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PHORBOL 12-MYRISTATE 13-ACETATE (TPA) BLOCKS CD3-MEDIATED Ca^{2+} MOBILIZATION IN JURKAT T CELLS INDEPENDENTLY OF PROTEIN KINASE C ACTIVATION

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Stimulation of Jurkat T cells with antibodies against the T cell receptor/CD3 complex induces a rise in the intracellular concentration of Ca^{2+} within seconds. The inositol phosphate-dependent Ca^{2+} mobilization induced by OKT3 was completely abrogated when Jurkat cells were pretreated for 1 min with the phorbol 12-myristate 13-acetate TPA (10nM), a concentration which activates protein kinase C (PKC). The effects of TPA on the Ca^{2+} fluxes were insensitive to treatment of the cells with known PKC inhibitors (H-7 and staurosporin) under conditions where the PKC-mediated phosphorylation was blocked. Furthermore, another activator of PKC, mezerein, inhibited the Ca^{2+} signal induced by OKT3. This inhibition, however, could completely be reversed by pretreatment with H-7 or staurosporine. We conclude that the TPA-mediated inhibition of Ca^{2+} fluxes in Jurkat T cells largely acts through a PKC-independent pathway.

Triggering of various cell surface receptors induces a rapid hydrolysis of inositol phospholipids to diacylglycerol and inositol phosphates, including inositol 1,4,5-trisphosphate (IP3). Diacylglycerol acts as an allosteric activator of protein kinase C (PKC) (1), while IP3 causes a release of Ca^{2+} from intracellular stores (2). PKC activation and Ca^{2+} mobilization are believed to be important signals for the induction of a number of cellular responses, including cell proliferation.

In several receptor systems TPA inhibits agonist-induced phospholipase C activation (3,4) and rise in cytosolic free calcium $[Ca^{2+}]_i$ (5,6). The mechanism behind this is unknown, but involvement of PKC has been suggested. PKC inhibitors like H-7 and staurosporin revert the TPA-mediated inhibition of phospholipase C in platelets (7,8), indicating that the inhibition is indeed mediated by protein kinase C. Thus, PKC would exert a negative feedback on receptor-stimulated inositol phospholipid turnover.

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Abbreviations; $[Ca^{2+}]_i$, intracellular concentration of free calcium; TPA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinelinesulfonyl)-2-methylpiperazine; IP3, inositol trisphosphate; PKC, protein kinase C.

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Stimulation of the leukemic T cell line Jurkat with anti-CD3 antibodies induces a number of rapid biochemical responses typical of T lymphocyte activation. These include inositol phospholipid turnover and a rapid increase in the cytoplasmic free calcium concentration $[Ca^{2+}]_i$ (9,10) . We have measured both inositol formation and Ca^{2+} fluxes in Jurkat T cells and show that anti-CD3-induced Ca^{2+} mobilization is blocked by TPA by a mechanism that is largely independent of protein kinase C.

MATERIALS AND METHODS

Cells : Jurkat, a human T-cell leukemia line (11), was cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with L-glutamine, antibiotics and 5% (vol/vol) FCS (Gibco). The cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell viability was monitored by trypan blue exclusion.

Reagents : OKT3, a monoclonal antibody against the CD3ε-chain, was used (1:5000 dilution) as ascites produced in Pristane treated BALB/c mice using hybridoma cells obtained from the American Type Culture Collection. Fura-2 (Molecular Probes Inc., Oregon) was dissolved in dimethyl sulfoxide and stored at -70°C. Phorbol 12-myristate13-acetate (TPA) was from Sigma, St. Louis, MO,

Measurement of $[Ca2+]_i$ with Fura-2: $[Ca^{2+}]_i$ was measured essentially as described by Tsien et al. (12) using the fluorescent calcium indicator Fura-2. The cells were loaded in 3 μM of the membrane permeable acetoxymethylester of Fura-2 for 20 min. After washing the cells were resuspended in 100mM NaCl, 10mM HEPES, 5.4mM KCl, 1.2mM MgCl2, 0.44mM KH_2PO_4, 4.2mM $NaHCO_3$, 0.4mM $CaCl_2$, 5mM glucose. Fluorescence was monitored with a Perkin-Elmer MPF-3 fluorescence spectrometer, at excitation wave length 340 nm and emission wave length 510 nm. During measurements the temperature of the cell suspension was kept at 37°C. After each experiment the intracellularly trapped Fura-2 was released with 1% Triton-X 100 and fluorescence at 0% (Fmin) and 100% (Fmax) Ca^{2+} saturation was measured. The concentration of free intracellular Ca^{2+}, $[Ca^{2+}]_i$, was calculated using the equation:

$$[Ca^{2+}]_i = Kd\ (F - Fmin) / (Fmax - F)$$

where Kd for Fura-2 (at 37°C) is 220 nM and F is the observed fluorescence from the intracellular Fura-2.

Measurement of PI turnover in JURKAT cells: 30 x 10^6 Jurkat cells were labeled in inositol-free culture medium (RPMI 1640 sine inositol, Pharmacy of Helsinki University, Finland) for 20-24 h with 30-50μCi/ml ^{3}H-inositol (NET 906, NEN DuPont). The washed cells were resuspended in inositol-free medium containing 10mM LiCl and preincubated for 20-30 minutes at 37°C. After treatment with TPA and/or mitogen 0.5ml methanol/chloroform/HCl (pH 5) was added, followed by 0.2ml chloroform and 0.2ml water. The inositol phosphates in 0.55 ml of the water phase were separated on a MonoQ anion-exchange column with a FLPC system (Pharmacia, Sweden), with a continous gradient of 0-1 M ammoniumformate in 50 ml at a flow rate of 0.4-0.8 ml/min as previously described (13).

Phosphorylation experiments: ^{32}P labelling and preparation of cell lysates was performed as previously described (14).

RESULTS

Treatment of Jurkat T cells with the anti-CD3 monoclonal antibody, OKT3, induced a rapid increase in $[Ca^{2+}]_i$ from 130 nM to 500 nM within 1 min (Fig 1A).

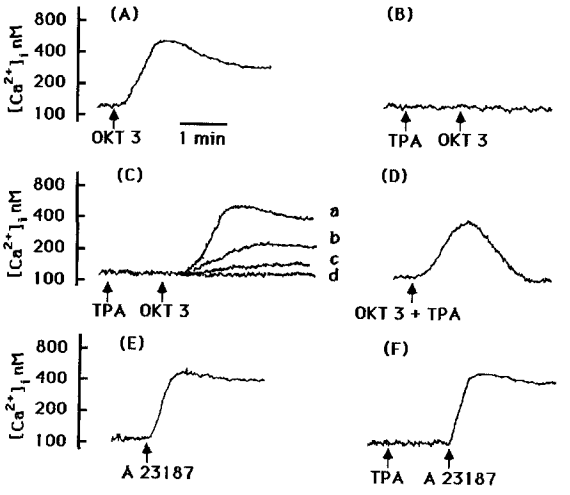
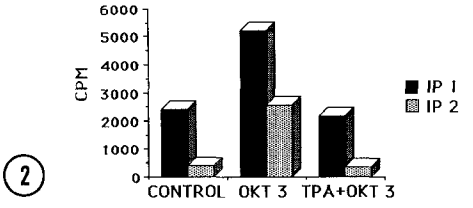


Fig.1. Effect of TPA on OKT3 and A23187-induced Ca^{2+} signals in Fura-2 loaded Jurkat cells.
Fura-2 loaded Jurkat cells were treated with: (**A**) OKT3 (**B**) OKT3 one min after 10 nM TPA , (**C**) OKT3 one min after (a) 0 , (b) 0.1, (c) 1.0, and (d) 10.0 nM TPA. (**D**) OKT3 and TPA 10 nM added at the same time. (**E**) 10 nM A23187. (**F**) 10 nM A23187 one min after 10 nM TPA, The traces shown were obtained in one experiment representative of at least five.

After the initial rise, the $[Ca^{2+}]_i$ declined within 1-2 min to a plateau at about 250 nM. When Jurkat cells were treated with 10 nM TPA for 1 min before the addition of OKT3 the Ca^{2+} response was completely abolished (Fig 1B). The inhibition was dose-dependent, with 50% inhibition of the Ca^{2+} response at 0.1 nM TPA (Fig 1C). The inhibition by TPA became effective after a lag of approximately 1min. Thus, when TPA was added simultanously with OKT3 the Ca^{2+} signal was initially intact. After the initial rise (approx. 1min), however, the signal returned to the basal level (Fig. 1F). Pretreatment with TPA had no effect on the increase in $[Ca^{2+}]_i$ seen when Jurkat cells were treated with 10 nM of the Ca^{2+} ionophore A23187 (Fig 1D and E). This indicates that TPA does not "freeze" the cell membrane or reorganize the Ca^{2+} pool in the cell. Furthermore, the formation of inositol phosphates was also completely inhibited (Fig 2) when the cells were stimulated with OKT3 one min after the addition of TPA. TPA alone did not have any effect on basal PIP_2 hydrolysis or on the basal $[Ca^{2+}]_i$ levels even after prolonged incubations (data not shown).

To investigate whether the suppression of the Ca^{2+} response and PIP_2 hydrolysis by TPA was mediated through activation of PKC, we tried to reverse the effect by preincubating the cells with PKC inhibitors. When Jurkat cells were pretreated for 1h with 100 μM H-7 or 100 nM staurosporin and then with TPA, followed by OKT3, the Ca^{2+} signal was still suppressed (Fig. 3A and 3B). H-7 and staurosporin by themselves effected neither the basal Ca^{2+} level nor the OKT3 induced Ca^{2+} signal (Fig.3C and 3D).

To ensure that the TPA-induced activation of PKC was inhibited by H-7 or staurosporin, we studied the TPA-stimulated incorporation of ^{32}P into a PKC dependent 19 kDa substrate. The 19 kDa protein has earlier been shown to be a



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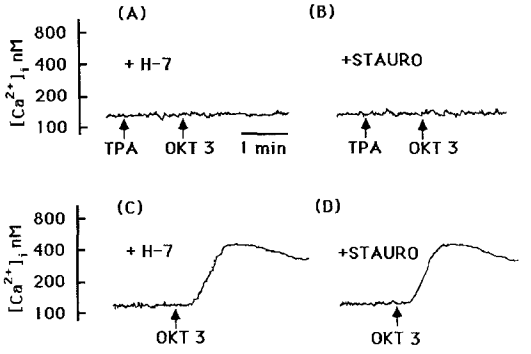


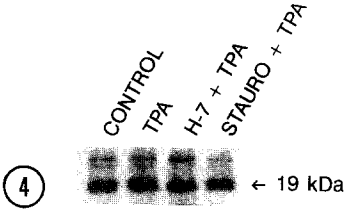
(3)

Fig.2. Effect of TPA on OKT3-induced inositol turnover in Jurkat cells. IP1 and IP2 formation in ^{3}H-inositol labeled Jurkat cells stimulated with OKT3 in the presence or absence of 10 nM TPA. One representative experiment out of two is shown.

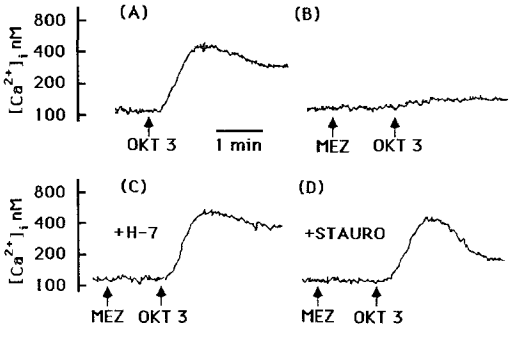
Fig.3. Effect of H-7, staurosporin and TPA on the OKT3 induced Ca^{2+} signal in Fura-2 loaded Jurkat cells.
Jurkat cells pretreated for one hour with 100 mM H-7 (**A** and **C**) or 100 nM staurosporin (**B** and **D**) were stimulated with OKT3 (1:500 dilution) in the presence (**A** and **B**) or absence (**C** and **D**) of 10 nM TPA.

sensitive marker for PKC activation in Jurkat cells (14). To achieve a reproducible increase in the phosphorylation of the 19 kDa band with 10 nM TPA, a preincubation of 5 min or more was required (data not shown). The TPA-induced increase in the phosphorylation of the PKC dependent 19 kDa substrate could be completely blocked by pretreatment with H-7 or staurosporin (Fig.4).

To further characterize the mechanism by which TPA affects the Ca^{2+} signal in this cell line we also tried to inhibit the OKT3-induced Ca^{2+} signal by other known protein kinase C activators. We found that 10 nM mezerein, a diterpene ester



(4)



(5)

Fig.4. Effect of H-7 and staurosporin on the TPA-induced phosphorylation of the PKC-dependent 19 kD substrate in Jurkat cells.
Incorporation of ^{32}P into the 19 kDa substrate in untreated cells ; or cells treated with 10 nM TPA ; 10 nM TPA after pretreatment for 20 min with 100mM H-7; 10 nM TPA after pretreatment for 20 min with 100 nM staurosporin. The incubation time with TPA was 5 min.

Fig.5. Effect of mezerein on the OKT3 induced Ca^{2+} signal in Fura-2 loaded Jurkat cells.
Fura-2 loaded Jurkat cells were treated with: (**A**) OKT3 , (**B**) OKT3 one min after 10 nM mezerein (MEZ),(**C**) 100μM H-7 for 30 min and then stimulated with OKT3 one min after 10 nM mezerein,(**D**) 100 nM staurosporin (STAURO) for 30 min and then stimulated with OKT3 one min after 10 nM mezerein.

structurally related to TPA, also could inhibit the calcium signal up to 80-90 % (Fig 5 A and B). Higher concentrations of mezerin did not further increase the degree of inhibition (data not shown). In contrast to TPA, the inhibition of the OKT3-induced calcium signal by mezerein, could be completely reversed by pretreatment with H-7 or staurosporin (Fig 5 C and D).

DISCUSSION

It is generally accepted that the inhibition of phospholipase C by TPA is mediated through protein kinase C. In this way PKC would exert some kind of feedback regulation on the activation signal. However, in this report we have described an effect of TPA on the signal transduction pathway in the T-cell leukemia line Jurkat that differs from this view. In contrast to earlier reports on various cell systems, we found that the TPA mediated inhibition of the OKT3-induced Ca^{2+} traffic in Jurkat cells could not be reversed by pretreatment with PKC inhibitors. On the other hand, the inhibition induced by mezerein, another well-known activator of PKC, could be completely reversed by pretreatment with H-7 or staurosporin. Thus, the inhibition by mezerein appeared to be mediated through PKC. We suggest that TPA, in addition to activating PKC, in this cell line, also affects other early steps in the signal transduction pathway upstream of phospholipase C activation. The molecular mechanisms by which TPA mediates the rapid inhibition of the phospholipase C dependent calcium traffic in this cell line are currently being investigated in our laboratory.

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