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RAPID COMMUNICATION

PHORBOL ESTERS AUGMENT SPERMIDINE TRANSPORT WITHOUT PROTEIN KINASE C ACTIVATION

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There are many recent studies dealing with the membrane transport systems as important targets of tumor promoting phorbol esters (1). It is widely assumed that phorbol esters modulate the transport systems via protein kinase C (PKC) activation (1-3). We have also recently observed that phorbol esters increased the spermidine transport in murine leukemia (L 1210) cells (4). Inhibition of both biosynthesis of polyamines and their uptake has been recently debated as important targets in cancer chemotherapy (5,6). In fact, some of the structural analogues of polyamines have been tested for their efficacy as inhibitors of polyamine uptake process and as anti-proliferative agents (5). H-7 (a PKC inhibitor), structurally unrealted to polyamines, has been found to inhibit the polyamine uptake (4). The present study was undertaken to elucidate whether phorbol ester stimulated polyamine uptake was PKC dependent. It was also thought worthwhile to test the effects of a more potent inhibitor of protein kinase C than H-7. These experiments were performed on *Xenopus laevis* oocytes which have recently been reported to possess polyamine transport pathways (7). We also used other synthetic diacylglycerols (DiC8 and OAG) which are known to mimic the effects of phorbol esters by activating the PKC in intact cells (8).

MATERIALS AND METHODS

Chemicals

^{14}C-Spermidine dihydrochloride (N - (3 - aminopropyl) - (1, 4 - ^{14}C) tetramethylene - 1, 4 - diamine trihydrochloride), Specific activity 100 mCi/mmol, 3.7 GBq/mmol) and ACS were obtained from Amersham, U.K. Soluene - 100 was purchased from Packard Ltd., The Netherlands. Phorbol ester (12-O-tetradecanoyl phorbol-13-acetate,TPA), synthetic diacylglycerols (*sn*-1,2-dioctanoglycerol, DiC8 and 1, 2 - oleoylacetylglycerol, OAG), and H-7 (1,5-isoquinolinyl-sulfonyl-2-methyl piperazine dihydrochloride) were purchased from Sigma, U.S.A. Protein kinase C pseudosubstrate (PKC-P) and anti-protein kinase C antibody were procured from Bachem Biochemicals, U.S.A.

Oocytes preparations

Ovaries of *Xenopus laevis* were removed from the female under anesthesia. The oocytes were treated with collagenase (20 mg/ml), and then the follicular layers around the oocytes were removed as described elsewhere (7). The oocytes were always stored in Barth's medium.The Barth's solution contained the followings : NaCl, 88 mM ; KCl, 1.0 mM ; $NaHCO_3$, 2.4 mM ; $CaCl_2$, 1.0 mM ; $MgCl_2$, 0.82 mM and Hepes, 5.0 mM.

^{14}C-Spd uptake assays

The oocytes were incubated for 90 min in the presence of ^{14}C-Spd (30 μM) as described elsewhere (7). The oocytes were washed with Barth's medium containing unlabelled Spd (1.0 M), and polyamines determinations were assayed by HPLC as described elsewhere (7). Briefly, 400 μl of ice cold 10 % $HClO_4$ was added to precipitate the protein. After shaking and centrifugation, supernatant was saved, and 100 μl of saturated aqueous Na_2CO_3 and 20 μl of dansyl chloride (5 mg/ml acetone) were added. After selective evaporation of acetone and extraction of dansylated polyamines, 100 μl of cyclohexane was added and subjected to further evaporation.The residues were redissolved in acetonitrile and subjected to HPLC. Measurement of radioactive Spd was carried out by entering the HPLC effluent into a simultaneous liquid scintillation beta detector for HPLC. Luma Flow III was used as scintillation fluid. Protein concentrations were determined by Lowry's standard method. All the experiments were carried out at 22±2°C.

Micro-injection of anti-PKC antibody or / and TPA

Oocytes were microinjected with 50 nl of the solution containing : anti-PKC antibody (monoclonal antibody raised against the catalytic subunit of PKC, 5 mg/ml) or TPA (7 mM) and just after microinjection (of TPA or / and anti-PKC antibody), oocytes were incubated in the presence of PKC-P (200 nM) or TPA (10 μM) for 90 min. The oocytes were washed, and Spd concentrations were determined by HPLC as described above. Stock solution of OAG (5 mM) was prepared in sterilized normal saline by evaporating the chloroform. Sonications of solutions improved the solubility of the compounds.

RESULTS AND DISCUSSION

Fig.1 shows that 1,2-oleoylacetylglycerol (OAG) and a protein kinase C pseudosubstrate, PKC-P (a pseudosubstrate corresponding to 19 - 36 residues from the regulatory domain of protein kinase C) respectively augmented and curtailed the uptake of ^{14}C-Spd in a dose dependent manner in Barth's medium. PKC-P has been reported to inhibit both autophosphorylation and protein substrate phosphorylation (9). Besides, PKC-P has been found to be more potent inhibitor of PKC activity than H-7 since the former exerted its effects in nanomolar range whereas the latter was effective in the micromolar range (9). In this study, TPA and DiC8 were used at 10 μM because this concentration was found to exert maximum stimulatory effects on PKC (4,10). The rationale of using DiC8, besides OAG, was that OAG bears a structural relationship to phorbol esters. Therefore, its effects on PKC could reflect this similarity with TPA rather than its structural relationship with *sn* -1, 2-diacylglycerols whereas DiC8 is a diacylglycerol with saturated acyl chains with 8 carbons (11). Fig. 2 (a) shows that 12-O-tetradecanoyl phorbol-13-acetate (TPA, phorbol myristyl acetate) and synthetic diacylglycerols (DiC8 and OAG) increased the Spd transport in oocytes.

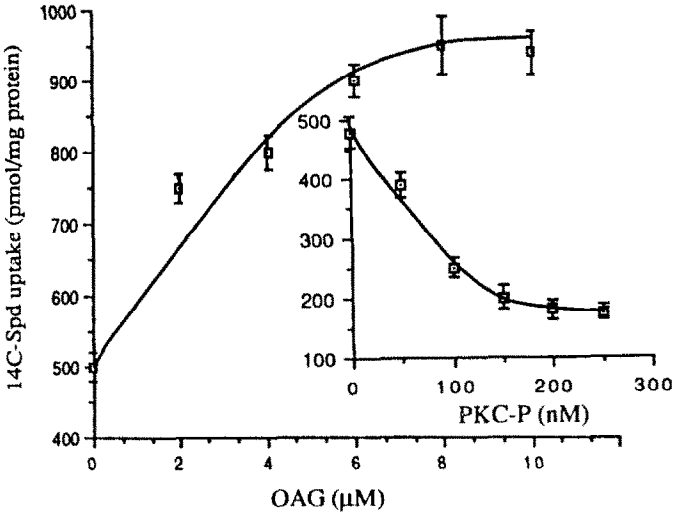

Fig.1. *Effects of different concentrations of OAG and PKC-P on Spd uptake by Xenopus laevis oocytes.* Fully grown defolliculated oocytes were incubated with increasing concentrations of OAG (0 - 10 μM) and PKC-P (0 - 250 nM) in the presence of ^{14}C-Spd (30 μM) for 90 min. The assays were terminated by washing with Barth's medium. Polyamine concentrations were determined as described in Materials and Methods.

Furthermore, PKC-P seems to be a more potent inhibitor of Spd uptake than H-7. The addition of TPA potentiated the Spd uptake in the oocytes receiving the exogenous diacylglycerols, and this indicates that TPA might be stimulating the Spd uptake process by other means than PKC. Therefore, to elucidate whether the target of TPA is PKC, we microinjected anti-PKC monoclonal antibodies and incubated simultaneously in the presence of PKC-P, and later, these oocytes were either injected or incubated in the presence of phorbol esters. Fig. 2 (b) shows that incubation of oocytes with TPA (receiving anti-PKC antibody + PKC-P incubation), but without microinjection, increased the Spd uptake. This effect of TPA cannot be attributed to PKC since we simultaneously are using anti-PKC antibody and a PKC inhibitor. To confirm this notion, we assayed the PKC activity, and observed that it was significantly (several fold) lower in the oocytes which received microinjection of anti-PKC antibody along with PKC - P and TPA incubation or TPA microinjection than the control oocytes (results are

not shown). These results corroborate to a very recent finding in which micromolar concentrations of phorbol esters exerted their effects by depressing calcium currents in chick sensory neurons independently of protein kinase C (11). This view has been recently supported in a recent commentary that some effects of phorbol esters are not mediated by PKC (12).

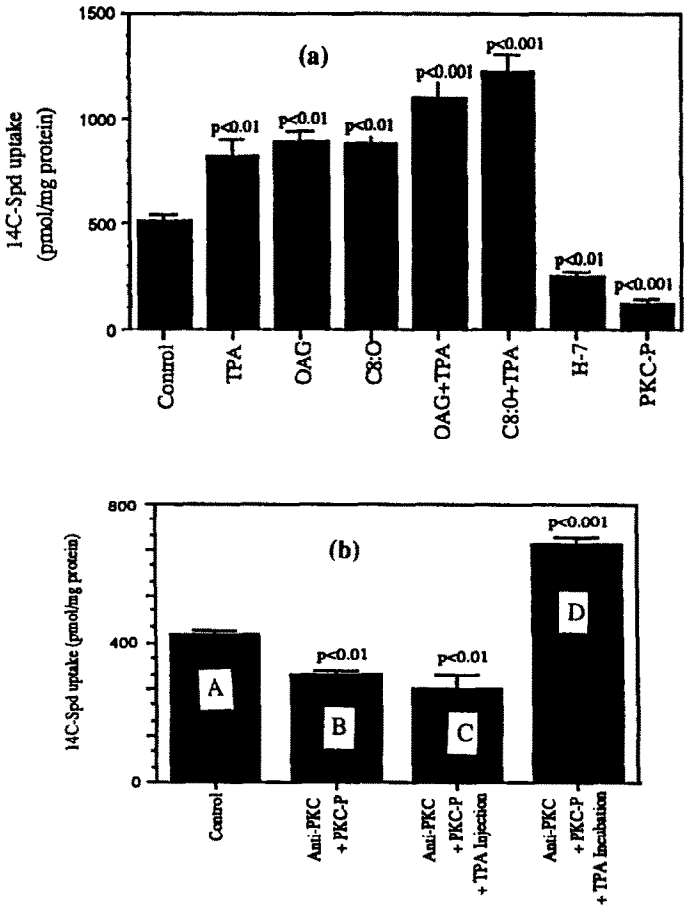

Fig.2. *Effects of PKC activators or inhibitors on Spd uptake by Xenopus laevis oocytes.* (a). Oocytes were incubated in Barth's medium in the absence (control) or presence of TPA, 10 μM; OAG, 10 μM; DiC_8, 10 μM; OAG+TPA, DiC_8+TPA, H-7, 50 μM or PKC-P, 200 nM, and ^{14}C-Spd (30 μM) for 90 min. (b). Oocytes were microinjected with 50 nl of the solution containing : IgG 5 mg/ml (A) ; anti-PKC antibody ; 5 mg / ml (B, C, & D) and then incubated in the presence of PKC-P (200 nM). Oocytes of group C and D, after 30 min incubation in the presence of PKC-P, were further injected (C, 7 mM) or incubated (D, 10 μM) with TPA. The final incubations were 90 min. The assays were terminated by washing with Barth's medium. Polyamine concentrations were determined as described in Materials and Methods. p values over the histograms are compared with control, following the Mann Whitney test of significance.

Microinjection of control antibody (IgG) into oocytes did not influence the Spd uptake as compared with non-injected or oocytes only injected with carrier (results are not shown). Incubation with control solutions of either glycerol or the solvant had no significant effects on the Spd transport. The results of the present study indicate that intracellular application of TPA is not effective to enhance the Spd uptake but incubation in the extracellular medium was able to stimulate polyamine uptake process. TPA is a tumor promoter (1). It seems that TPA induced increase of Spd uptake might be a part of mechanisms involved in TPA induced tumor promotion. Tumorigenesis is always accompanied with higher polyamine biosynthesis (5), and with higher rate of polyamine transport (13). By depletion of intracellular polyamine contents, cancer cells can be growth arrested, but, later, addition of polyamines to these cells can restore the normal rate of cell proliferation (5). It seems that TPA serves as a signal for higher polyamine uptake which is independent of PKC activation.

With this study, it is apparent that PKC inhibitors (H-7 and PKC-P) are potent inhibitors whereas PKC activators (OAG and DiC8) are potent activators of Spd transport. Hence, PKC might be involved in modulating the transport process, but TPA, a widely well known tumor promotor and PKC activator, activates the Spd transport independently of PKC, and the target of TPA may be present on the outer surface of the plasma membrane. Further studies to characterize the membrane target of phorbol esters are in progress in our laboratory.

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