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CALCIUM FREE INOSITOL (1,4,5)-TRISPHOSPHATE STIMULATES PROTEIN KINASE C DEPENDENT PROTEIN PHOSPHORYLATION IN NUCLEI ISOLATED FROM MITOGEN-TREATED SWISS 3T3 CELLS

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As a step towards the elucidation of the role played by nuclear polyphosphoinositides, we have investigated the effect of exogenous calcium free inositol (1,4,5)-trisphosphate on the in vitro phosphorylation of proteins in nuclei prepared from Swiss 3T3 cells treated with bombesin and insulin-like growth factor I. When present in combination with phosphatidylserine , inositol (1,4,5)-trisphosphate enhanced the phosphorylation of two nuclear proteins, Mr 21,000 and 31,000, as well as of exogenous histone H1, to the same extent as a combination of phosphatidylserine and diacylglycerol . Inositol (1,4,5)-trisphosphate alone had no effect. This stimulation could be abolished by the protein kinase C inhibitor sphingosine and by EGTA, while could be restored by a combination of phosphatidylserine and exogenous Ca++ ions.These results raise the possibility that inositol (1,4,5)-trisphosphate is capable of liberating Ca++ ions from a nuclear store thus stimulating protein kinase C activity.

The response of cells to mitogenic signals requires a highly coordinated series of events that can be stimulated by the specific binding of a growth factor to its receptor on the plasma membrane. Some information is available on the very early steps of the response in which there is a rapid and transient appearance of cytoplasmic second messengers such as IP_3 and DAG from the hy-

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ABBREVIATIONS ARE: IP_3: inositol (1,4,5)-trisphosphate; IGF-I:insulin-like growth factor I; PS: phosphatidylserine; DAG: diacylglycerol; PKC: protein kinase C; EGTA: [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; D-MEM: Dulbecco's modified minimum essential medium; IP_2: inositol (1,4)-bisphosphate; IP_4: inositol (1,3,4,5)-tetrakisphosphate; Sph: sphingosine.

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drolisis of phosphatidylinositol (4,5)-bisphosphate (1,2). However, the mechanisms by which the mitogenic stimulus is eventually transmitted to the cell nucleus is completely unknown. We have recently provided evidence that treatment of quiescent 3T3 cells with a mitogenic combination of bombesin and IGF-I also results in a rapid and transient stimulation of nuclear polyphosphoinositide metabolism (3,4). We have further demonstrated that following these early changes a nuclear PKC activity appears, which is capable of phosphorylating in vitro two proteins, Mr 21,000 and 31,000 in size, as well as exogenously added histone H1. We proposed that the appearance of this kinase activity could be somehow related to the availability at the nuclear level of molecules like DAG and IP_3 (5,6). Here we have addresssed the question of whether calcium free IP_3 can stimulate the in vitro phosphorylation of proteins in nuclei prepared from 3T3 cells treated with bombesin and IGF-I. We find that IP_3 enhances the phosphorylation of the same two proteins we had previously demonstrated to be substrates for nuclear PKC in Swiss 3T3 cells stimulated by bombesin and IGF-I.

MATERIALS AND METHODS

Cell culture: Swiss 3T3 mouse fibroblasts were grown in D-MEM supplemented with 10% newborn calf serum at 37°C in a humidified atmosphere containing 5% CO_2. After 6 days, when cells were confluent, they were washed twice with serum free D-MEM containing 1% bovine serum albumin and then incubated in the same medium for 45 min in the presence of bombesin (1 nanomolar) and IGF-I (20 nanogram/ml).

Isolation of nuclei: nuclei were isolated as previously described (5).

Purification of PKC: PKC was partially purified from rat brain according to Kikkawa et al. (7). The specific activity of the enzyme at the end of the purification was similar to that originally described.

In vitro phosphorylation of nuclear proteins: the reaction mixture (300 μl) contained: 20 mM Tris-HCl pH 7.3, 10 mM $MgCl_2$, 100 mM KCl, 45 mM 2-mercaptoethanol, 50 μM ATP, 2.5 μCi [^{32}P]-γ-ATP (5000 Ci/mmole, Amersham) and 100 μg of nuclear protein. When present, histone H1 (Sigma type III-S) was 125 μg, $CaCl_2$ was 0.1 or 1.0 mM, while PS and DAG were 75 and 4.5 μg, respectively. Sphingosine was equimolar with DAG. Other additions (i.e. Ca++ free IP_3, IP_2 and IP_4, kindly supplied by Dr. R.F. Irvine, AFRC Babraham, Cambridge, U.K.) were as indicated in the figure legends. Incubation was for 10 min at 30°C and was stopped by adding trichloroacetc acid to a final concentration of 25% (w/v). Samples were kept at 4°C overnight and protein was recovered by

centrifugation at 12,000 x g for 15 min. Pellets were washed twice with acetone, once with ethyl ether and air dried.

Other procedures: electrophoretic analysis, autoradiography and protein assay were as previously reported (5).

RESULTS

As described previously (5) the addition of PS and DAG to nuclei isolated from Swiss 3T3 cells stimulated with bombesin and IGF-I enhanced the in vitro phosphorylation of two proteins, Mr 21,000 and 31,000 (the latter identified as endogenous histone H1) (Fig. 1). If only PS was present, the enhancement of the phosphorylation level was almost negligible. However, if the reaction mixture contained both calcium free IP_3 and PS, the phosphorylation of the two bands was stimulated to the same levels obtained with the combination of PS and DAG. This phenomenon was observable in a concentration range from 20 to 50 μM, reaching the optimal stimulation at 50 μM, while lower amount of IP_3 (i.e. 2 μM) induced only a weak increase of the phosphorylation of the 21 kDa band. We next determined whether other inositol phosphates were able to enhance the phospholipid dependent phosphotransfera-

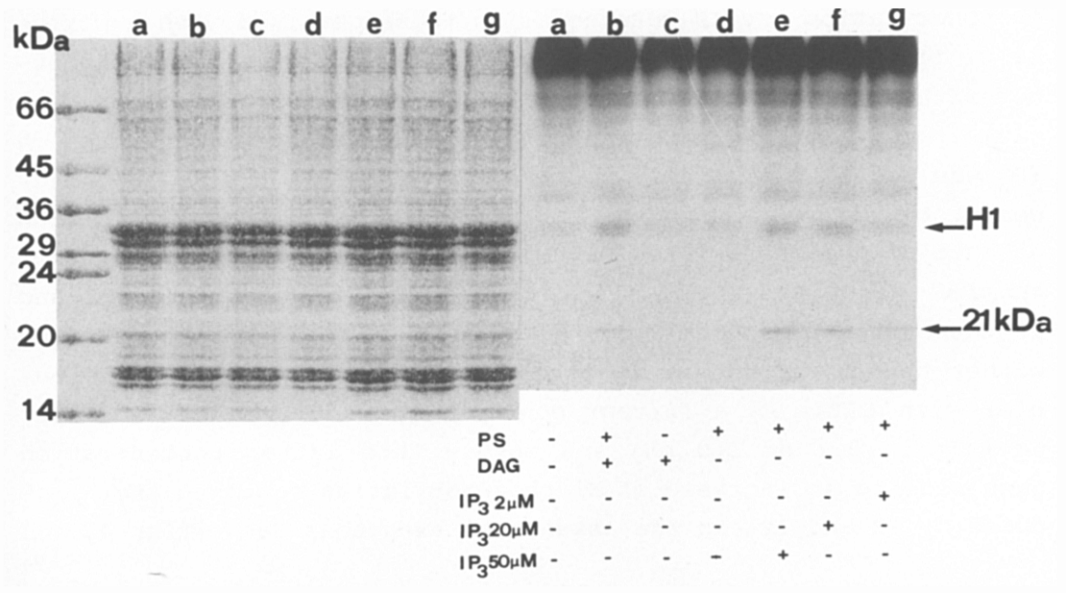

Fig.1. In vitro phosphorylation of purified nuclei from 45 min mitogen-stimulated 3T3 cells empolying endogenous substrates. Reactions were carried out in the absence of exogenous Ca++. Left panel is the Coomassie Blue staining. Right panel is the corresponding autoradiogram. The first lane on the left corresponds to molecular weight standards.

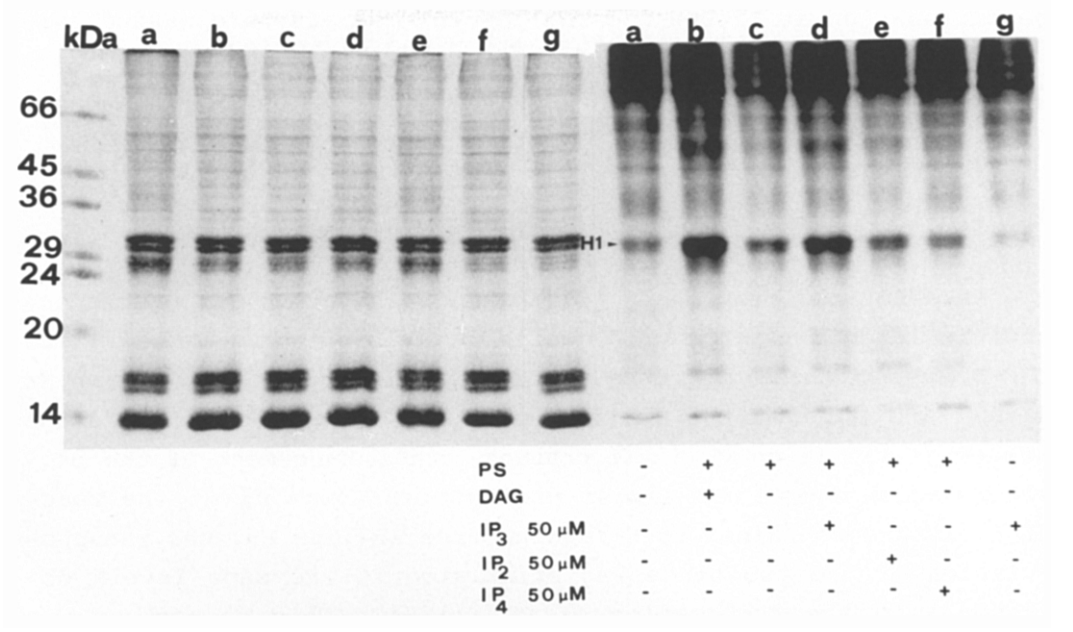

Fig.2. In vitro phosphorylation of purified nuclei from 45 min mitogen-stimulated 3T3 cells in the presence of histone H1 as exogenous substrate for PKC activity. Other specifications as in Fig.1 .

se activity. As a substrate for PKC we used exogenous histone H1 (8). In Fig. 2 we show that IP_3 was effective in enhancing the phosphorylation of histone H1 only in combination with PS. The phosphorylation levels were equal to those obtained with a mixture of PS and DAG. All of other inositol phosphates tested together with PS were ineffective. A direct stimulatory effect of calcium free IP_3 on PKC was also ruled out by an experiment in which IP_3 was added to partially purified PKC from rat brain: no enhancement of exogenous histone H1 phosphorylation was seen in the absence of cofactors which PKC activity depends on, i.e. PS, Ca++ and DAG (not shown). In Fig. 3 we demonstrate that the PS and IP_3 dependent phosphorylation of histone H1 could be abolished by either the PKC inhibitor sphingosine or by preincubating the nuclei with EGTA. As a further control, the addition of exogenous Ca++ ions (0.1 or 1.0 mM) and mostly this latter concentration gave rise to an increase of H1 phosphorylation equal to that produced by PS and IP_3 in the absence of exogenous Ca++ (Fig. 4).

DISCUSSION

Recently, attention has been focused on the presence of PKC in isolated nuclei (9-13). We have demonstrated that nuclei pu-

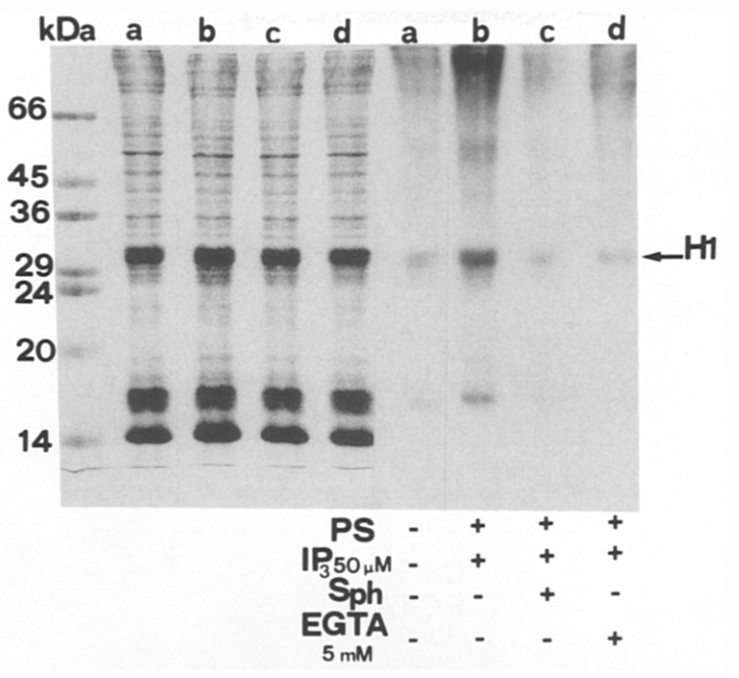

Fig. 3. In vitro phosphorylation of purified nuclei from 45 min mitogen-stimulated 3T3 cells employing histone H1 as exogenous substrate for PKC activity and in the presence of Sph and EGTA. Other specifications as in Fig.1 .

rified from Swiss 3T3 cells treated with a mitogenic combination of bombesin and IGF-I contain a PKC activity which selectively phosphorylates in vitro two proteins of Mr 21,000 and 31,000. The same proteins are hyperphosphorylated in vivo within 45 min of exposure of quiescent 3T3 cells to the same combination of mitogens (5). These events follow the earlier changes in nuclear polyphosphoinositide mass which accompany growth factor stimulation (3,4). Here we have addressed the question of whether IP_3 is capable of enhancing in vitro nuclear PKC activity by mechanisms analogous to those found in the cytoplasm. It is worth noting that in our system no exogenous Ca++ was required to demonstrate nuclear PKC activity. This suggest that the enzyme can utilize endogenous Ca++ stored within the nucleus (14,15). This suggestion is strenghtened by recent observations dealing with the presence of Ca++ binding proteins in nuclei and subnuclear fractions (16,17). The addition of both PS and IP_3 caused an impressive enhancement of the phosphorylation of the Mr 21,000 and 31,000 protein species as well as of exogenous histone H1. The requirement for the combination of these two molecules is demonstrated by the evidence that the stimulatory effect was not seen if PS and IP_3 were added separately. It is worth noting that al-

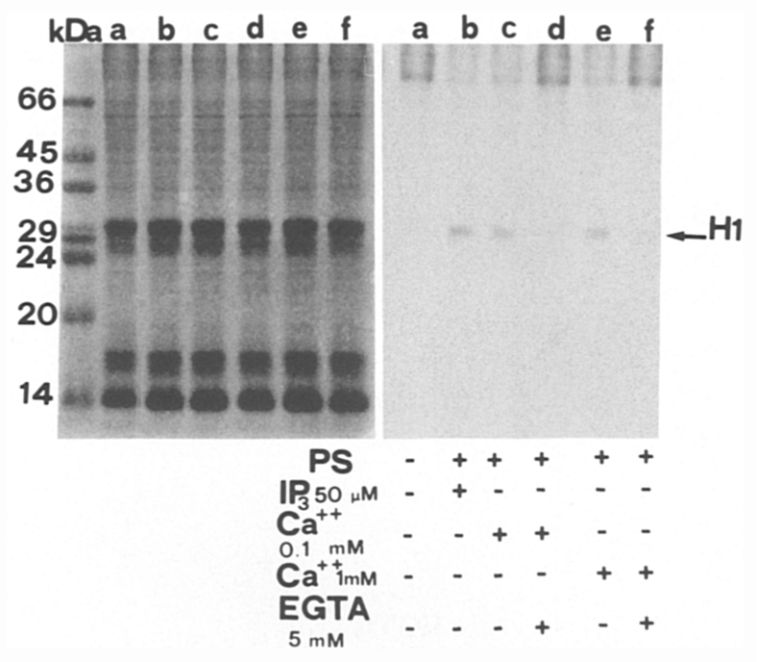

Fig.4 In vitro phosphorylation of purified nuclei from 45 min mitogen-stimulated 3T3 cells in the presence of histone H1 as exogenous substrate for PKC activity and of exogenous Ca++ and EGTA. Other specifications as in Fig.1 .

though the maximum stimulation was seen at 50 μM IP_3 , a weak increase over the control was observed at 2 μM, i.e. an IP_3 concentration not very different from that which is estimated to be present in the cells (18). Other inositol phosphates which are ineffective in liberating Ca++ from cytoplasmic intracellular stores (1) did not increase the phosphorylation of exogenous histone H1. The IP_3 dependent effect was inhibited by the PKC inhibitor sphingosine and by EGTA. The ability of EGTA to abolish the stimulation of PKC by IP_3 suggests a direct involvement of Ca++. It is of interest that the IP_3 receptor has been shown recently to be located also in the nuclear membrane of Purkinje cells (19). Therefore it is not unreasonable to propose that following the very early polyphosphoinositide breakdown caused in the nuclear compartment by bombesin and IGF-I, second messengers like DAG and IP_3 are produced and that these could act as stimulatory molecules for the activation of PKC in the nucleus. The reciprocal relationship between Ca++ and DAG in these preparations of isolated nuclei is not clear. It is possible that much of the endogenous nuclear DAG is lost on purification and this would explain the stimulatory effect of subsequent addition. If exogenous DAG is not present PKC activity is barely detectable. It is known

that DAG lowers the threshold of PKC for Ca++ (20); it is conceivable that IP_3, by liberating additional Ca++ from nuclear stores, could obviate the need for additional DAG in these preparations.

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