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TEMPORAL CHANGES IN INTRACELLULAR DISTRIBUTION OF PROTEIN KINASE C IN SWISS 3T3 CELLS DURING MITOGENIC STIMULATION WITH INSULIN-LIKE GROWTH FACTOR I AND BOMBESIN:TRANSLOCATION TO THE NUCLEUS FOLLOWS RAPID CHANGES IN NUCLEAR POLYPHOSPHOINOSITIDES

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Using a polyclonal antibody raised against a synthetic peptide of the catalytic region of protein kinase C, we have carried out a combined immunocytochemical and immunochemical analysis to follow the subcellular localisation of this enzyme in response to mitogenic stimulation with insulin-like growth factor I and bombesin. These investigations show a time dependent translocation of protein kinase C from the cytoplasm to the nucleus since 5 min stimulation reaching a maximal effect after 45 min. These results show clearly that mitogen induced translocation of protein kinase C to the nucleus follows temporally the earlier changes in nuclear polyphosphoinositide metabolism previously demonstrated.

Recent findings suggest the existence of a nuclear phosphoinositide signalling system based on evidence that highly purified nuclei synthesize inositol lipids *in vitro* and that their metabolism changes during erythroid differentiation of Friend cells and when Swiss 3T3 fibroblasts are stimulated with growth fac-

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ABBREVIATIONS ARE: PtdInsP, phosphatidylinositol 4-phosphate; PtdInsP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; IGF-I, insulin-like growth factor I; InsP3, inositol (1,4,5)-trisphosphate; PDGF, platelet-derived growth factor; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; NGS, normal goat serum; BSA, bovine serum albumin; PS, phosphatidylserine; DAG, diacylglycerol; PMSF, phenylmethylsulfonylfluoride; EDTA, (ethylenedinitrilo) tetraacetic acid.

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tors (1-3). The latter studies showed that changes in nuclear PtdInsP and PtdInsP2 levels are determined by IGF-I, acting at the plasma membrane through a tyrosine-kinase receptor (4,5) and synergising with bombesin, so that the events generated at the nucleus are quite distinct from the signalling pathway evoked at the cell surface.The same mitogenic stimulation gives rise to a dramatic increase of nuclear PKC activity within 45 min (6).This observation was supported by evidence that calcium free InsP3 stimulates PKC in nuclei isolated from mitogen-treated 3T3 cells thus raising the possibility that InsP3 itself is capable of liberating calcium ions from a putative nuclear calcium store (7). A crucial question is whether IGF-I and bombesin at mitogenic concentrations activate PKC molecules already localised at nuclear level or induce PKC translocation from the cytoplasm to the nucleus. This latter possibility is supported by the evidence that phorbol ester mediates the association of PKC to the nucleus in several cell types (8-10) and by an immunochemical analysis showing a PDGF-mediated translocation of PKC to the nuclear envelope (11). Employing an anti PKC antibody in a combined immunocytochemical and immunochemical analysis we have shown a time dependent PKC translocation to the nucleus following the earlier breakdown of PtdInsP and PtdInsP2.

MATERIALS AND METHODS

Cell culture: Swiss 3T3 mouse fibroblasts were grown to confluency and then stimulated with IGF-I (20 nanogram/ml) and bombesin (1 nanomolar) as previously described (6,7).
Isolation of nuclei, partial purification of PKC and protein assay: all the procedures were carried out exactly as previously described (6,7).

Anti-PKC polyclonal antibody preparation: The antibody was prepared by injecting rabbits with the synthetic peptide CYVNPQFVHPILQSAV derived from the C-terminal sequence of protein kinase C as described by Parker et al. (12). The peptide was conjugated to purified protein derivative of tubercolin and administered as described by Lachmann et al. (13). Serum was stored at -70° C until use.

Immunocytochemical analysis: confluent cells, grown on coverslips, were fixed in 4% paraformaldehyde/PBS for 30 min at room temperature. Cells were then permeabilized in 0.2% Triton X-100 for 10 min. Aspecific binding was blocked with 3% NGS, 2% BSA dissolved in PBS. Fibroblasts were incubated with anti PKC polyclonal serum diluted 1:25 in 2% BSA, 3% NGS in PBS for 3 hrs at 37°C.Coverslips were rinsed with PBS and incubated with an alkaline phosphatase conjugated goat anti rabbit IgG diluted 1:300 in 2% BSA, 3% NGS in PBS, for 1 hr at 37° C. After washing with PBS, they were further rinsed and reacted with 200 mM Tris-

HCl pH 9.6, 10 mM $MgCl_2$, 2 mM levamisole, containing 0.33 mg/ml p-nitro-blue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate. The reaction was allowed to proceed for 15-60 min at 37° C and was stopped by transferring the coverslips to PBS containing 20 mM EDTA. After several rinses in PBS, coverslips were air dried and mounted in Aquamount (BDH,Poole, England).Isolated nuclei were cytocentrifuged at 250 x g for 7 min on glass microscopy slides coated with poly-L-lisine (14). Slides were air dried overnight and then nuclei were fixed in 4% paraformaldehyde, 50 mM Tris HCl pH 7.4, 5 mM $MgCl_2$, 0.5 mM PMSF. After 30 min fixation at room temperature, nuclei were treated for immunochemical staining as described above.

Immunoblotting analysis: proteins from whole cells and purified nuclei were separated by 8% SDS-PAGE and then trasferred to nitrocellulose paper (15).Binding sites were saturated by incubating nitrocellulose for 1 hr at 37° C in PBS, 3% BSA. Incubation with the primary antibody (1:50 dilution) was for 7 hr at room temperature in PBS, 0.1% BSA. After 4 washes in PBS, 0.1% Tween 20, nitrocellulose sheets were reacted for 2 hr at room temperature with a 1:500 dilution of an alkaline phosphatase conjugated goat anti rabbit IgG in PBS, 0.1% BSA, 0.1% Tween 20. Nitrocellulose sheets were washed as above in PBS-Tween 20 and antibody binding was detected as described above.

RESULTS

Fig.1 shows the redistribution of PKC during IGF-I and bombesin stimulation of quiescent 3T3 cells. It is worth mentioning that rabbit preimmune serum does not produce any appreciable staining. Unstimulated cells have a uniform distribution of PKC through the cytoplasm, but after 5 min stimulation the immunostaining begins to concentrate around the nucleus. After 15 min PKC translocation to the nucleus is more evident and a discrete staining is also visible inside the nucleus itself. At the same time it is possible to observe an intense immunostaining at the plasma membrane. At 45 min PKC is almost entirely translocated to the nucleus, which now appears to be uniformly stained in a large number of cells. In order to better define the localisation we decided to carry out the same immunocytochemical analysis on isolated nuclei. Because nuclear localisation of the PKC reactivity *in situ* appears to be most obvious at 45 min stimulation nuclei from cells treated to that extent have been used. The highly pure nuclei, stripped of their outer envelope, were judged to be free of cytoplasmic markers according to criteria described previously (1,2,6,7). While the preimmune serum does not show aspecific immunostaining and nuclei from unstimulated cells display only a faint reactivity, at 45 min stimulation isolated nuclei have an intense ring shape staining at the level of the nuclear periphery as well as a diffuse immunoreactivity in the

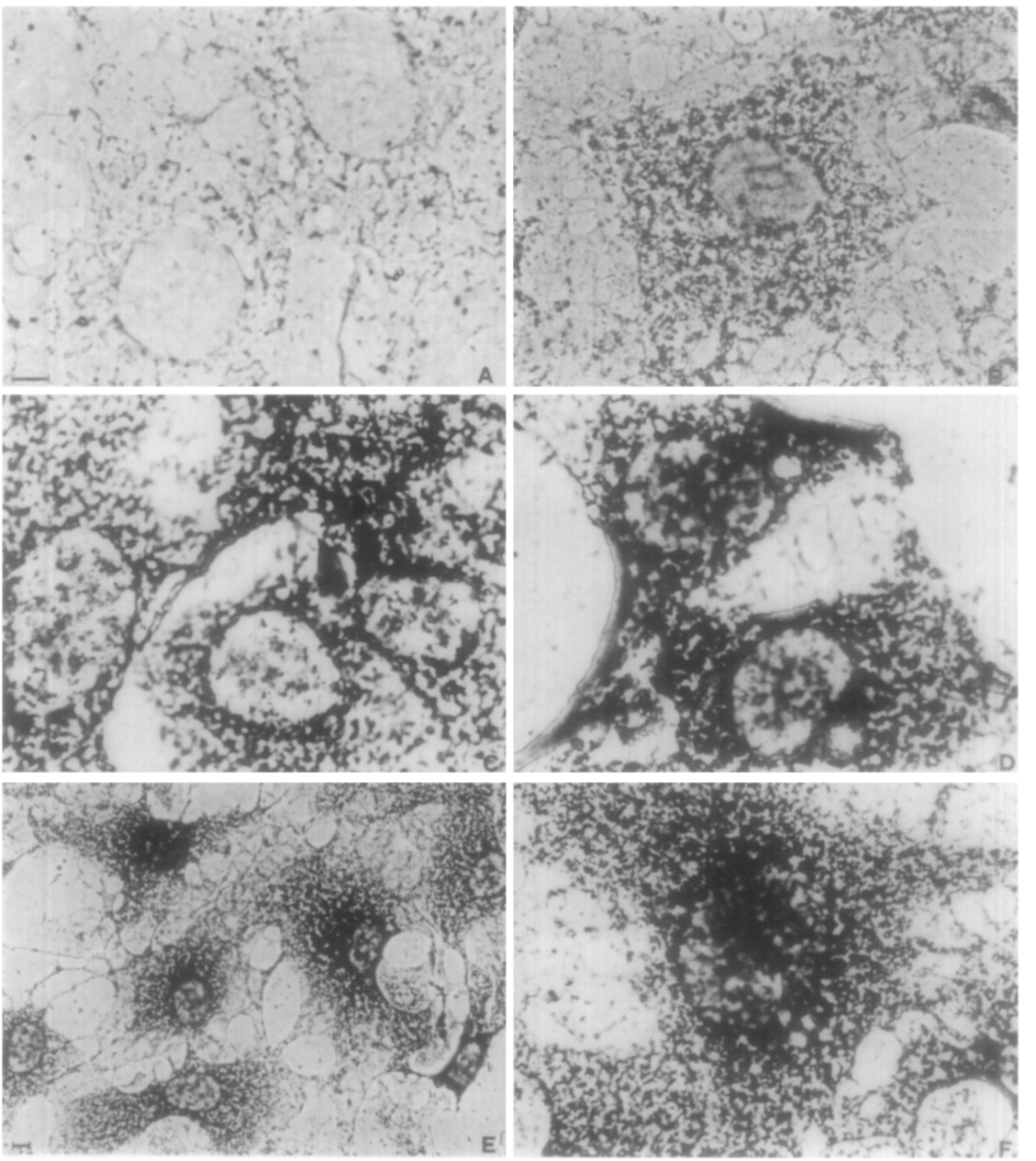

Fig. 1. Immunocytochemical analysis of in situ Swiss 3T3 fibroblasts showing PKC translocation to the nucleus during stimulation with IGF-I and bombesin. A, cells at 45 min stimulation incubated with rabbit preimmune serum; B, C, D, F, cells at 0, 5, 15, 45 min of stimulation respectively incubated with anti PKC antibody.E is the same as in F at a lower magnification. Bar: 5 μm.

inside (Fig.2). Western Blots of nuclear proteins (Fig.3) clearly show PKC translocation to the nucleus after 45 min stimulation . In addition to a major staining band at approximately 80 kDa corresponding to native PKC a second band is detected at 50

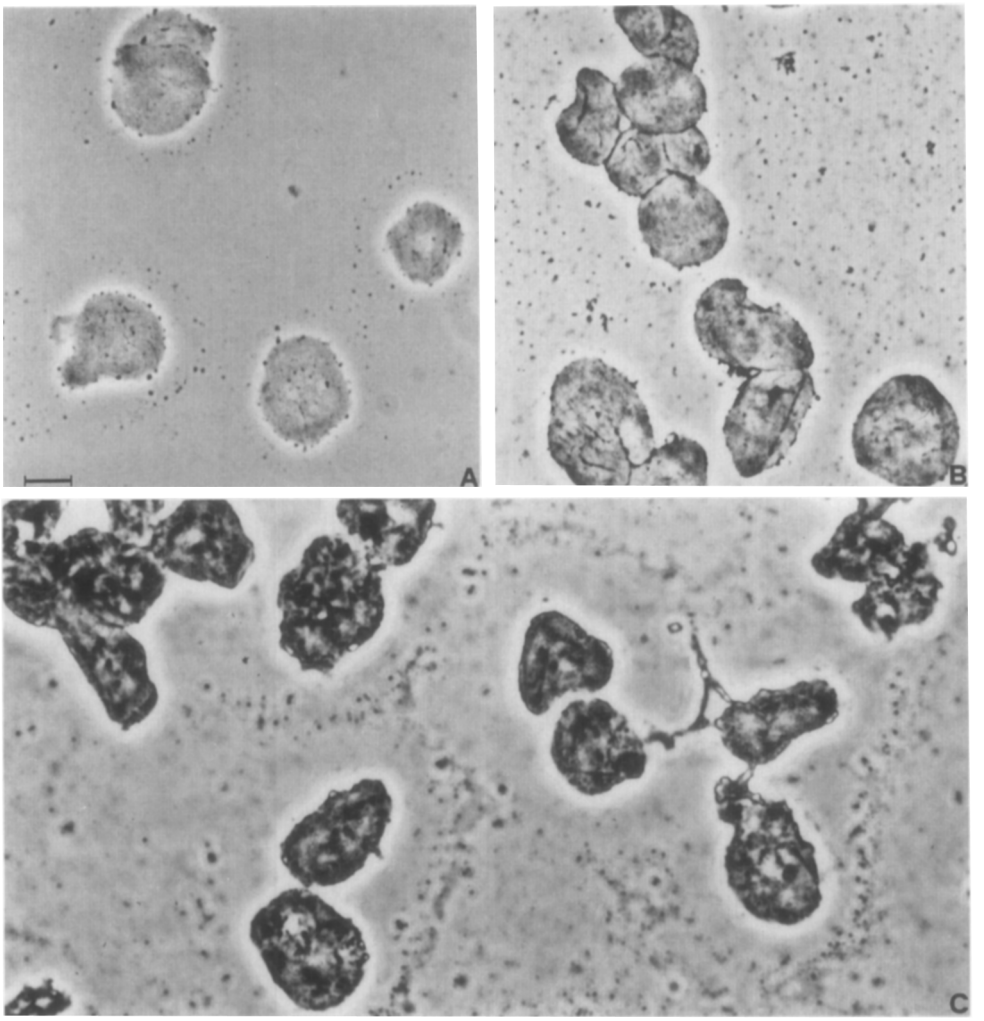

Fig. 2. Immunocytochemical analysis of isolated nuclei from Swiss 3T3 cells quiescent (B) and stimulated with IGF-I and bombesin for 45 min (A,C). A, nuclei incubated with rabbit preimmune antiserum. B and C nuclei incubated with anti PKC antibody. Bar: 5 μm.

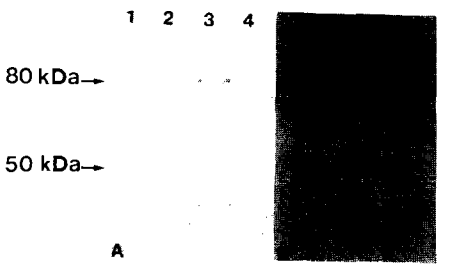

Fig. 3. Panel A: immunoblotting of nuclear proteins from control and IGF-I/bombesin stimulated 3T3 cells using the anti PKC antibody. Lane 1, partially purified PKC; lane 2, nuclear proteins from unstimulated cells; lane 3, nuclear proteins from 45 min stimulated cells; lane 4, nuclear proteins from the same cells incubated with rabbit preimmune serum.Panel B: corresponding Coomassie stained gel. 20 μg of protein was loaded for each lane.

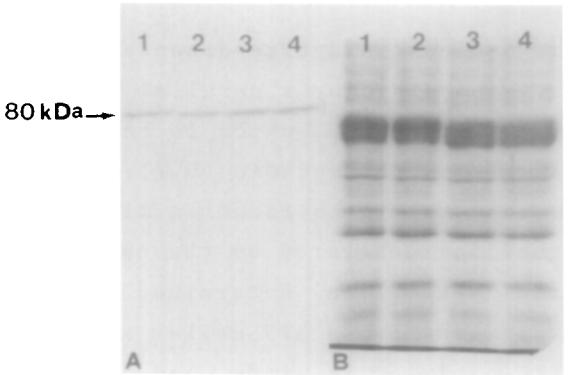

Fig. 4. Panel A: total cellular proteins of quiescent and IGF-I/bombesin stimulated 3T3 fibroblasts were subjected to immunoblotting using the anti PKC antibody. Lane 1, quiescent cells; lane 2, cells stimulated for 5 min; lane 3, cells stimulated for 15 min; lane 4 cells stimulated for 45 min. Panel B: corresponding Coomassie stained gel. 80 μg of protein was loaded for each lane.

kDa, presumably corresponding to the proteolytically derived truncated PKC (PKM) because the antiserum used recognizes the carboxy-terminal region (catalytic domain) of the protein. To establish whether the mitogenic stimulus induced only translocation or *de novo* synthesis of PKC we have subjected to immunoblot analysis total proteins from control and stimulated intact cells. Fig.4 shows that the amount of total cellular PKC remains constant through the entire time course of the stimulation.

DISCUSSION

The data described here, for the action of IGF-I in concert with bombesin, raise the question of whether PKC translocation to the nucleus might be part of the mechanism by which these two peptides stimulate cell division in Swiss 3T3 fibroblasts. Previously it has been shown that IGF-I on its own or in association with bombesin acts as an agonist that affects nuclear, but not cytoplasmic, inositide metabolism (2,3). Since this effect takes place after 2 min stimulation it precedes temporally the PKC translocation to the nucleus described here.

Although it is well documented that phorbol ester mediates the translocation of PKC to the nuclear region (8,9) and that PDGF stimulates the association of this kinase, as determined by immunoblot analysis, to the nuclear envelope (11), this is, to our knowledge, the first evidence, obtained by means of a coupled immunocytochemical and immunochemical analysis of both intact cells and purified nuclei, that a mitogenic stimulus induces translocation of native PKC and to a lesser extent of its trun-

cated form to the nucleus in a time dependent way. Moreover the translocation is accompained by a parallel increase of the biochemical activity in vitro, which is totally dependent on the presence of calcium ions, PS and DAG (6,7). Thus it seems reasonable to propose that this translocation might be the downstream stage of the signalling generated by the changes of nuclear polyphosphoinositide metabolism determined by IGF-I and bombesin stimulation of quiescent Swiss 3T3 cells. Recent reports showing stimulation of PKC activity in vitro and changes in nuclear calcium induced by InsP3 (7,16) point to PKC as a target at the nuclear level too for the second messengers generated by polyphosphoinositide hydrolysis, and strengthen previous biochemical and immunochemical evidences concerning the nuclear localisation of this enzyme (6,8,9,17). Moreover as PKC activation is generally considered to be dependent on binding to DAG (18) it is worth mentioning that changes in nuclear DAG are associated with changes in nuclear polyphosphoinositides during erythroid differentiation of Friend cells and after treatment of Daudi cells with interferon (19,20). The observation that a mitogenic response caused by IGF-I and bombesin correlates both with a nuclear inositide signalling system and a subsequent translocation of PKC to the nucleus offers a novel insight into the early nuclear events which lead to the onset of DNA synthesis.

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