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REDISTRIBUTION OF PROTEIN KINASE C DURING MITOGENESIS OF HUMAN B LYMPHOCYTES

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G0 human tonsillar B-lymphocytes were stimulated to divide by the polyclonal mitogen Staphylococcus Aureus Cowan strain 1 (SAC) and by the combined use of 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the calcium ionophore ionomycin. The activities of protein kinase C, which requires Ca++ and phospholipid as co-factors, and a proteolytically cleaved form of this enzyme (protein kinase M), which is independent of calcium and phospholipid control, were determined in soluble and particulate fractions obtained from activated B cells. Treatment of G0 B cells with SAC or TPA together with ionomycin caused redistribution of protein kinase C from the soluble to the particulate fraction where the 80,000-Dalton protein kinase C was cleaved to give rise to a 50,000-Dalton form of the kinase which was also found in the cytoplasm. These data suggest that redistribution and proteolytic cleavage of protein kinase C are key signal transduction events in B cell mitogenesis.

Purified G0 human B cells provide a good model system for investigating biochemical pathways involved in the control of cell proliferation. Recently, we have shown that when these cells are activated by SAC and anti-immunoglobulin, which both cross-link cell surface immunoglobulin, hydrolysis of phosphatidyl 4,5-bisphosphate (PI 4,5-P_2) occurs within minutes (1). Similarly, other workers have shown that PI 4,5-P_2 is

The abbreviations used are: Da, Dalton; DG, 1,2-diacylglycerol; DiC8, sn-1,2-dioctanoylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(B-amino ether)-N,N,N^1,N^1,-tetraacetic acid; OAG, 1-oleoyl-2-acetyl-glycerol; PI 4,5,-P2, phosphatidylinositol 4,5 bisphosphate; PKC, protein kinase C (EC. 2.7.1.37); PKM, protein kinase M; PMSF, phenylmethylsulfonylfluoride; SAC, Staphylococcus aureus Cowan strain 1; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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hydrolysed when cell surface receptors of purified T cells and T cell lines are crosslinked by polyclonal mitogens (2,3). Hydrolysis of PI 4,5-P2 gives rise to two internal signals; diacylglycerol (DG), which activates PKC, and inositol 1,4,5-trisphosphate (1,4,5-IP_3) which liberates Ca++ from intracellular stores (rev. in 4). The involvement of mobilised Ca++ and DG as signals that synergise to provoke lymphocyte mitogenesis is also revealed by the ability of DG analogues (TPA, OAG or DiC8) and calcium ionophores (ionomycin or A23187) to stimulate the growth of B cells (5,6) and T cells (7) when used in combination but not when used alone.

Both Ca++ and DG are required for the optimal activation of protein kinase C (8). During 3T3 fibroblast mitogenesis, stimulated by adding Ca++ or fresh serum to media, PKC is redistributed from the soluble to the particulate cellular fraction (9,10). This process of PKC insertion into the particulate phase appears to depend on Ca++ and DG, as shown by the experiments of Wolf et. al. in which movement of PKC from a soluble to particulate phase, in a cell free assay, is caused by the addition of Ca++ and DG which act synergistically (11). While attached to the plasma membrane, PKC (Mol. Wt. 80KDa) can be proteolytically cleaved to give a 50KDa form (PKM) originally described by Kishimoto et al (12). Recent papers by Tapley and Murray (13,14) and Melloni et al (15) have questioned the physiological significance of PKM, which is active even in the absence of Ca++ and phospholipid (12). In this study, the soluble and particulate distributions and activities of PKC and of PKM were investigated during B cell mitogenesis.

MATERIALS AND METHODS

SAC was obtained from Calbiochem-Behring, Cambridge, U.K., ionomycin from Calbiochem, La Jolla, CA, USA and TPA, leupeptin,

phosphatidylserine (PS), histone type lll-S and diolein were obtained from Sigma, Poole, Dorset, U.K. Gamma labelled ^{32}P-ATP was obtained from Amersham International, Amersham, U.K. The purification of resting B cells and their growth analysis were as described previously (16). During the purification procedure cells were stored overnight at 4°C. The protein kinase C and M activities were assayed using modifications of existing assays (14,17) as follows. The cells were washed once in RPMI 1640 medium and suspended in cold (4°C) sample buffer containing 20mM Tris/HCl, 0.3M sucrose, 2mM EDTA, 2.5 mM EGTA, 10mM β-mercaptoethanol, 0.01% leupeptin and 2mM PMSF, at pH 7.5. The cells were disrupted by sonicating twice for 10 seconds at 0.7 maximal output of a Branson sonicater. The sonicate was spun at 100,000 X g for 1 hour, the soluble (cytosolic) fraction removed for analysis and the residue (particulate fraction) solubilised in the sample buffer containing 1% Nonidet P-40 by stirring on ice for 1 hour. The Nonidet P-40 treated material was centrifuged at 15,000 X g for 20 minutes and the supernatant, containing the previously membrane bound components of PKC and PKM, was removed for analysis. The soluble and particulate fractions were in turn applied to a 1 X 5 cm DE-52 ion exchange column which had been previously equilibrated with 20mM Tris/HCl, 2mM EDTA, 2.5mM EGTA, 10mM β-mercaptoethanol, 2mM PMSF and 0.01% leupeptin at pH 7.5. This buffer was used to elute the column and after the protein returned to base level the PKC (80KDa) and PKM (50KDa) forms were eluted by a 40ml 0-0.5M NaCl gradient in eluting buffer. One ml fractions were collected and 50µl aliquots of each fraction were assayed by adding 200µl of Tris/HCl, 10µM ATP, 10mM Mg acetate, 0.01% leupeptin at pH 7.5 plus 20µg of histone with either 1mM EGTA or 5mM $CaCl_2$ and 20µg/ml PS and 1.2µg/ml of diolein. The amount of ^{32}P gamma labelled ATP was adjusted in each assay to 8.8 X 10^6 dpm/ml. Assays were carried out at 30°C for 10 minutes after which 30µl aliquots were applied to 1 X 1 cm squares of filter paper previously soaked in 1µM ATP and 1mM Mg acetate and dried. After applying the aliquots, the filters were dropped into ice cold 5% trichloroacetic acid containing 1mM phosphate. After washing and drying, toluene based scintillant was added to each paper in scintillation vials prior to counting. All procedures were carried out at 4°C unless specified.

RESULTS AND DISCUSSION

Amounts of TPA and ionomycin which were synergistically mitogenic for human tonsillar B cells were added separately and together to aliquots of purified G0 B cells (10^8 cells per aliquot). At various timepoints the distribution and levels of activity of protein kinases C and M were investigated. Fig 1 shows the data obtained after 30 minutes application of 0.8µg/ml ionomycin, 0.1ng/ml TPA and a combination of these two agents, as compared to control cells. In G0 B cells 82% of the kinase activity exists as the 80KDa proenzyme form in the cytoplasm.

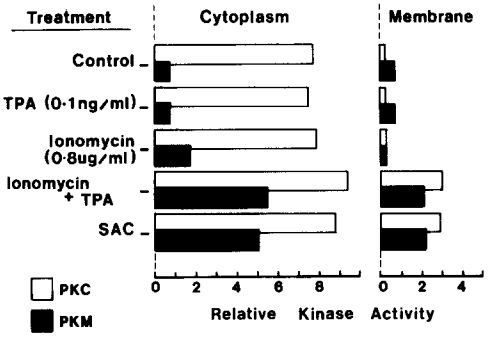

Fig 1. Distribution and levels of kinase activity in stimulated B cells. Aliquots of G0 B cells (10^8) were treated with 0.8µg/ml ionomycin, 0.1ng/ml TPA, TPA plus ionomycin for 30 minutes and SAC for 4 hours. Protein kinase C (□) and protein kinase M (■) activities were assayed in soluble and particulate fractions as described in Materials and Methods and are expressed as relative kinase activity. The mean value for peak fraction, control cytoplasmic PKC activity was 13,845 d.p.m. of ^{32}P incorporated into histone for the assay as described. The data are the means of triplicate values obtained from two experiments.

When cells were treated with TPA, at the non-mitogenic dose of 0.1ng/ml, essentially no change in the distribution and level of activity of the enzyme was observed. A similar result was obtained when G0 B cells were treated with 0.8µg/ml ionomycin, although a small increase in the soluble PKM occurred. Addition of these two agents caused several changes. The level of protein kinase (C and M forms) increased indicating either induction of enzyme synthesis or that treatment of cells with the two agents reveals a cryptic enzyme activity. Incorporation of the pro-enzyme (PKC) into the membrane was observed and the Ca++ and phospholipid independent PKM form of the enzyme appeared in both the membrane and cytosolic fractions obtained from activated B cells. The most likely interpretation of this data is that PKC is cleaved by a Ca++ dependent protease (15) in the membrane and the PKM form returns to the cytoplasm. In the case of both B cells and platelets (14) an increased amount of PKM is observed in the particulate fraction obtained from activated cells even though this form of the enzyme no longer requires phospholipids

for its activity. Gel filtration chromatographic analysis of the membrane and cytoplasmic M kinases showed them to have a molecular weight of approximately 50KDa, which is similar to the value obtained for PKM in platelets (14). After treating cells for 4 hours with TPA and ionomycin a similar pattern of kinase distribution was observed.

Stimulation of B cells with SAC or 0.8µg/ml ionophore together with 0.1ng/ml TPA showed similar kinetic profiles of RNA and DNA synthesis (1). It was of interest therefore whether SAC stimulated B cells showed a similar activation and redistribution of protein kinase C. Fig 1 also shows the effect of simulating G0 B cells with SAC as regards distribution and levels of activity of the two forms of kinase. After four hours treatment with SAC, the distribution of PKC and PKM forms between the membrane and cytoplasm was similar to that seen in B cells treated for 30 minutes with a combination of 0.8µg/ml ionomycin and 0.1ng/ml TPA. After 30 minutes treatment with SAC, although the changes were smaller in magnitude, a similar trend as to incorporation of PKC into the membrane and proteolytic cleavage was observed (data not shown).

TPA when used alone at a higher dose than used above is mitogenic for human B cells though the response is less than that seen when these cells are treated with SAC or with 0.1ng/ml TPA together with 0.8µg/ml ionomycin. Fig 2 shows that when G0 B cells were treated with 5ng/ml TPA for 30 minutes 87% of the protein kinase C was incorporated into the membrane and a small amount of PKM was produced. Hence the increased amount of TPA overcomes the requirement for Ca++ in causing redistribution of PKC from the cytoplasm to the membrane. Four hours after the addition of TPA (5ng/ml), conversion of PKC to the PKM form was observed. At four hours PKM was observed to a greater extent in

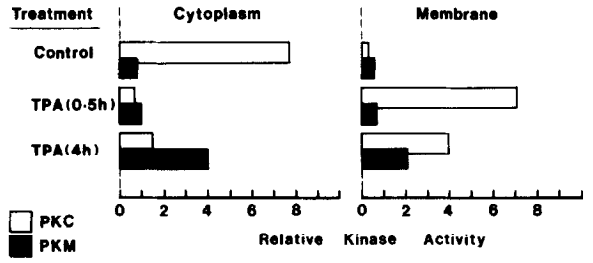

Fig 2. Distribution and levels of kinase activity in B cells treated with 5ng/ml TPA. G0 B cells were treated with TPA for 30 minutes and 4 hours and protein kinase C (□) and protein kinase M (■) activities were assayed in soluble and particulate fractions of cells. The results are expressed as relative kinase activity. The mean value for peak fraction cytoplasmic PKC activity was 15,102 d.p.m. of ^{32}P incorporated into histone for the assay as described above. The data are the means of triplicate values obtained from two experiments.

the cytoplasmic fraction. The slow conversion of PKC to PKM can be attributed to the low level of activity of membrane proteases which require calcium for their activation.

The above data, together with consideration of other studies (11,14,15), leads to a model, shown in Fig 3, for the role of protein kinase C in B cell mitogenesis. The model describes initial membrane and cytoplasmic events in the biochemical pathways which instigate cell growth (DNA synthesis) in response to external stimuli. As shown in Fig 3, interaction of SAC or

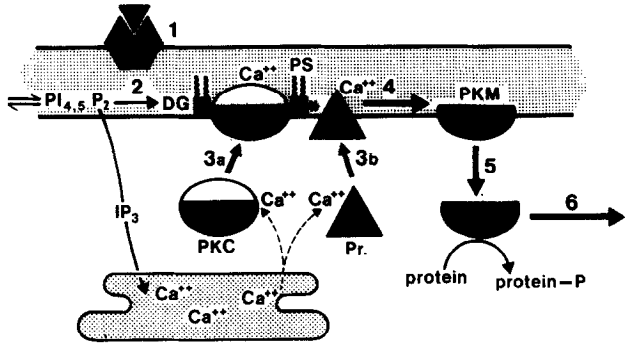

Fig 3. A model for the role of protein kinase C in B cell mitogenesis. The figure shows Ca++ and phospholipid dependent incorporation of protein kinase C (PKC) into the membrane where PKC is cleaved by a Ca++ activated protease (Pr) to give the PKM form of protein kinase which returns to the cytoplasm and is independent of Ca++ and phospholipid control.

anti-immunoglobulin with cell surface immunoglobulin molecules (Fig 3 stage 1) leads to hydrolysis of PI 4,5-P_2 to generate DG and 1,4,5-IP_3 which mobilises Ca++ (Fig 3 stage 2). DG and mobilised Ca++ act synergistically causing incorporation of PKC into the plasma membrane (Fig 3 stage 3a) where the enzyme is activated which requires phosphatidylserine as an additional cofactor. The PKC is then proteolytically cleaved (Fig 3 stage 4). Incorporation of the protease into the plasma membrane and its activation are also Ca++ and phosphatidylserine dependent (15) (Fig3 stage 3b). The 50KDa form of the enzyme moves back to the cytoplasm (Fig3 stage 5) and phosphorylates, as yet unidentified, cytoplasmic substrates which are required for initiating RNA synthesis prior to DNA synthesis.

At present, it is unclear whether inositol phospholipid hydrolysis is essential for redistribution and activation of protein kinase C. Growth factors such as IL2, IL3, and EGF induce mobilisation of Ca++ and redistribution of protein kinase C from a soluble to a particulate phase without any reported inositol phospholipid hydrolysis. However, whatever the reason for PKC relocation, the Ca++ and phospholipid independent PKM that appears in the cytoplasm when B cells are stimulated by the above agonists may play a crucial role in signal transduction during mitogenesis. A number of cytoplasmic proteins have been shown to be substrates for activated PKC which indicates a physiological role for the PKM form of this enzyme which is active in the cytoplasm (18,19,20,21). In normal B cells it is expected that PKM, once formed, is subject to strict proteolytic control (Fig 3 stage 6). Of particular interest is whether Epstein-Barr virus, which transforms B cells (22), might encode an enzyme with cytosolic kinase activity or perturb production or inactivation of a Ca++ and phospholipid independent protein kinase.

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