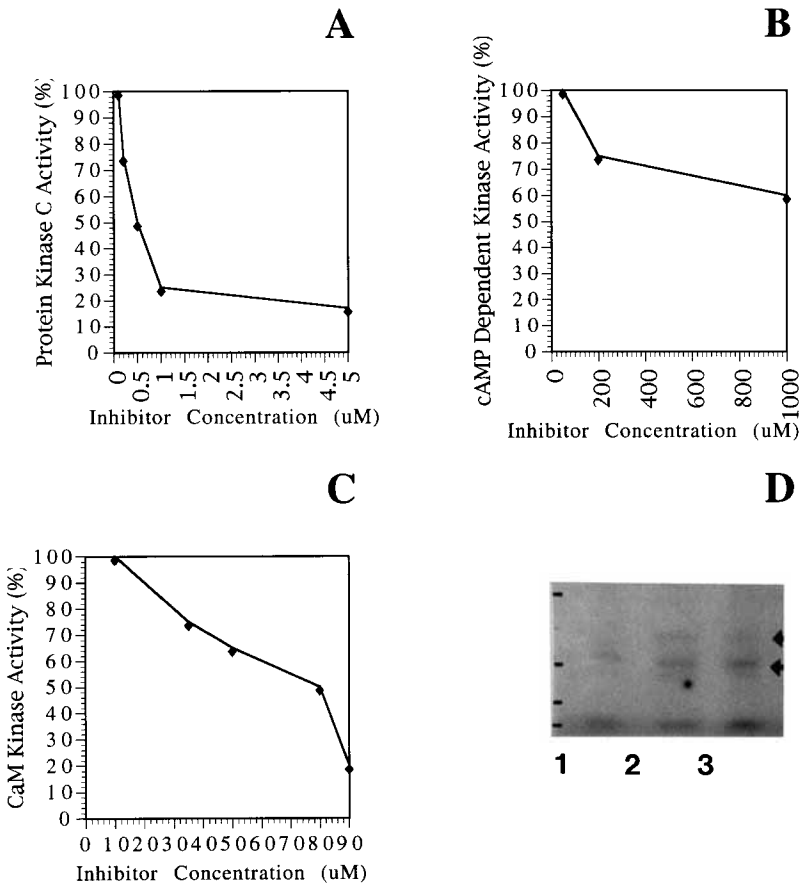

Fig. 1. *In vitro* determination of the IC_{50} for the inhibition of three different kinases by the PKC pseudosubstrate inhibitor and *in vivo* control of its inhibition specificity. **A:** Inhibition of PKC. The IC_{50} is approximately 0.4 μM. **B:** Inhibition of cAMP dependent kinase. The IC_{50} is approximately 1 mM. **C:** Inhibition of Ca^{2+}/calmodulin kinase. The IC_{50} is approximately 80 μM. **D:** Immunoprecipitation of CaM kinase. *Lane 1* is ala2 (which expresses a peptide inhibitor of CaM kinase under the control of a heat shock promoter), *lane 2* is w^2 control, and lane 3 is *PKCi4A/GAL4*. The two arrows show bands of 55 and 60 kDa.

Phosphorylation of ACh-R In Vitro and In Vivo by PKC

To assess the effectiveness of the peptide in blocking an identified endogenous substrate, the nicotinic acetylcholine receptor (nAchR) (Lindstrom, 1981), we used a monoclonal antibody to the nicotinic Ach-R, described in the embryonic chick brain, which clearly recognizes and immunoprecipitates the homologous fly receptors. Four subunits for nicotinic Ach-R for *D. melanogaster* ARD, SBD, Da2, and ALs (E.D. Gundelfinger et al. 1992) have been described. For Da2 and ALs, the putative size of the gene product is 54 kDa, but the immunoblot shows a band of about 50 kDa [Gundelfinger and Hess, 1992]. The putative gene products for Da2 and ALs are about 58 and 60 kDa, respectively, and the immunoprecipitated product for ALs is reported at 42 kDa [Schloss et al. 1992]. Only ALs and Da2 both have two potential phosphorylation sites for PKC [E.D. Gundelfinger and Hess, 1992]. Figure 3 shows that the antibody MAb35 recognizes the native pentameric nAch-R complex in detergent soluble form and/or isolated subunits Als or Da2. We observed that there was little expression of these two subunits in embryos. In the presence of PKA inhibitor, (PKI), nAch-R are strongly phosphorylated by PKC, and the transfer of the radioactive phosphate is inhibited by the pseudosubstrate. The band here is at 45 kDa, suggesting that this is the processed precursor (after cleavage of the signal peptide) of ALs and/or Da2.

To assess the effect of inhibition in vivo, a parallel experiment of in vivo phosphorylation of flies in sealed vials was performed (Fig. 4A,B).

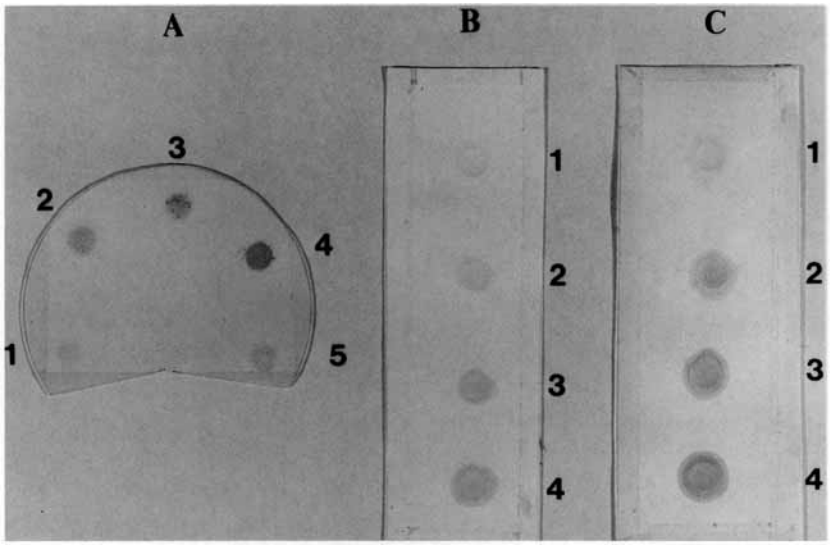

Fig. 2. Extraction of the pseudosubstrate inhibitor from 100 progeny of the cross PKCi4 × GAL4 in 6M guanidinium chloride. The extract was diluted and passed through a Sepak column, washed with 1% TFA, and then the peptide was released by 60% acetonitrile/1% TFA. The eluates were dried up, solubilized in a solution of 1% bovine serum albumin (BSA), and cross-linked with glutaraldehyde, before spotting on nitrocellulose paper using a vacuum chamber. **A:** The dots represent the amount of peptide extracted before heat shock (*1*), immediately after 1 hour heat shock (*2*), and 2 hours (*3*), 4 hours (*4*), and 6 hours (*5*) after 1 hour heat shock. **B:** 25 third instar larvae were ground in 6M guanidinium chloride, and then passed through a Sepak column. Then the following steps were carried out as described above. From top to bottom: *GAL4/w*^{z} (*1*), *PKCi4A/w*^{z} (*2*), *PKCi2A/GAL4* (*3*), *PKCi4A/GAL4* (*4*). **C:** Standards. The dots represent (*1*) 1, (*2*) 5, (*3*) 10, and (*4*) 20 μM peptide concentrations in 200 μl.

We observed a significant decrease of nAch-R phosphorylation after heat shock of *PKCi4A/GAL4* and *PKCi2A/GAL4* flies. A visible higher band could be an imperfectly separated species such as a dimer, as reported by other researchers [Schloss et al. 1992].

Phosphorylation In Vitro and In Vivo of PKC

Since PKC also serves as its own substrate [Choi et al., 1991], we examined the pseudosubstrate's effect on autophosphorylation. We took advantage of the fact that PKC is attached to the membrane and can be released by the complexing of Ca^{++} with EGTA. We incubated membranes with ^{32}P ATP, DAG, and phosphoserine. To avoid diluting the pseudosubstrate inhibitor, isolation and incubation were carried out in minimum volume. The radioactive material released by EGTA from membranes and analyzed in an SDS gel showed that a band of approximately 84 kDa is missing after heat shock, whereas the other bands acting as internal controls showed the same intensity (Fig. 5A,B). We show in Figure 5 that six membrane proteins phosphorylated in vitro following the incorporation of ^{32}Pi into membranes of *PKCi4A/GAL4* flies showed the same intensity of labelling.

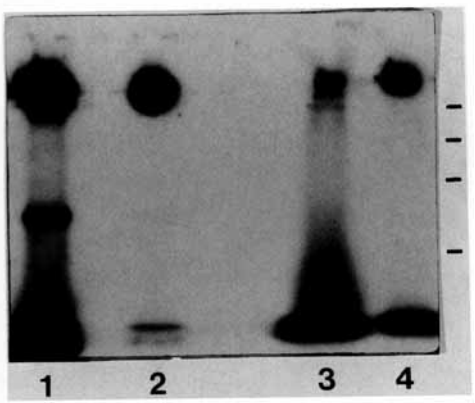

Fig. 3. Immunoprecipitation of nicotinic acetylcholine receptors in the *D. melanogaster* brain phosphorylated in vitro. Fifty heads (*lane 1*) and ten third instar larvae (*lane 2*) were ground in 1 mM EDTA, 25 mM Tris pH 7.4, aprotinin, leupeptin, 1 μg/ml PMSF, and centrifuged for 1 min at 2,000 rpm in a microcentrifuge tube. The supernatant was centrifuged for 10 min at 10,000 rpm and the pellet resuspended and complemented with radioactive ^{32}y ATP diluted with cold ATP (50 μM), Mg (10 mM), Ca^{++}, 10 nM of PKI (mammalian inhibitor of PKA), phosphatidylserine, and diacylglycerol for 5 minutes at room temperature. Lanes (*3*) and (*4*) are the same as lanes (1) and (2) except that 100 μM of the inhibitory peptide was preincubated for 20 minutes before adding radioactivity, Mg and Ca. These materials were immunoprecipitated with an anti-n AChR, and analyzed on an acrylamide gel. The markers represent from bottom to top: 38, 54, 65, and 88 kDa.

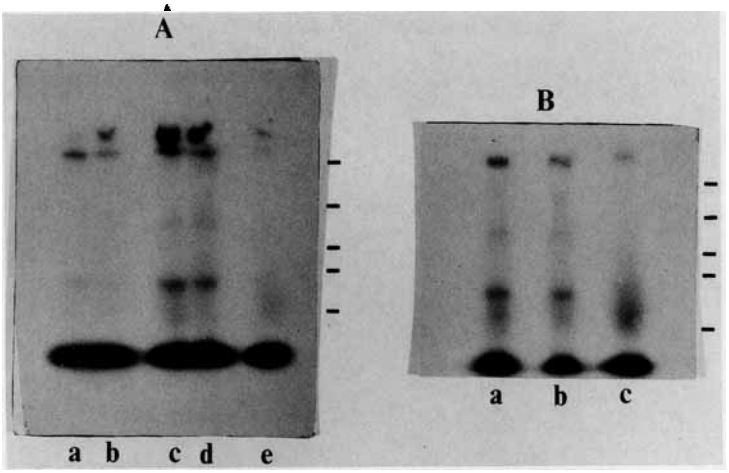

Fig. 4. Immunoprecipitation of AchR phosphorylated in vivo. **A:** *Lanes a* and *b* represent PKC inhibited flies (the progeny of the cross between *PKCi2A* or *4A* × *GAL4*) after heat shock, and *lanes c* and *d* represent the same flies before heat shock. Ten flies were ground and the centrifuged pellet solubilized and immunoprecipitated with the antibody, and finally analyzed in a 7.5% acrylamide gel. *Lane e* is the control without the antibody. **B:** Wild-type flies (w^2) were analyzed with the same antibody. *Lane a* after heat shock and lane *b* before heat shock. *Lane c* is the control.

These six bands can be viewed as internal controls which show clearly that the expression of the pseudosubstrate inhibitor is specifically affecting the incorporation of phosphate into one band. The size of this missing band is consistent with the autophosphorylation of PKC itself. To test this hypothesis, immunoprecipitation on solubilized membranes was carried out just after the phosphorylation step with a rabbit anti-PKC antibody recognizing the consensus sequence 543–550, which is highly conserved between species [E. Schaeffer et al. 1989]. The band observed at 84 kDa is significantly reduced after heat shock for *PKCi2A/GAL4* and *PKCi4A/GAL4* (Fig. 6A,B), as is a second band, which is probably a higher molecular weight isoform of PKC. Finally the immunoprecipitation of solubilized membranes which were phosphorylated in vivo, was performed using the same antibody (Fig. 6C). Although we saw less labeled material after heat shock, the PKC band is present in PKC-inhibited flies (*PKCi/GAL4*), suggesting either partial inhibition or, alternatively, a low turnover of phosphorylation, which may mask the efficiency of the peptide's inhibitory effect.

Analysis of the Morphology of Wild-Type Embryonic Cultured Maturing Neuroblasts, Using Direct Labeling, Visible in the Scanning Electron Microscope

Embryos entering the early gastrula stage when a set of neuroblasts delaminate from the ectoderm layer were cultured. The technique we used allows only for the attachment of myoblasts and neuroblasts to the surface of plastic Primaria Petri dishes. These precursor cells undergo maturation and division, providing an excellent model for studying the process of neuronal differentiation *in vitro*. We have developed a technique to directly observe mature neurons and processes in the scanning electron microscope (SEM). By labelling with gold particles, increased in size by silver enhancement (Fig. 7), we can clearly see clusters of cells of different sizes. The largest cells (>5 μm) with a chaotic surface and asymmetric shape are the original neuroblasts. Each cluster is presumed to orginate from the attachment of embryonic neuroblasts. These cells undergo asymmetric division to generate intermediate-sized cells (about 5 μM) called ganglion mother cells (GMCs) (Figs. 7, 8). These divide once to give mature neurons with branching processes (Fig. 7). In our model, we can clearly observe type I processes (Fig. 7) which are thick, sometimes in bundles of axons, and often connected to another cluster or a myotube. Type II processes are thinner, highly branched, and show small varicosities (<1 μm) (Fig. 8). Type II processes are never seen to be connected to another cell cluster or myotube, and appear to "run" on the plastic as if in search of some target. Unconnected type I processes often show lateral type II branches and a highly branched type II terminus. Connected type I processes usually show few varicosities and few

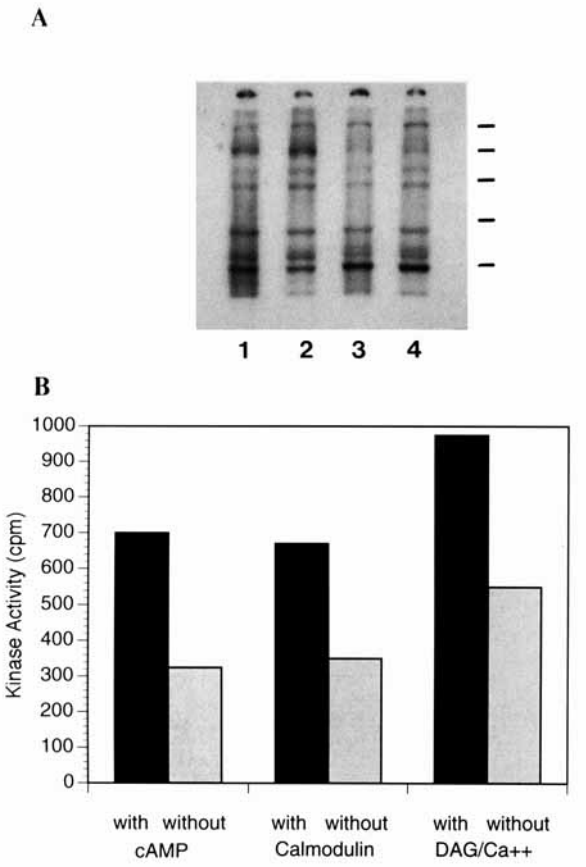

Fig. 5. Gel electrophoresis of proteins from EGTA treated membranes of the progeny of the crosses *PKCi2A* × GAL4 and *PKCi4A* × GAL4. For each genotype, fifty heads were ground in 1 mM EDTA, 25 mM Tris pH 7.4, and 1 μg/ml PMSF in a minimum volume (100 μl) and immediately incubated with radioactive ATP, Mg^{++}, Ca^{++}, DAG, and phosphoserine for 5 min at room temperature. PKC, which is normally attached to membranes, was separated from the cytosol by centrifugation, then released (along with other membrane-bound proteins) from the membrane by incubation in 5 mM EGTA for 1 h at 4°C. Next, the samples were centrifuged in a Beckman centrifuge for 5 min at 10,000 rpm and the supernatant analyzed on an acrylamide gel. Markers are 125, 88, 65, 54, and 38 kDa. **A:** *Lanes 1* and *2* represent the progeny of the crosses *PKCi2A* × *GAL4* and *PKCi4A* × *GAL4* before heat shock. *Lanes 3* and *4* represent the same flies after heat shock. This material was released from membranes by EGTA. **B:** Different kinase activities found in the material released from membranes of wild type flies by EGTA.

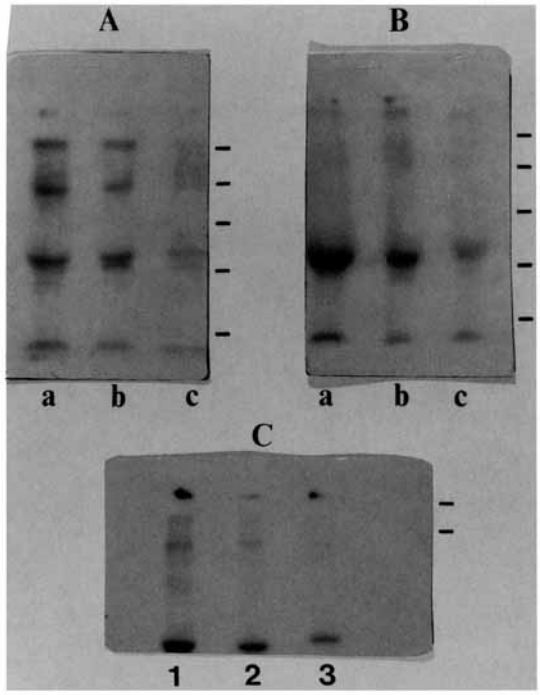

Fig. 6. Immunoprecipitation of PKC after and before heat shock from progeny of the crosses *PKCi2A* × *GAL4* and *PKCi4A* × *GAL4*. The phosphorylation protocol described in Figure 3 was followed. The membranes were then directly solubilized and immunoprecipitated using an antibody directed against the conserved domain of PKC in different species, consensus sequence 543–550. Calbiochem 539550 antibody was used at a final 1 in 5 dilution. **A:** Progeny of *2A* × *GAL4* and *4A* × *GAL4* in *lanes a* and *b* before heat shock. *Lane c* is a wild-type control without antibody. **B:** Progeny of *2A* × *GAL4* and *4A* × *GAL4* in *lanes a* and *b* after heat shock. *Lane c* is a wild-type control without antibody. **C:** Immunoprecipitation of PKC phosphorylated in vivo after animals were fed with inorganic ^{32}P phosphate. Ten whole flies were ground, membranes were separated, solubilized, and immunoprecipitated using the same Calbiochem antibody. *1*) Wild type after heat shock, *2*) *4A/GAL4* progeny after heat shock, *3*) w^z control without antibody.

branches, and are either connected to other neuronal clusters or run on the surface of myotubes.

Analysis of Cultured Neuroblasts From Transgenic, Control, and *turnip* Embryos

The GMC:mature neuron ratios for all genotypes (Table II), except homozygous *turnip* (which expresses a defective form of PKC), do not differ significantly from the w^z control (Fig. 9A), suggesting that partial inhibition of PKC activity does not effect neuronal differentiation. A comparison of each genotype with the control, however, shows that both expression of the pseudosubstrate inhibitor (*PKCi/GAL4*) and the presence of a defective form of PKC (*tur/FM7* and *tur/tur*) significantly reduced the number of type I and type II processes (Table III and Fig. 9B,C).

The majority of clusters in the non-heat shock *PKCi/GAL4* cultures showed a reduction in the number of processes. However, a few showed a more normal morphotype compared to the control. This heterogeneity can be explained by the nature of the heat shock promoter, the site of insertion of which in the genome can affect the

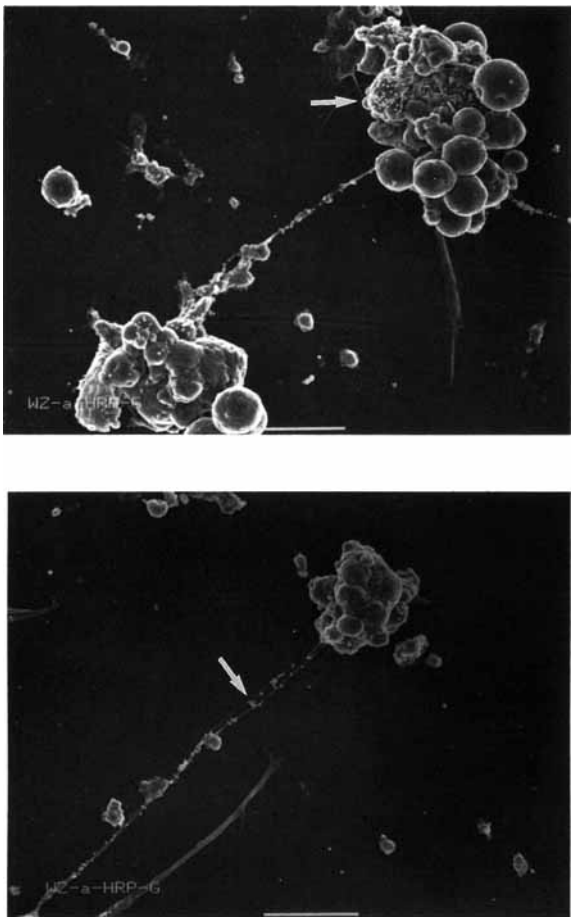

Fig. 7. Scanning electron micrograph of *Drosophila* embryonic cell culture and identification of neuronal cells using a surface marker. Bright beads (*arrows*) are silver enhanced gold particles. Cells were first incubated with anti-HRP (marker for processes and mature neurons) and then with anti-rabbit gold particles of 10 nm diameter. White bars represent 10 μm.

level of its leaky expression depending on cell type. Therefore, in a small subpopulation of neuroblasts, there may be little or no leaky expression of GAL4. This assumption is confirmed by our data, shown in Figure 2, in which embryos that were not heat shocked express the pseudosubstrate inhibitor. Furthermore, this variation in the pattern of leaky expression of GAL4 has been confirmed in adult brain slices by Feveur et al. [1994]. Note that Table III, which summarizes the analysis of process morphology from different genotypes, includes two types of *turnip* flies, heterozygous *tur/FM7* and homozygous *tur/tur,* the genotype of the latter being more extreme suggesting a gene dosage dependency.

The reduction in the number of processes was accentuated in the *PKCi/GAL4* cultures following heat shock, suggesting that the high level of expression of GAL4 is the cause. However, it should be noted that the increased expression of GAL4 protein after heat shock in the GAL/w^z cultures resulted in a toxic effect on myotubes, possibly via non-specific binding of the GAL4 transcription factor to DNA due to the high levels of the protein following heat shock. The more dramatic effect on cell clusters observed may be due to the partial disappearance of myotubes which serve as a site of attachment for processes and clusters. Table IV summarizes the data on the effect of heat shock on the transgenic embryos. Notice that the morphotype of *tur/tur* is similar to that of *PKCi/GAL4* after heat shock with a dramatic reduction in the number of type 1 and 2 processes. The morphotype of *PKCi/GAL4* without heat shock, although still significant, is less dramatic, which correlates with a low level of expression of the pseudosubstrate inhibitor due to the leaky GAL4 promoter.

DISCUSSION

The only early gastrula stage embryonic precursor cells that can undergo division and differentiation in culture are those that differentiate into morphologically defined neurons or myotubes [Salvatera et al., 1987, and us, data not shown]. In our system, the expression of the PKC pseudosubstrate inhibitor allows us to examine the effect of a partial inhibition of kinase activity over a 24 h period, during which time the neuroblasts mature into neurons and complex processes. This approach avoids the use of chemical activators or inhibitors of kinases that are used for short-term experiments such as studies of the regulatory mechanisms of channels [Knox et al., 1992]. Drugs, such as TPA, are only useful for such short-term experiments because of the down-regulation of PKC that follows its activation. This contradictory action makes the analysis of the effect of these drugs on neuronal growth over a 24 h period difficult. Furthermore, there is the possibility that chemical inhibitors, such as H-7, may interact non-specifically because the Ki's for PKC, cAMP kinase, and cGMP kinase are all in the μM range in vitro [Bixby, 1989]. Such chemicals also interact with ATP binding sites, raising the possibility of an interaction with ATP-ase molecules such as the cytoskeletal proteins actin and tubulin, which has not, to our knowledge, been investigated. Another problem that may occur with

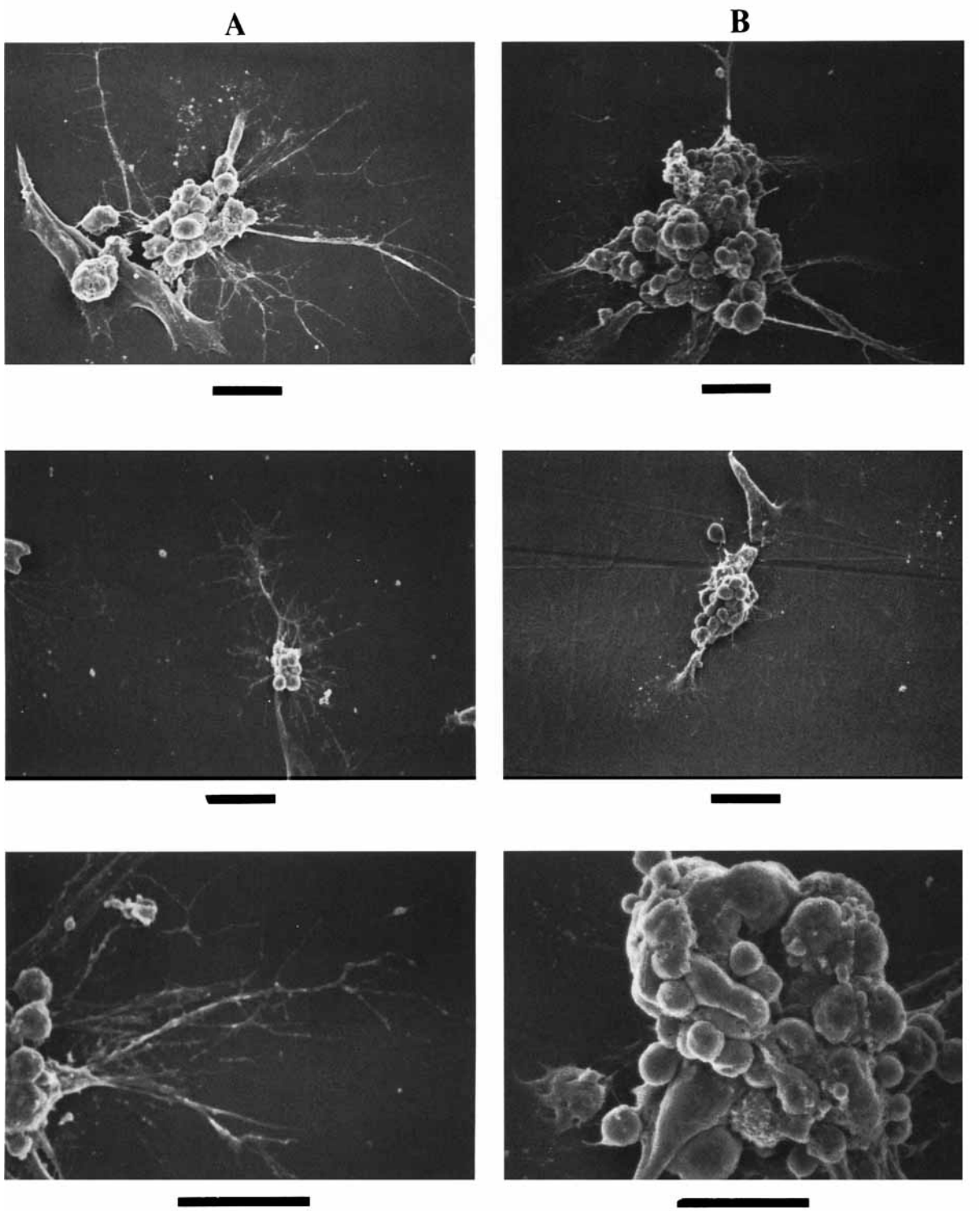

Fig. 8. Typical morphology of w^z control (*column A*) and *PKCi/GAL4* (*column B*) cultures. One hundred embryos at early gastrula stage were homogenized and seeded on a Petri dish as described in Materials and Methods. The detailed analysis of these clusters is presented in Tables III and IV. Only myoblasts and neuroblasts adhere and differentiate on plastic. A myotube (flat, elongated cell) can be seen in the top micrograph in column A. Neuroblasts differentiate and divide to form cell clusters which include the original neuroblast (not always present), intermediate ganglion mother cells (GMCs) which are >5 μm in size, and mature neurons which are spherical and about 3 μm in diameter. Two types of processes were observed. Type I are thick, dense with few varicosities. Type II are thin and beaded,and are highly branched. Black bars represent 10 μm.

TABLE II. Morphological Composition of Non-Heat Shocked Cultured Neuronal Cell Clusters*

Genotype	% of clusters with neuroblast	Mean number of GMCs per cluster	Mean number of mature neurons per cluster	Mean GMC:mature neuron ratio
GAL4/w²	70	3.5 ± 1.5	12.8 ± 4	0.3
PKCi/GAL4	75	2.9 ± 1.28	14 ± 4.7	0.25
PKCi/w²	75	2.7 ± 1.95	12 ± 3.6	0.27
w²	75	3.3 ± 1.58	12.5 ± 3.2	0.28
turnip/FM7	70	2.8 ± 1.37	11.5 ± 5	0.32
turnip/turnip	96	5.2 ± 2.5	8.5 ± 4.5	0.8

*Cells were identified by expression of a HRP-like antigen, directly visible on the scanning electron microscope by gold particle labeling. For each genotype, 15 clusters from 3 different cultures were analyzed. This table shows the mean number of each cell type per cluster ± standard deviation.

TABLE III. Analysis of Process Number and Morphology for Each Genotype*

	Type I processes		Type II processes		
Genotype	Total number per cluster	Number per cluster with type II terminus	Total number per cluster	Number per cluster that initiate from a type I process	Number per cluster that initiate from the cell cluster
GAL4/w²	10.4 ± 5.4	4.3 ± 2	9.43 ± 5.7	6.6 ± 3.5	3 ± 2.7
PKCi/GAL4					
No heat shock	3 ± 1.8	1 ± 1.1	4.4 ± 3	0.7 ± 1.1	4 ± 2.6
Heat shock	2 ± 1	(N.D.)	1.3 ± 1.3	(N.D.)	(N.D.)
PKCi/w²					
No heat shock	6.7 ± 3.8	3.3 ± 2	10.3 ± 6.3	6.2 ± 4	4.3 ± 3
Heat shock	7 ± 3.1	(N.D.)	9.5 ± 4.2	(N.D.)	(N.D.)
turnip/FM7	5.5 ± 2.5	(N.D.)	5.5 ± 2.6	(N.D.)	(N.D.)
turnip/turnip	3.3 ± 2.1	2 ± 1.2	2.9 ± 1.9	1.5 ± 1.1	1.4 ± 1.1
w²	9.3 ± 3	6.8 ± 2.5	10.3 ± 6.2	6.8 ± 3.5	3.6 ± 3.7

*The structures were identified by gold particle labeling using anti-HRP antibody. As in Table II, 15 clusters for each genotype were analyzed and the table shows the number of processes ± standard deviation.

chemicals is that they may become "trapped" in the cell membrane of processes, so modifying the fluidity of the lipid bilayer.

Without heat shock, the results obtained from the *PKCi/GAL4* flies showed that a very low concentration of GAL4 protein may trigger substantial synthesis of the pseudosubstrate inhibitor. This production of the GAL4 protein is due to the nature of the heat shock promoter which is often leaky. However, without the presence of GAL4 protein, no detectable expression of inhibitory peptide occurs. Therefore, one of the advantages of using the GAL4/UAS system is that there is no basal expression of the pseudosubstrate inhibitor in the parental strains which could conceivably have some effect on egg development. Also, the progeny of the cross between flies homozygous for hspGAL4 and flies homozygous for UAS-PKC inhibitor are all heterozygous and so any insertional effects of the P elements can be discounted.

To address the question of the identity of cell types in culture, we used a useful neuronal cell type specific antigen that is recognized by antibodies raised against HRP [Katz et al., 1988, Jan and Jan, 1982]. We have confirmed that this antigen is highly represented in mature neurons and processes, absent in neuroblasts, and only weakly present on intermediate cells. We observed that heavily beaded type II processes only weakly recognized the antibody. This point is interesting because type II processes were seen to extend out from the type I process along its length or to form a highly arborized terminus to the untargeted type I process (in which case the type II terminus may actually represent the filopodia of the growth cone). We also observed that type II beaded branches most often ran on the plastic, unconnected or untargeted to cell clusters or myotubes. Type I processes that were seen to connect two cell clusters or one cluster and one myotube (and were possibly undergoing

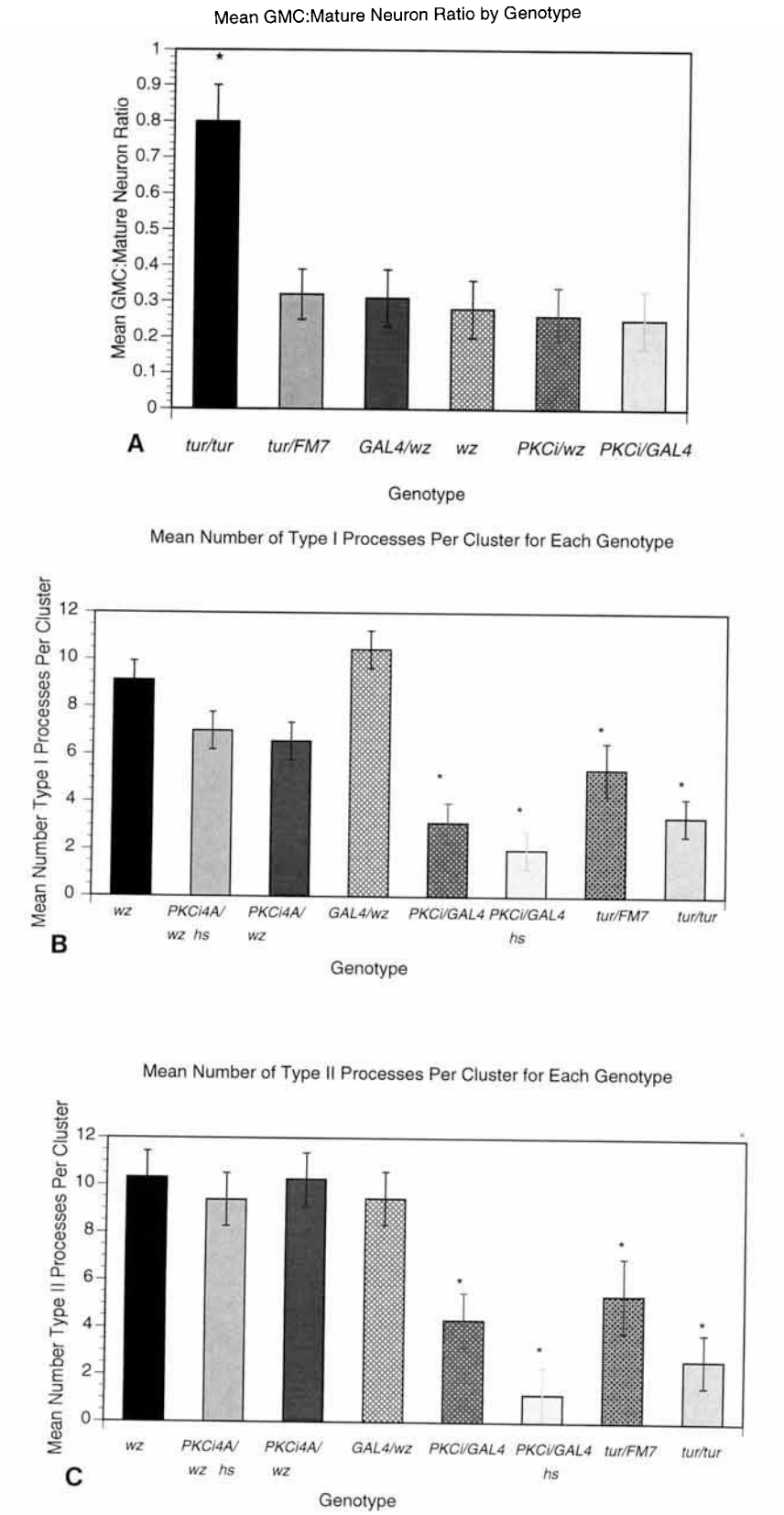

Fig. 9. Statistical analysis. Data for all genotypes were tested for normal distribution using a Shapiro-Wilk W test. Comparisons with the w^z control were performed using Dunnet's method. Genotypes that differed significantly ($P < 0.05$) from the control are marked by an asterisk and error bars represent SEM. **A:** Statistical analysis comparing the mean of the GMC:mature neuron ratio for each genotype with the w^z control. **B:** Statistical analysis comparing the number of type I processes per cluster for each genotype with the w^z control. **C:** Statistical analysis comparing the number of type II processes per cluster for each genotype with the w^z control.

TABLE IV. The Effect on Cell Culture Morphology of Heat Shocking Transgenic Embryos*

	PKCi4A/GAL	*PKCi2A*/GAL	*GAL4*/w^z	*PKCi4A*/w^z	*PKCi2A*/w^z
Reduction in the number of type I processes	+++	+++	+	+/−	+/−
Reduction of the number of type II processes	+++	+++	+	+/−	+/−
Increase in the GMC:mature neuron ratio	++	++	−	−	−
Reduction of the number and size of myotubes	++	++	++	−	−

*Embryos were heat shocked at 37°C for 30 min and allowed to recover for 4 h at 25°C before grinding for culture.

or had undergone synaptogenesis) labelled more heavily for anti-HRP. These data raise questions regarding the timing of molecular trafficking in the processes before and after functional synapses have been created on a specific target. Another interesting question raised by our data concerns the ability of type I processes to navigate to their targets in our in vitro system. This phenomenon occurred at a very low density of cells, making the distance between two connected cell clusters quite long, suggesting that secretion of active soluble factors by the differentiated cell clusters may be involved in guidance. Such diffusible protein gradients produced by targets towards receptive neurons have been described elsewhere [Zheng et al., 1994]. In addition, NGF through the activation of its tyrosine kinase receptor, has been shown to activate PKC [Hama et al., 1986].

Our results showed that inhibition of PKC inhibits neurite growth of *D. melanogaster* neurons in vitro, which is in agreement with the reported relationship between activation of PKC and neurite outgrowth in chick ciliary ganglion neurons [Bixby, 1989]. This inhibition or stimulation of neurite outgrowth may, of course, be mediated by more than one of the many substrates of PKC in the cell, and the effect of a partial inhibition of PKC on such substrates will depend on the sensitivity of the particular mechanism involved. It should, of course, be noted that the potential pleiotropic effects of partially inhibiting PKC may involve such mechanisms as the regulation of channels and the electric properties of the membrane which could indirectly affect process growth. A role for PKC in endocytosis has been suggested in *Drosophila* from studies of a mutant, *Shibire* [Kim and Wu, 1987]. *Shibire* has a temperature-sensitive mutation in dynamin, which is a microtubule binding protein with GTPase activity that is essential for endocytosis in neurons and other cells. PKC phosphorylates dynamin, increasing its GTPase activity 12-fold, and excitation of the cell dephosphorylates it. It has been suggested that such regulation of dynamin causes synaptic vesicles to be rapidly endocytosed following exocytosis [Robinson et al., 1993]. When placed at the restrictive temperature, adult *Shibire* flies are paralyzed due to the disruption of synaptic transmission that may be caused by altered membrane fusion and retrieval. In culture, Kim and Wu [1987] showed that developing *Shibire* larval CNS neurons showed reduced neurite growth and arborization, and state that such changes are correlated with the adhesive properties of the cell membrane. *Shibire* neuron cell bodies and neurites at the restrictive temperature showed decreased adhesion to the substratum, and growth cones showed altered morphology with no lamelipodia. In addition, PKC has been implicated in long-term potentiation observed in the hyppocampus [for review see Bourne and Nicoll, 1993], and has been shown to promote calcium influx, which is associated with a change in the morphology of processes characterized by an extension of lamellipodium [Knox et al., 1992]. These studies suggest that one role of PKC that may be similar in neurite elongation and synaptic plasticity is the activation of dynamin, which allows membrane recycling, and, in turn, the internalization of soluble factors bound to their receptors and the redistribution of growth cone adhesive contacts to the substratum. PKC may, therefore, play roles in synaptic rearrangement, involving the loss of many initial synapses and the formation of new ones, in the adult [Purves et al., 1986], that are similar to its roles in neurite elongation during development.

One of the primary substrates for PKC in the growth cone, GAP 43, is at its highest level in

embryonic neurons, suggesting that both molecules are crucial to neuronal development [Baetge and Hammang, 1991]. Extracellular matrix proteins have been reported to promote neurite growth by a mechanism involving the activation of PKC [Bixby, 1989]. Laminin promotes growth in a concentration-dependent manner, and collagen and fibronectin stimulate PKC to a lesser extent than laminin, which correlates with their less efficient promotion of neurite growth. PKC has also been shown to play a major role in the regulation of the cytoskeleton by acting on a number of different substrates, including MAP2 [Williams et al., 1994, Tang et al., 1993, Cissielski et al., 1991].

Our work suggests that activation of PKC is a mandatory step in neurite elongation, which contrasts with our findings that suggest a role for CaM kinase in neurite trimming [Wang et al. 1994, Broughton et al., submitted]. In the future, we will investigate the molecular differences between type I and II processes in our in vitro system using similar genetic tools, since these morphological differences in processes have been reported in vivo on the segmental muscle of third instar larvae [Littleton et al., 1993, Johansen et al., 1989].

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