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# TRANSLOCATION OF PROTEIN KINASE C IS NOT REQUIRED TO INHIBIT THE ANTIGEN-INDUCED INCREASE OF CYTOSOLIC CALCIUM IN A MAST CELL LINE

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Cross-linking of receptor bound IgE antibodies by multivalent antigen ($DNP_8$-BSA) on PB-3c cells leads to an increase of cytosolic calcium ($(Ca^{2+})_i$). Active tumor promoting phorbol esters and teleocidin which specifically activate the phospholipid $Ca^{2+}$-sensitive protein kinase (PKC), inhibited the antigen-mediated rise in $(Ca^{2+})_i$ and induced a time and dose-dependent translocation of cytosolic PKC to membranes of the PB-3c cells as determined by enzyme activity or immunoblotting using a polyclonal anti-PKC antibody. This TPA concentration did not affect the subcellular distribution of PKC, although 1nM of 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited to 50 % the antigen-mediated increase in $(Ca^{2+})_i$. The concentration of TPA required to induce a half-maximal subcellular redistribution of immunodetactable PKC activity was an order of magnitude greater than the half-maximal dose required to inhibit the antigen-mediated increase in $(Ca^{2+})_i$. These data demonstrate that the TPA-dependent activation of PKC is not directly coupled to its translocation to membranes. 

Survival and proliferation of the bone-marrow derived mast cell line PB-3c cells is strictly dependent upon interleukin 3 (IL-3) which is also responsible for the maintenance of the

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Abbreviations
BSA, Bovine serum albumin;
$(DNP_8)$-BSA, Dinitrophenol-bovine serum albumin;
IL-3, Interleukin 3; IgE, Immunoglobulin class E;
PDBu, 4ß-phorbol-12,13-dibutyrate;
TPA, 12-O-tetradecanoylphorbol-13-acetate.

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antigen mediated release of vasoactive amines and the transient increase of intracellular calcium ($(Ca^{2+})_i$))(1-3). TPA, a tumor promoter known to activate and bind to PKC (4,5) blocked the antigen-mediated increase in $(Ca^{2+})_i$ preceding the serotonin release. TPA and a variety of hormones and growth factors are known to induce the translocation of PKC to membranes which is believed to reflect the intracellular activation of the enzyme (4). Antigen challenge of mast cell, however, has been shown to activate specifically only the membrane-bound PKC activity without affecting the cytosolic enzyme (6). Therefore it remained to be clarified whether the TPA-induced translocation of cytosolic PKC to membrane was required to inhibit the antigen-mediated increase of $(Ca^{2+})_i$ observed in PB-3c cells. Our data suggest that tumor promoter induced activation of PKC without its translocation appears to be sufficient to inhibit the antigen-mediated increase in intracellular calcium.

## MATERIALS AND METHODS

### Materials

Phorbol esters were bought from SIGMA. Teleocidin was a generous gift of Dr. Sugimura (NCCRI, Tokyo).

### Cell culture

Culture conditions of the PB-3c cells have been described earlier (1,2,7).

### Measurement of $(Ca^{2+})_i$

Cells were sensitized with anti-$DNP_8$ monoclonal IgE antibodies for 1 hour at 4°C (8). Before challenge with 1.0 ng of antigen $DNP_8$-BSA the PB-3c cells (0.5 to $1.0 \times 10^6$ cells/ml) were loaded with 20 µM QUIN-2-AM (CIBA-GEIGY) for 15 min at 37°C in Tyrode buffer. Changes of $(Ca^{2+})_i$ were monitored and quantitated as described earlier (9).

### Determination of PKC in subcellular fractions

Subcellular fractions of PB-3c were prepared as described (10). PKC activities were analyzed by non denaturating PAGE, assayed and quantitated exactly as reported (16).For immunological quantitation of PKC, the cytosol was supplemented with 3 % SDS and immediately boiled. The 100'000 x g pellet ("membrane" fraction) was directly solubilized in SDS sample buffer (10mM Tris-HCl, pH 7.4, 2 % ß-mercaptoethanol,3% SDS, 2mM EGTA, 2mM EDTA).

### Purification of PKC and production of polyclonal anti-PKC antiserum

PKC was purified from pig brain exactly as described by Uchida and Filburn (11) followed by electroelution of PKC from prepa-

rative SDS-PAGE (12). Purified PKC (50 μg) was injected into the back of a rabbit in complete Freund's adjuvant. Every three weeks, the rabbit was given intradermal injections of 25-30 μg of the enzyme in incomplete Freund's adjuvant. Antisera were collected after the second boost. Anti-PKC antibody against the 77 kD PKC was purified exactly as reported by Olmsted (13). Competition of the anti-PKC antibody with purified PKC was tested as described (12).

Immunoblotting

Cytosol and membrane fractions were subjected to polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and electrophoretically transferred to nitrocellulose (NC)(3,14,15). The NC was blocked in TN buffer (50mM Tris-HCl, pH 7.4, 200mM NaCl) containing 3% BSA, 10% heat-inactivated horse serum (GIBCO) and 0.2% Triton X-100, incubated for 90' with purified anti-PKC antibody (1:50) followed by the addition of 0.2 μCi/ml of $^{125}$I-donkey-anti-rabbit antibody (AMERSHAM: S.A. 5-20 μCi/μg) for 90'. The NC was then extensively washed with TN containing 0.2%Triton X-100 and autoradiographed at -70°C. Immunodetectable PKC was quantitated from autoradiograms(12).

Other analytical methods

Protein was determined by the method of Bradford (17) using BIO-RAD reagents with BSA as standard. Statistical significance was analyzed by the Wilcoxon rank-sum test.

## RESULTS

Changes in cytoslic free calcium concentrations ($(Ca^{2+})_i$) were monitored after the addition of $DNP_8$-BSA to sensitized PB-3c cells. The antigen $DNP_8$-BSA induced a rapid increase of $(Ca^{2+})_i$ from 90 to 500 nM in 30 seconds which returned gradually to basal levels within 10 minutes (Fig.1A). Treatment of PB-3c cells with 100 nM TPA, PDBu or teleocidin did not affect the basal $(Ca^{2+})_i$ (data not shown). However, if these compounds were added shortly before the antigen, there was a complete inhibition of the antigen-mediated increase of $(Ca^{2+})_i$ (Fig.1A). Addition of tumor promoters shortly after the antigen resulted in an enhanced decay of the fluorescent calcium signal (Fig.1B). A specific and dose-dependent inhibition of the antigen-mediated calcium signal was only observed with active tumor promoters (Fig.2A). TPA was most effective with an $IC_{50}$ of about 1 nM as compared to teleocidin ($IC_{50}$:10nM) or PDBu ($IC_{50}$:100nM) whereas the inactive phorbolester analog 4α-phorbol 12,13-didecanoate and phorbol did not affect the antigen-mediated calciun signal. Polyacrylamide gel electro-

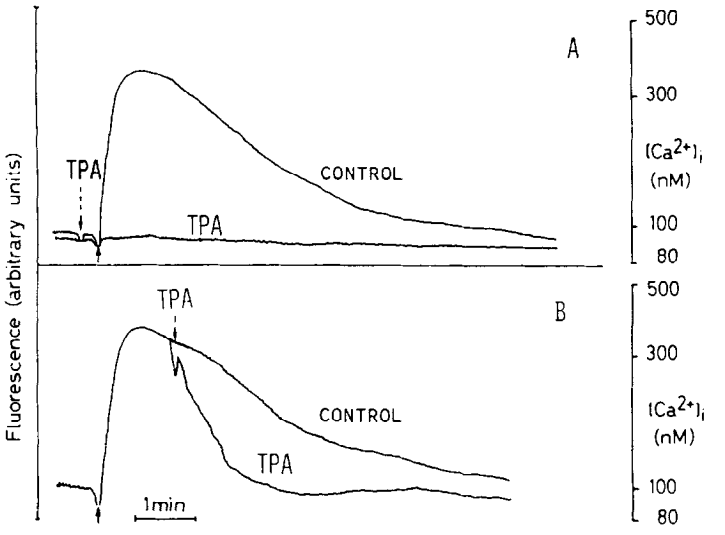
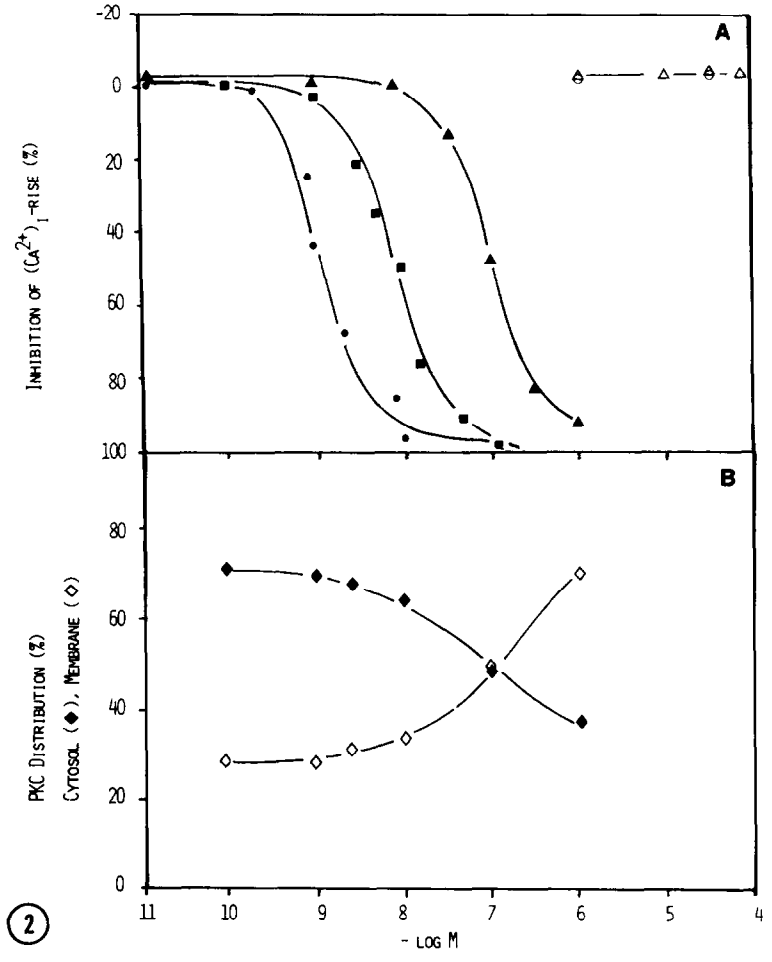
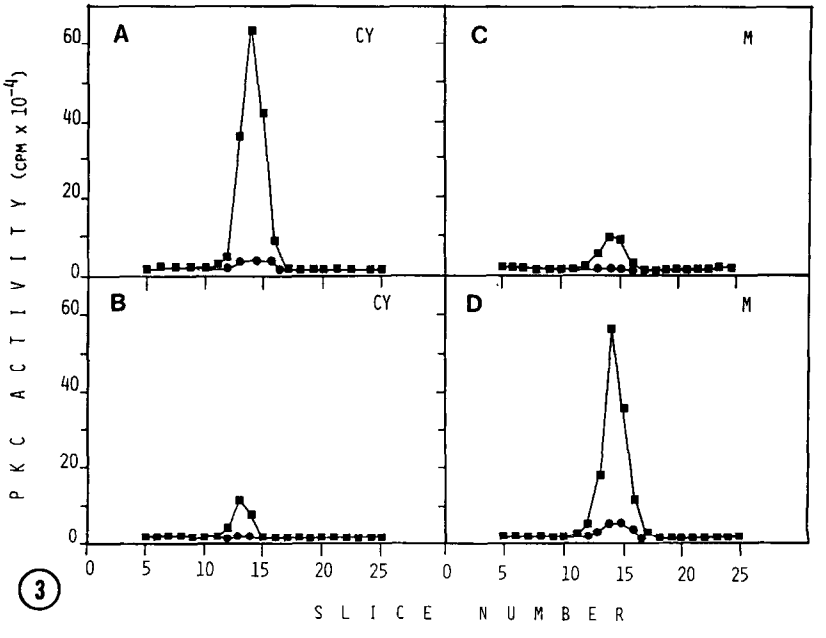



Fig.1. Effects of TPA on the antigen-induced changes in $(Ca^{2+})_i$.
A: Effects of 50nM TPA added 1 min before the antigen (arrow).
B: Effects of 50nM TPA added 1 min after addition of antigen (arrow). The antigen concentration was 0.5μg/ml of $(DNP_8)$-BSA. $(Ca^{2+})_i$ was determined as described in methods.

phoresis of cytosol and membrane fractions of PB-3c resolved a single peak of phospholipid and $Ca^{2+}$-sensitive protein kinase activity(Fig.3A/3C). Treatment with TPA for 30 minutes caused a decrease of cytosolic PKC activity which was paralleled by an increase of PKC activity in the membrane fraction (Fig.3B/3D). Similar results were obtained when the subcellular fractions of PB-3c cells were subjected to immunoblotting (Fig.4). The rabbit polyclonal anti-PKC antibody, raised against the porcine brain 77 kD PKC holoenzyme clearly recognized on immunoblots a protein with a molecular weight of 80 kD. This 80 kD band of PB-3c cells was identified by several criteria as a PKC (3). There was a rapid loss of the 80 kD protein band in the cytosol of the respective TPA-treated cells that was paralleled by an increase of the 80 kD band in the corresponding membrane fractions (Fig.4). In addition there was a dose-dependent translocation of immunodetectable cytosolic PKC activity to


A
B
INHIBITION OF $(Ca^{2+})_I$-RISE (%)
PKC DISTRIBUTION (%)
CYTOSOL (◆), MEMBRANE (◇)
- LOG M
-20
0
20
40
60
80
100
11
10
9
8
7
6
5
4

2


A
CY
B
CY
C
M
D
M
PKC ACTIVITY (CPM x $10^{-4}$)
SLICE NUMBER
0
20
40
60
5
10
15
20
25

3

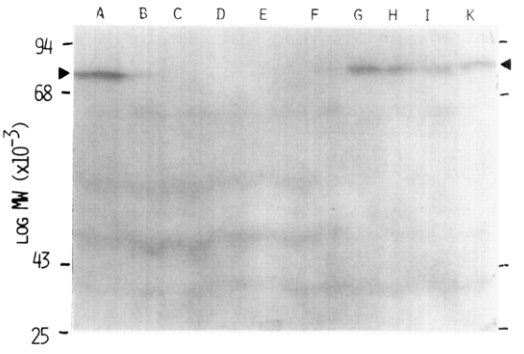



Fig.4. Immunoblot of PKC from PB-3c cells.
PB-3c were treated with 300 nM TPA. At indicated times cells were subjected to subcellular fractionation followed by Western blot analysis and immunological detection as described in methods. Cytosol from control cells (lane A) and from cells treated for 5,10,30,and 60 min with 300nM TPA (lanes B-E) as well as corresponding control membrane (lane F) and TPA treated membranes (lanes G-K). The arrow indicates the position of the 80 kD PKC polypeptides. Lines indicate MW markers (94,68,43 and 30 kD respectively).

membranes by TPA (Fig.2B). It should be noted that the TPA-dependent subcellular redistribution of PKC activity matched the immunodectatble PKC (data not shown). Athough 1nM TPA induced a half-maximal inhibition of the antigen-mediated $(Ca^{2+})_i$ rise it did not affect significantly the subcellular distribution of PKC activity(Fig.1B). A significant PKC translocation was only observed at 30 to 100 nM TPA. Thus, the concentration of TPA

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Fig.2. Quantitation of the inhibition of the antigen-mediated $(Ca^{2+})_i$ and PKC translocation by tumor promoters
Inhibition of the antigen-induced calcium signal as well as quantitation of immunodetectable PKC were performed as described in methods.
A:Inhibition of antigen-mediated calcium signal by a short preincubation (30 sec) of PB-3c cells by either TPA ( ), Teleocidine (■), PDBu (▲), 4α-phorbol 12,13-didecanoate(△) or phorbol (○).
B: Quantitation of TPA-dependent translocation of immunodetectable PKC. PB-3c cells were exposed to increasing concentration of TPA for 10 min. After the incubation subcellular fractions were prepared and subjected to immunoblotting as described in methods.

Fig.3. PAGE analysis of PKC activity of TPA-treated PB-3c cells
PB-3c cells were treated for 30 min with 300 nM TPA and subjected to subcellular fractionation as described in methods. Aliquots (300µg) of cytosol (Cy) and membranes (M) were analyzed by PAGE using a 10% final gel concentration as described in methods. PKC activity was determined in presence (■) or absence (●) of Ca2+, phosphatidylserine and diolein.

required to induce a half-maximal inhibition of the antigen-mediated calcium signal was clearly an order of magnitude lower than the doses required to induce a half-maximal translocation of cytoslic PKC to membranes. A similar discrepancy between the doses of tumor promoters required to block the calcium signal and those to induce PKC translocation were ob- served for both teleocidin and PDBu (data not shown).

## DISCUSSION

These results demonstrate that the tumor promoter dependent inhibition of the antigen $DNP_8$-BSA-induced increase in $(Ca^{2+})_i$ is specifically mediated by PKC. TPA was more effective in the activation and translocation of PKC as compared to teleocidine and PDBU. The relative potencies of the phorbol diester analogs and teleocidin in inhibiting the antigen-induced rise in $(Ca^{2+})_i$ correlated with their capacity to promote docking of PKC to membranes (10). PKC translocation is thought to be causal for its activation (4).

However there is good evidence indicating that PKC translocation is not responsible for the blockage of the antigen-mediated calcium rise in PB-3c cells. First, the concentration of the tumor promoter required to induce the integration of PKC to membrane was an order of magnitude higher than the doses required to inhibit the antigen-mediated calcium signal. Second, the effects of active tumor promoters on the calcium signal occurs almost immediately (30 seconds) whereas PKC translocation is only observed if PB-3c cells are exposed for more than 5 min to 1μM TPA. It appears that the tumor promoter-dependent activation of PKC is not necessarily coupled with its translocation to membranes. Thus, tumor promoter mediated subcellular redistribution of PKC appears to be part

of those mechanisms which eventually leads to the down modulation of the phorbol ester receptor (18,19).

ACKNOWLEDGEMENT

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