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Tumor Promoter 12-0-Tetradecanoyl-Phorbol-13-Acetate (TPA) Can Reduce the Ca-Transporting Ability of Ca-Ionophores in T Lymphocytes: The Involvement of Intracellular Heavy Metal Ions

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12-0-tetradecanoyl-phorbol-13-acetate (TPA) can significantly reduce the Ca-ionophore-induced rise in the intracellular calcium concentration (Ca_i) of T lymphocytes measured by quin2 or fura-2 fluorescence. This counteraction of TPA is maximal at a preincubation of 90 min at TPA concentrations higher than 20 nM. ^{45}Ca uptake and efflux measurements directly indicate that TPA does not activate the calcium extrusion systems in thymocytes but impairs the Ca-transporting ability of Ca-ionophores. TPA causes no immobilization of the Ca-ionophores as it is demonstrated by the lack of significant changes in fluorescence and fluorescence polarisation of A23187 during TPA incubation. Similarly the energy transfer between the Tyr, Try groups of membrane proteins and A23187 shows no significant difference in control and TPA treated thymocytes. This indicates that A23187 is not in a membrane protein-bound form after TPA preincubation. The intracellular heavy metal chelator, N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) restores the ionophoretic ability of Ca-ionophores in TPA pretreated cells to the control level. Diacyl-glycerols also impair the Ca-transporting ability of Ca-ionophores. TPEN prevents this effect as well. These findings suggest that TPA and diacyl-glycerols may cause an increase in the availability of intracellular heavy metal ions. Our results may reflect a new, physiologically important mechanism of the action of diacyl-glycerols and phorbol esters.

The tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) is mitogenic for T lymphocytes of wide origin (Whitfield et al., 1973; Mastro and Mueller, 1974; Wang et al., 1975; Mastro and Smith, 1983; Gelfand et al., 1985) and synergistically enhances the mitogenic effects of lectins (Mastro and Mueller, 1974; Wang et al., 1975; Gelfand et al., 1985) or calcium ionophores (Wang et al., 1975; Mastro and Smith, 1983). However, in the past years several reports have demonstrated that TPA can *inhibit* the DNA synthesis of mitogen activated T lymphocytes. The exact mechanism of this antiproliferative effect of TPA is not known (Gescher, 1985; Dröge, 1986).

The presence of extracellular calcium is an important requirement for T lymphocyte activation (Alford, 1970; Imboden et al., 1985). In the past years it has been proved that, indeed, a rise in the intracellular calcium concentration (Ca_i) is one of the earliest events of the activation process (Tsien et al., 1982; Hesketh et al., 1983; Imboden et al., 1985). Recently numerous reports have demonstrated that TPA is able to block the agonist-induced rise in Ca_i in various cell-types (Rickard and Sheterline, 1985; Poll and Westwick, 1986; Mendoza et al., 1986 and references therein). Mastro and Smith (1983) have shown that the synergistic, co-mitogenic effect of TPA depends on the duration of TPA incubation and on the sequence of addition of TPA and the calcium ionophore, A23187.

In the light of these findings the possibility arises that the antiproliferative and "Ca-blocking" effect of TPA can be linked. However, in case of antigens or lectins several other changes also occur besides the rise in Ca_i (Imboden et al., 1985). Therefore the aim of this study was to investigate the effect of TPA in a "model-system", where *only* a rise in the intracellular calcium

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Abbreviations used: Ca_i, intracellular calcium concentration; Ca_o, extracellular calcium concentration; DMSO, dimethyl sulfoxide; EGTA, ethylene-glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid; OAG, oleoylacetyl-glycerol; TPA, 12-0-tetradecanoyl-phorbol-13-acetate; TPEN, N,N,N′N′-tetrakis(2-pyridylmethyl)-ethylene-diamine.

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concentration served as an initial signal for increased cell proliferation. In this way we have studied whether TPA can affect the Ca-ionophore-induced rise in Ca_i. In the present paper we report that TPA can greatly reduce the Ca-ionophore-induced rise in Ca_i of thymocytes. This effect is not due to the TPA-induced activation of Ca-extrusion processes or immobilization of Ca-ionophores. The counteraction of TPA can be explained mostly by an increased availability of intracellular heavy metal ions. These findings have been published earlier in a preliminary form (Csermely and Somogyi, 1986).

MATERIALS AND METHODS

Materials

A23187, ionomycin, TPEN and fura-2 acetoxymethyl ester were from Calbiochem. Calmidazolium (R24571), dioctanoyl-glycerol, Hepes, oleoyl-acetyl-glycerol, quin2 acetoxymethyl ester and TPA were Sigma products. Triton X-100 was from Serva, dimethyl-sulfoxide from Fluka. ^{3}H-quin2 acetoxymethyl ester (370 MBq/mmol) was an Amersham product. $^{45}CaCl_2$ (4.3 GBq/mmol) was from the National Institute of Isotopes (Budapest, Hungary). Cell culture media and the supplements used were from Gibco. Diphenyl-oxazole and 1,4–di(2–(5–phenyl)-oxazoyl)benzene were from Koch-Light Laboratories (Colnbrook, Berks, England). $CaCl_2$ and $MgCl_2$ were Merck ultrapure products. All the other reagents used were of best analytical purity.

Isolation of mouse thymocytes and TPA-incubation

Mouse (CLFP, 6–8 weeks old, male) thymocytes were prepared by the method of Kleiman et al. (1984). After isolation, cells were incubated with TPA (at concentrations and times indicated) or with the solvent dimethyl sulfoxide (DMSO) at a final concentration less than 0.2% (v/v). Cell density was 5×10^6 cells/ml, the incubation was performed at 37°C in Eagle's minimal essential medium supplemented with 10 mM Hepes (pH 7.4), essential amino acids, streptomycin and penicillin 100 U/ml each. In the experiments viability was never below 95% as judged by trypan blue exclusion.

Measurement of intracellular calcium concentration

Loading of quin2 and measurement of its fluorescence was carried out as described earlier (Csermely and Somogyi, 1987). Briefly, thymocytes were incubated with quin2 acetoxymethyl ester or with fura-2 acetoxymethyl ester at a final concentration of 20 μM or 2 μM respectively at 37°C. The medium of incubation contained 143 mM NaCl, 1 mM Na_2SO_4, 5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 10 mM Hepes, pH 7.4 (standard medium). In the first 20 min of incubation, the cell density was 5×10^7 cells/ml, then the cells were diluted to tenfold with standard medium and incubated further for 20 min. The amount of extracellular indicator was routinely checked by the method of Hesketh et al. (1983) and Pollock et al. (1986). The intracellular concentration of quin2 was determined using ^{3}H-quin2 acetoxymethyl ester. The intracellular volume of mouse thymocytes was taken as 104 fl (Hesketh et al., 1983).

In case of TPA preincubation up to 90 min, cells were *first* loaded with quin2 *then* incubated with TPA. In case of incubations longer than 45 min the experiments were (also) done under conditions when TPA preincubation *included* the quin2 loading procedure. (In this case TPA concentration was maintained at a constant throughout the incubation.) At time points where the two experimental protocols were overlapping (45, 60 and 90 min) there was no significant difference in the results (not shown).

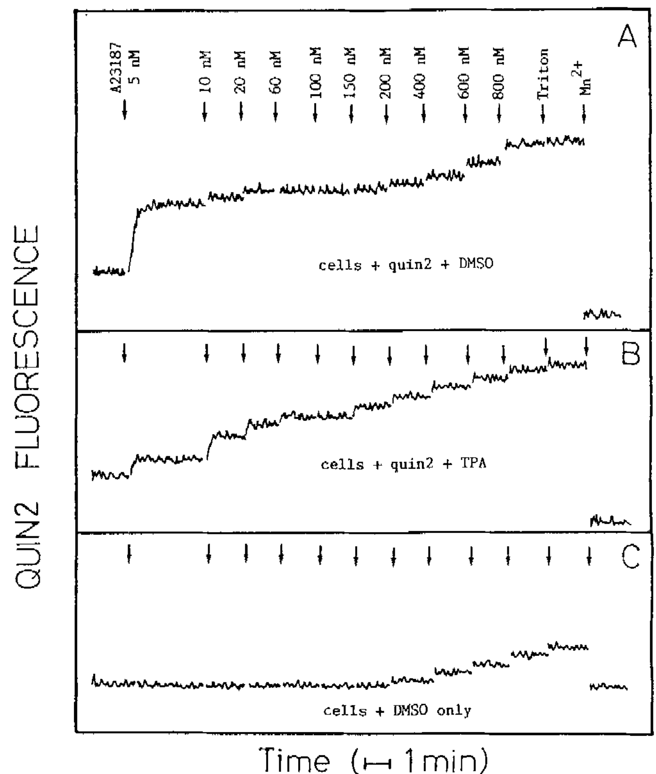

Fig. 1. The effect of TPA preincubation on the A23187-induced rise in Ca_i of mouse thymocytes. Mouse thymocytes were incubated with DMSO (*panel A, C*) or with TPA at a final concentration of 50 nM (*panel B*) for 90 min as described in Materials and Methods. In case of panels *A* and *B* quin2 loading and measurement of Ca_i was done as described in Materials and Methods. *Panel C* shows the autofluorescence of cells before and after the addition of A2187. At the arrows A23187 (in the final concentrations indicated), Triton X-100 (0.05% v/v) and $MnCl_2$ (10 μM) were added. The fluorescence scales in panels *A-C* are identical. The traces shown are representatives of six separate experiments. The intracellular quin2 concentration was in the range of 0.8–1.2 mM.

Fluorescence measurements were performed in a Spectrofluo JY3 (Jobin Yvon, Longjumeau, France) spectrofluorimeter. The excitation/emission wavelength pair was 339/492 nm in case of quin2 measurements and 340/510 nm in case of fura-2 measurements, respectively (4 nm slits). The samples were measured in 1 cm square quartz cuvets thermostatted to 37°C. The calibration of the fluorescence signal and the calculation of the intracellular calcium concentration was done as described earlier (Pollock et al., 1986, Csermely and Somogyi, 1987).

^{45}Ca uptake and efflux measurements

^{45}Ca uptake was measured by incubation of mouse thymocytes in Eagle's minimum essential medium supplemented with 10 mM Hepes (pH 7.4), essential amino acids, streptomycin and penicillin 100 U/ml each and 40 kBq/ml (2–2.5 $\times 10^6$ cpm/ml) $^{45}CaCl_2$. Incubation was

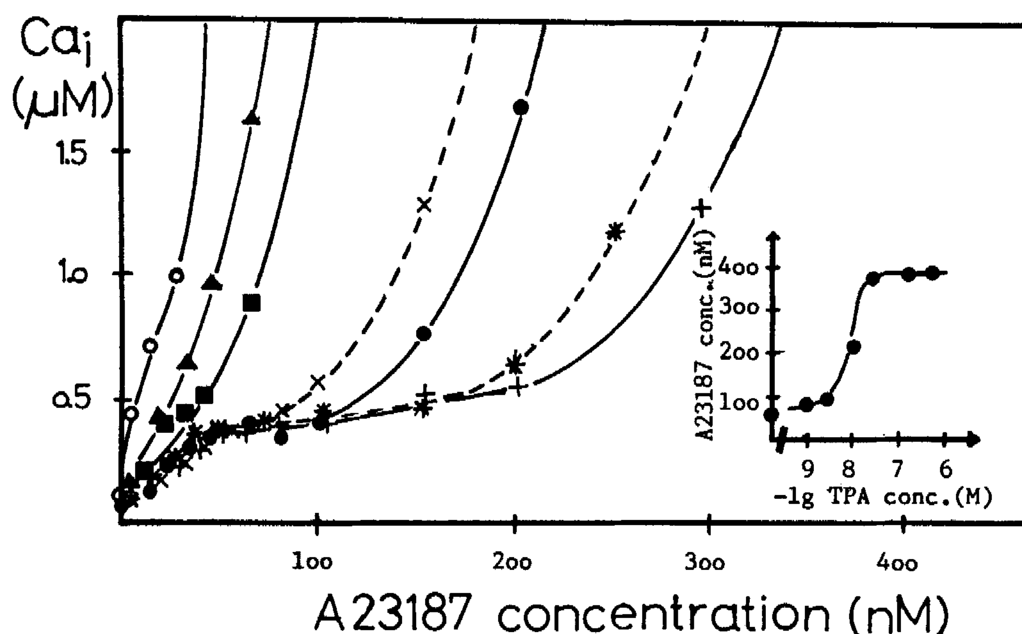

Fig. 2. The time and concentration dependence of the effect of TPA preincubation on the A23187-induced rise in Ca_i. Treatment of mouse thymocytes with TPA, measurement and calculation of Ca_i was done as described in Materials and Methods. The fluorescence signal of quin2 was followed for 5–10 min after each addition and the Ca_i was calculated from the final, steady-state value. Data points are values after serial additions of A23187. However remeasurement of some data points after prompt addition of A23187 in appropriate concentration resulted in no significant differences in Ca_i. Samples were incubated with DMSO for 150 min (○—○); or with TPA (at a final concentration of 20 nM) for 15 min (▲—▲), 30 min (■—■), 45 min (●—●), 90 min (+—+), 120 min (*—*) or 240 min (×—×). The *inset* shows the TPA concentration dependence of A23187 concentrations necessary to reach calcium equilibration (incubation time: 90 min). Data are representative of three separate experiments. The intracellular quin2 concentration was in the range of 0.6–1.3 mM.

done in Eppendorf tubes at a cell density of 5×10^6 cells/ml at 37°C for the times indicated. The final volume of the sample was 1 ml. After the incubation cells were centrifuged in a microfuge. After the removal of the supernatant the tips of the Eppendorf tubes were cut and placed into scintillation vials. Radioactivity was determined by liquid scintillation; the scintillation cocktail contained 5.9 g/l diphenyl-oxazole, 160 mg/l 1,4-di(2-(5-phenyl)-oxazoyl)benzene, 30% (v/v) Triton X-100 and 70% (v/v) toluene.

For ^{45}Ca efflux measurements cells were loaded with ^{45}Ca by incubation with 160 kBq/ml (10^7 cpm/ml) $^{45}CaCl_2$ for 30 min. Other conditions of incubation were the same as described for ^{45}Ca uptake measurements. The excess of ^{45}Ca was removed by centrifugation (400 g, 5 min, 4°C) and cells were washed twice with the same volume of Eagle's medium. Cells were resuspended in the same medium (with no ^{45}Ca added) at a cell density of 5×10^6 cells/ml. At times indicated 0.5 ml aliquots of the cell suspension were withdrawn and centrifuged in a microfuge. Radioactivity of 2×0.2 ml of the supernatant was determined.

Measurement of A23187 fluorescence and fluorescence polarisation

A23187 fluorescence was measured after Pfeiffer et al. (1974). The excitation-emission wavelength pair was 370 nm and 430 nm, respectively. Fluorescence polarisation measurements were made using polarization filters. (Oriel Co., Stamford, CT, USA) by the general procedure of Chen and Bowman (1965). For the calculation of the emission anisotropy we used the formula of Jablonski $r = (I_{vv} - G \times I_{vh})/I_{vv} + 2G \times I_{vh})$ where I_{vv} and I_{vh} is the fluorescence intensity at vertical-vertical or vertical-horizontal filter positions respectively. The grating factor (G, the ratio of the fluorescence intensities measured at horizontal-vertical and horizontal-horizontal filter positions) was 0.90 in our experiments.

Energy transfer measurements

Energy transfer measurements were done after Hyono et al. (1985). Excitation wavelength was 270 nm and the emission spectra were recorded after serial addition of A23187 at final concentrations indicated. Both the excitation and emission slits were 4 nm, cell density was set to 2×10^6 cells/ml. Emission spectra were corrected to the fluorescence of A23187 observed at 270 nm excitation. A23187 fluorescence was measured in the presence of soybean lecithin liposomes mimicking the hydrophobic environment of plasma membranes. Liposomes were prepared by sonication using a Sonic 300 dismembrator (Artex Inc. Co., Farmingdale, NY, USA). The optimal density of liposomes (0.2–2 μg/ml) was set to get the same increase in A23187 fluorescence (measured at different A23187 concentrations) as by addition of mouse thymocytes at a cell density of 2×10^6 cells/ml.

RESULTS

TPA can reduce the A23187-induced rise in the intracellular calcium concentration

In Figure 1 the effect of TPA preincubation on the A23187-induced rise in the intracellular calcium concen-

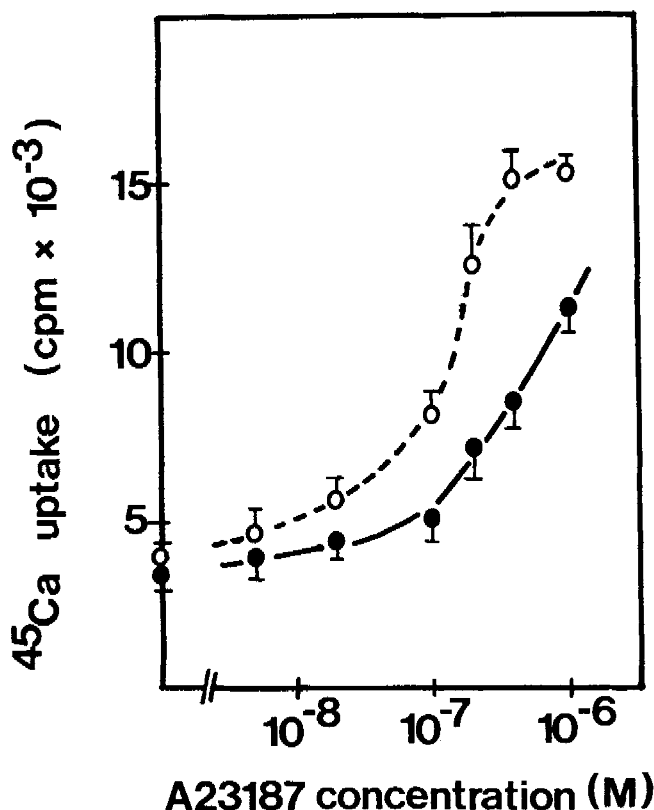

Fig. 3. The effect of TPA preincubation on the A23187-induced ^{45}Ca-uptake. Mouse thymocytes were incubated with DMSO (*open circles*) or with TPA at a final concentration of 20 nM (*closed circles*) for 90 mn. ^{45}Ca uptake measurements were done as described in Materials and Methods. Preliminary experiments indicated maximal ^{45}Ca uptake at 2 min of incubation in the presence of A23187 (not shown). Therefore this incubation time was chosen for ^{45}Ca uptake measurements in the presence of Ca-ionophores. Data are means ± SDS of three separate experiments done in triplicates.

tration (quin2 fluorescence) is shown. Control cells were incubated with the solvent of TPA, dimethyl sulfoxide (less than 0.2% v/v, panel A). Panel C shows the autofluorescence of cells before and after the addition of A23187. (The fluorescence of A23187 was identical if we measured it in TPA-preincubated cells, data not shown.) If panel A and C are compared it can be seen that after the addition of 20 nM of A23187 the increase in the fluorescence in panel A is due to the autofluorescence of A23187. The addition of Triton X–100 does not cause a further increase in quin2 fluorescence. Thus Ca_i is equilibrated with the extracellular calcium concentration (Ca_o) at A23187 concentrations higher than 20 nM.

The preincubation of mouse thymocytes with TPA (50 nM, 90 min) markedly reduces the ionophoretic ability of A23187 (panel B). The minimal A23187 concentration sufficient to equilibrate Ca_i with Ca_o is increased to 200 nM which is tenfold of the control value.

In Figure 2 the time and concentration dependence of the TPA-induced attenuation of the Ca-ionophoretic ability of A23187 can be seen. As it has been already shown in Figure 1, in control cells (○—○) Ca_i is equilibrated with Ca_o (=1 mM) at A23187 concentrations higher than 20 nM. After 9 min preincubation with TPA at a final concentration of 20 nM (+—+) this equilibrating, "minimal effective concentration" of A23187 was in the range of 200–400 nM. This TPA-induced increase in the minimal effective concentration of A23187 is time dependent and transient: it can be hardly detected if the preincubation time is less than 30 min and it declines if mouse thymocytes are preincubated with TPA for more than 90–120 min.

Examining the concentration dependence of this TPA-induced increase in the minimal effective concentration of A23187 (inset in Figure 2) it can be seen that it is maximal at TPA concentrations higher than 20 nM where protein kinase C is fully activated (Castagna et al., 1982).

TPA reduces the ionophore-induced uptake of ^{45}Ca

In Figure 3 the uptake of ^{45}Ca can be seen as the function of A23187 concentration. If the cells were preincubated with TPA (20 nM, 90 min) the uptake of ^{45}Ca is markedly decreased compared to the values measured in untreated cells. The data of Figure 3 give direct

TABLE 1. ^{45}Ca uptake and efflux in control and TPA treated mouse thymocytes[1]

Incubation time uptake/efflux	^{45}Ca uptake (cpm × 10 ^{3}/5 × 10^6 cells) Control cells	TPA treated cells	^{45}Ca efflux (cpm × 10 ^{3}/5 × 10^6 cells) Control cells	TPA treated cells
5 min	4.0 + 0.4	3.9 + 0.3	n.d.[2]	n.d.
10 min	7.9 + 0.8	6.0 + 0.4	1.5 + 0.3	1.5 + 0.4
+10 μM calmidazolium	17.2 + 1.4	18.0 + 0.6	0.8 + 0.3	0.8 + 0.2
20 min	13.1 + 1.0	10.2 + 0.8	3.5 + 0.3	2.3 + 0.4
60 min	18.1 + 2.0	15.3 + 1.3	7.8 + 0.8	7.0 + 0.9
+10 μM calmidazolium	n.d.	n.d.	2.5 + 0.5	2.3 + 0.4

[1]Isolation of cells, TPA preincubation (20 nM TPA, 90 min), ^{45}Ca uptake and efflux measurements were done as described in Materials and Methods. The values of ^{45}Ca efflux correspond to the radioactivity that appeared in the supernatant. Data are corrected to the value of "O min incubation" and expressed as means ± SDs of three separate experiments run in triplicates.
[2]n.d. = not determined.

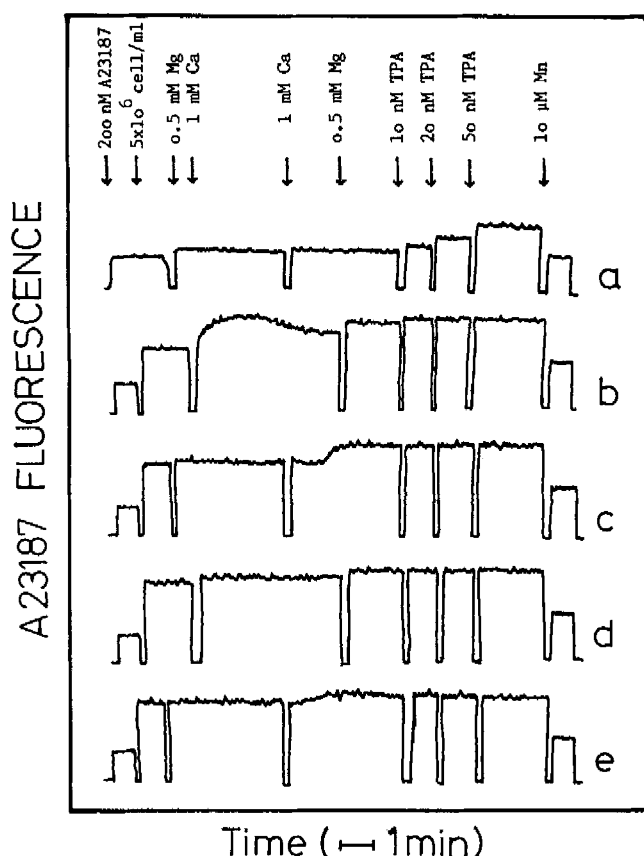

Fig. 4. The effect of TPA and TPA preincubation on the fluorescence of A23187 in mouse thymocytes. A23187 fluorescence was measured as described in Materials and Methods. Traces show the A23187 fluorescence in Ca^{2+} and Mg^{2+} free standard medium. Thymocytes were incubated with DMSO (traces *b*, *c*) or TPA (50 nM final concentration, 90 min; traces *d*,*e*) as described in Materials and Methods. After incubation cells were centrifuged at 200×g for 5 min and resuspended in Ca^{2+} and Mg^{2+} free standard medium at a cell density of 5 × 10^7 cells/ml. The fluorescence scales of traces *a*–*e* are identical. Traces are representatives of five experiments.

evidence for the assumption that TPA changes not only the steady state value of Ca_i but it impairs the Ca-transporting ability of Ca-ionophores, too. TPA treatment cannot totally abolish the A23187-induced increase in the uptake of ^{45}Ca. On the other hand, a significant inhibition can be observed in any concentration of A23187 tested. This shows that TPA diminishes the activity of some/all of the A23187 molecules.

TPA does not alter the uptake and efflux of ^{45}Ca

The attenuation of the A23187-induced rise in the Ca_i after TPA treatment can also be explained (on the analogy of neutrophils, Rickard and Sheterline, 1985) by the activation of Ca-extrusion systems. Therefore we have investigated the effect of TPA on the ^{45}Ca uptake and efflux in thymocytes (Table 1).

Our data show no significant differences between the ^{45}Ca uptake and efflux of control and TPA treated cells. The changes are insignificant, even in the case when the plasma membrane calcium pump is blocked with the selective calmodulin-antagonist, calmidazolium (R24571). Thus neither the calmidazolium inhibited, nor the remaining Ca-extrusion systems are activated after TPA treatment.

Fluorescence properties of A23187 after TPA treatment

The TPA-induced impairment of the activity of Ca-ionophores can be a consequence of their immobilization in the plasma membrane after TPA treatment. This possibility can be examined most easily utilizing the fluorescent properties of A23187.

Figure 4 shows the fluorescence of A23187 in buffer, in control and TPA treated cells. In trace *a* A23187 is added to Ca^{2+} and Mg^{2+} free medium. Apolar environment is known to enhance the A23187 fluorescence to severalfold (Pfeiffer et al., 1974). This might be the reason why the addition of the hydrophobic TPA enhances the fluorescence intensity of A23187 in standard medium (Fig. 4, trace *a*). TPA has no autofluorescence under these conditions (not shown) thus in a polar solvent a direct interaction between TPA and A23187 may occur showing the mutual exclusion of TPA and A23187 from the structure of water. Manganese quenches the fluorescence of A23187 (traces *a*–*e*); this is in agreement with earlier data of Pfeiffer et al. (1974).

In Figure 4 traces *b* and *c* show the fluorescence of A23187 in control (DMSO incubated) mouse thymocytes. The increase in the fluorescence upon addition of the cells is due to the insertion of A23187 to the hydrophobic plasma membrane. Upon addition of Ca^{2+} either in absence (trace *b*) or in presence (trace *c*) of Mg^{2+} a transient increase of the A23187 fluorescence can be seen. Under these conditions there is no fluorescence change upon the addition of TPA.

Traces *d* and *e* were recorded in the presence of TPA preincubated (50 nM, 90 min) mouse thymocytes. The

TABLE 2. The emission anisotropy of A23187 fluorescence in standard medium, in control and TPA incubated mouse thymocytes[1]

A23187 concentration (nM)	Emission anisotropy: Standard medium	Cells + DMSO (90 min)	Cells + TPA (20 nM, 90 min)
50	0.141 + 0.021	0.215 + 0.018	0.204 + 0.023
100	0.129 + 0.020	0.208 + 0.029	0.191 + 0.020
200	0.118 + 0.025	0.176 + 0.016	0.164 + 0.014
400	0.126 + 0.017	0.169 + 0.010	0.151 + 0.009

[1]Cells were incubated with DMSO or TPA and the emission anisotropy was measured as described in Materials and Methods. Cell density of the samples was 5×10^6 cells/ml. Data are means ± SD of five separate experiments.

increase in the A23187 fluorescence after addition of the TPA preincubated cells is not significantly different from the increase observed at the addition of control cells (compare traces *d* and *e* with traces *b* and *c*). Extending the investigations we could not find any difference between the A23187 fluorescence (from 20 nM to 800 nM) in control and TPA preincubated cells (data not shown). This indicates that there is no significant change in the "hydrophobicity" of the environment of A23187 during TPA incubation of mouse thymocytes.

TPA preincubation prevents, or greatly diminishes the Ca^{2+}–induced transient rise in A23187 fluorescence (compare traces *d* and *e* with traces *b* and *c*). The prevention of the Ca^{2+}–induced rise in A23187 fluorescence can also be achieved by the previous addition of 10 μM calmidazolium (R24571), a known inhibitor of the plasma membrane Ca^{2+}–pump (not shown). This suggests that the Ca^{2+}–induced transient increase in the A23187 fluorescence might be related to the Ca^{2+}–induced activation of the plasma membrane Ca^{2+}–pump. Therefore the Ca^{2+}–induced transient increase in A23187 fluorescence might be regarded as an "active-state" of the Ca-ionophore, A23187. This "active-state" does not occur after TPA preincubation of mouse thymocytes, which can be regarded as an additional sign of the TPA-induced impairment of Ca-ionophoretic activity.

Mn^{2+} quenches the A23187 fluorescence. Examining the concentration dependence of this quenching effect we have found that there is no difference between the "quenching activity" of Mn^{2+} in control and TPA-preincubated cells. Half-maximal quenching occurs in both cases at 0.3 μM Mn^{2+} concentration in the presence of 1 mM Ca^{2+} (not shown). This means that the relative affinity constant of A23187 in T lymphocytes for Mn^{2+} versus Ca^{2+} remains approximately 3×10^3 if cells are incubated with TPA. Thus TPA preincubation does not shift the preference of A23187 to heavy metal ions compared to Ca^{2+} in T lymphocytes.

Table 2 shows the emission anisotropy values of A23187 fluorescence. The emission anisotropy is a sensitive marker of the rotational mobility and so the possible immobilization of any fluorophore. The emission anisotropy of A23187 significantly increases upon addition of mouse thymocytes in any A23187 concentrations tested. This indicates a significant immobilization of A23187 upon insertion into the plasma membrane. However, there is no further increase in the emission anisotropy if the cells have been preincubated with TPA. On the contrary a slight ($P < 0.2$ in case of 50–200 nM A23187, $P < 0.025$ in case of 400 nM A23187) decrease in the emission anisotropy can be demonstrated. This might reflect an increased membrane fluidity after TPA preincubation. Thus the fluorescent properties of A23187 demonstrate that no major immobilization of A23187 occurs after TPA incubation of mouse thymocytes.

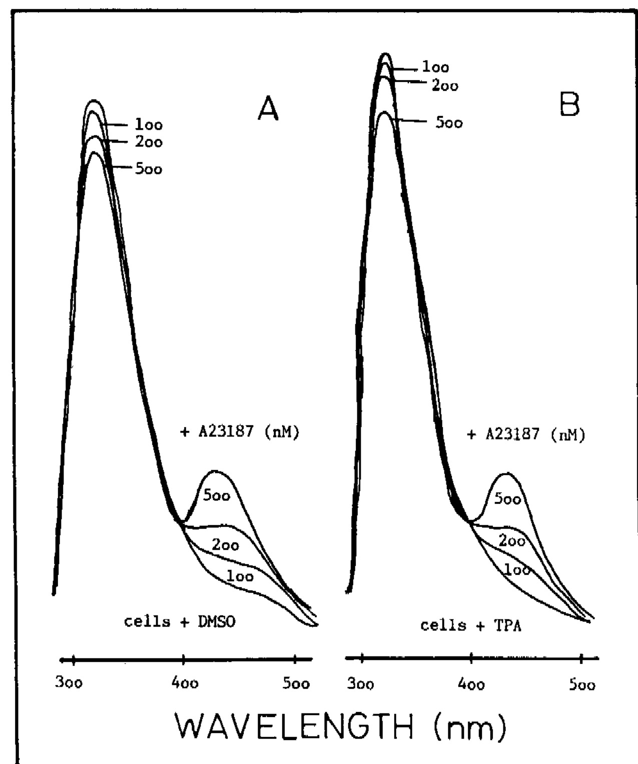

Fig. 5. Energy transfer between the Tyr, Try groups of membrane proteins and A23187 in mouse thymocytes. Mouse thymocytes were incubated with DMSO (panel *A*) TPA (20 nM final concentration, 90 min; panel *B*) as described in Materials and Methods. After incubation cells were resuspended in standard medium at a cell density of 2×10^6 cells/ml. The Ca-ionophore, A23187 was added at final concentrations of 100, 200 and 500 nM as indicated. The energy transfer measurements were done as described in "Materials and Methods". Emission spectra were corrected to the fluorescence of A23187 and the spectra are representatives of five separate experiments. The fluorescence scales of panels A and B are identical.

Energy transfer measurements indicate no immobilization of A23187

A23187 can be immobilized not only by a decrease in the overall membrane fluidity but by binding to certain proteins in the plasma membrane. This possibility may be examined using the phenomenon of the energy transfer. Energy transfer may occur between two fluorophores if the fluorescence emission spectrum of the first (the donor) is overlapping with the fluorescence excitation spectrum of the other (the acceptor). At the emission maximum of the Tyr, Try fluorescence (330 nm) A23187 can be excited; its secondary emission occurs at 430 nm. The rate of this energy transfer strictly depends on the average distance between the two fluorophores (Tyr, Try and A23187); as the fluorophores are getting closer and closer the rate of energy transfer dramatically increases. Thus changes in the energy transfer indicate very sensitively the binding of A23187 to membrane proteins after TPA treatment.

In Figure 5, the energy transfer between the Tyr, Try groups of membrane proteins and A23187 is demonstrated. Since spectra of Figure 5 were carefully corrected for the autofluorescence of A23187 (see Materials and Methods) the secondary emission of A23187 indicates a real energy transfer in the system. This is supported by the fact that the overall decrease in the Tyr, Try emission intensity is roughly corresponding to the overall increase in the secondary emission of A23187 (Fig. 5). Thus the insignificant differences between the energy transfer occurring in control (panel A) and TPA

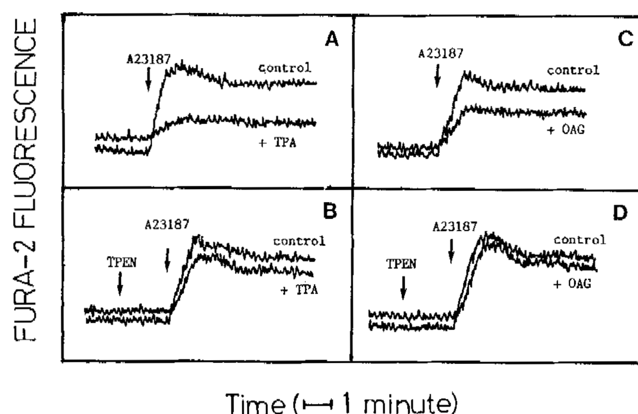

Fig. 6. Intracellular heavy metal chelator, TPEN restores the TPA-reduced ionophoretic ability of Ca-ionophores to the control level. Mouse thymocytes were incubated with DMSO (traces "control"), with TPA (20 nM final concentration, 90 min; traces "+TPA"), or with oleoyl-acetyl-glycerol (50 μg/ml final concentration, 20 min; traces "+OAG"). The cells were loaded with fura-2 and Ca_i was measured as described in "Materials and Methods". Panels *A* and *C* show the Ca-ionophoretic effect of A23187 without, panels *B* and *D* with TPEN. At the arrows A23187 and TPEN were added as indicated at final concentrations of 20 nM and 50 μM respectively. Traces are representatives of three separate experiments. The fluorescence scales of panels A-D are identical.

preincubated cells (panel B) indicate a roughly equivalent energy transfer, i.e., average distance between membrane proteins and A23187 in these cells.

Involvement of intracellular heavy metal ions in the effect of TPA and oleoyl-acetyl-glycerol (OAG)

Figure 6 demonstrates the involvement of intracellular heavy metal ions in the TPA-induced attenuation of the Ca-ionophoretic ability of A23187. In *panel A* the effect of 20 nM A23187 can be seen in control and TPA preincubated (20 nM, 90 min) mouse thymocytes. *Panel C* shows that preincubation with oleoyl-acetyl-glycerol (OAG) also diminishes the A23187-induced rise in Ca_i. In preliminary experiments the maximal effect of oleoyl-acetyl-glycerol was observed after 20 min preincubation (data not shown). However the effect of OAG is significantly smaller than the effect of TPA (compare panels A and C in Fig. 6). It seems plausible that OAG has been metabolised by the time its effect would be fully developed. (This requires 90 min preincubation in case of TPA.) In *panels B and D* the intracellular heavy metal chelator TPEN (reintroduced by Arslan et al., 1985) was added to the thymocytes preincubated with either TPA (panel B) or with OAG (panel D). In the presence of this chelator the efficiency of A23187 was restored to the control level. These results can be essentially reproduced using the ten-hundredfold more efficient Ca-ionophore, ionomycin (Csermely and Somogyi, 1987; not shown). We have gotten similar results using rabbit thymocytes instead of mouse thymocytes (not shown).

The data of Table 3 indicate that the intracellular heavy metal chelator, TPEN restores the ionophore-induced ^{45}Ca uptake to the control level after TPA treatment. Oleoyl-acetyl-glycerol induces similar changes as TPA in the uptake of ^{45}Ca, these effects can be antagonized by TPEN as well. These results give further evidence for the assumption that intracellular heavy metal ions play a role in the counteraction of TPA, diacylglycerols and Ca-ionophores.

DISCUSSION

Our initial question has been whether TPA can affect the Ca-ionophore-induced rise in the intracellular calcium concentration (Ca_i). We have found that TPA is able to diminish greatly the Ca-ionophore-induced rise in Ca_i (Fig. 1). This observation is surprising since it cannot be explained by the common assumption that protein kinase C (the major phorbol ester receptor) phosphorylates and thus inactivates the putative receptor-linked Ca-channels, or enzymes (receptors) of the inositol trisphosphate pathway (Poll and Westwick, 1986; Mendoza et al., 1986).

On the basis of Figures 1 and 2 we cannot conclude that TPA preincubation impairs the Ca^{2+}-transporting ability of Ca-ionophores since the reduction of the fluorescence signal (apparent Ca_i) can be the consequence of the quenching of quin2 induced by heavy metals (Hesketh et al., 1983). However, the same phenomenon can be observed using fura-2 (Fig. 6). Fura-2 is known to be much less sensitive to heavy metals than quin2 (Grynkiewicz et al., 1985). Therefore the reproducibility of the data of quin2 measurements (Figs. 1 and 2) by fura-2 (Fig. 6) indicates that TPA treatment causes real changes in the Ca-ionophore-induced rise in Ca_i.

Data of Figures 1, 2 and 6 suggest that TPA treatment diminishes the Ca^{2+}-transporting ability of A23187. However to *prove* this hypothesis *direct* measurements of the Ca^{2+}-fluxes are necessary. Figure 3 gives a direct

TABLE 3. The effect of the heavy metal chelator, TPEN on the uptake of ^{45}Ca[1]

A23187 concentration (nM)	Control thymocytes	^{45}Ca uptake (cpm × 10^{-3}) + TPA	+ TPA, TPEN	+ OAG	OAG, + TPEN
0	3.7 + 0.3	3.5 + 0.4	3.2 + 0.3	3.6 + 0.2	3.8 + 0.4
100	11.6 + 0.8	7.4 + 0.7	11.1 + 0.9	8.1 + 0.9	10.7 + 1.3
200	14.9 + 1.3	8.1 + 1.0	13.9 + 1.5	10.2 + 1.2	13.1 + 1.1

[1]Isolation of mouse thymocytes, preincubation and ^{45}Ca uptake measurements were done as described in Materials and Methods. Cells were preincubated with DMSO (control cells), with TPA at a final concentration of 20 nM for 90 min, with OAG at a final concentration of 50 μg/ml for 20 min or with TPEN at a final concentration of 100 μM for 90 or 20 min respectively as indicated. The uptake of ^{45}Ca was terminated after 2 min (see legend of Fig. 6). Data are means ± SDs of three separate experiments run in triplicates.

evidence that TPA indeed impairs the Ca^{2+}–transporting ability of Ca-ionophores since a marked decrease in the uptake of ^{45}Ca can be demonstrated after TPA treatment at various A23187 concentrations.

Thinking about the possible explanations of the phenomenon observed at least five different reasons can be suspected: (1) the Ca^{2+} extrusion systems are activated and so the increased Ca^{2+}–efflux is also responsible for the impaired ability of Ca-ionophores to raise Ca_i; (2) TPA directly interacts with the Ca-ionophores and thus blocks their action; (3) the membrane fluidity is drastically reduced upon TPA treatment, Ca-ionophores become "frozen" in the membrane; (4) the Ca-ionophores are immobilized by binding to certain membrane proteins and finally (5) the availability of intracellular heavy metal ions increases and the excess of heavy metals is able to block the Ca-ionophores.

Tsien et al. (1982) have reported a TPA-induced decrease of quin2 fluorescence in mouse and pig lymphocytes. The TPA-induced decrease in quin2 fluorescence has been regarded as a sign showing the TPA-induced activation of Ca-extrusion systems by several authors (see e.g., Rickard and Sheterline, 1985). Some reports have directly shown a TPA-induced increase in the Ca-efflux in neutrophils (Rickard and Sheterline, 1985 and references therein). However, this observation can not be generalized since in astrocytoma cells (Orellana et al., 1985) and in two secretory cell lines (PC 12 and RINm5F, Di Virgilio et al., 1986) TPA does not effect the efflux rate of ^{45}Ca.

The results of Table 1 show that in mouse thymocytes there is no significant difference between the ^{45}Ca uptake and efflux in control and TPA treated cells. These data are in agreement with the results of Grubbs and Maguire (1986) who report also an unchanged ^{45}Ca uptake and efflux after TPA treatment of S49 lymphoma cells. The effect of TPA is also insignificant if we compare the calmidazolium-induced increases in ^{45}Ca uptake or calmidazolium-induced decreases in ^{45}Ca efflux (Table 1). TPA does not activate the calmidazolium-dependent or calmidazolium-independent Ca efflux, this makes explanation 1 ("TPA activates the Ca-extrusion systems") unlikely and so TPA prevents the rise in Ca_i solely by the attenuation of Ca^{2+}–uptake in T lymphocytes.

TPA is able to block the effect of 5–10-times higher A23187 concentrations the effect is time dependent, being maximal after 90 min of preincubation (Fig. 2). TPA changes the fluorescence of A23187 in aqueous solutions while it fails to do so in mouse thymocytes (Fig. 4). These observations make explanation 2 (i.e., "TPA directly interacts with the Ca-ionophores") unlikely, because no direct interaction between TPA and A23187 can be demonstrated.

There are conflicting data about the effect of TPA on the membrane fluidity. Stocker et al. (1982) report a TPA-induced decrease in the membrane fluidity in polymorphonuclear leukocytes while in rat embryo cells (Fischer et al., 1974) and in artificial membranes (Deelers et al., 1981) a TPA-induced increase in the membrane fluidity has been observed. A recent report (Goppelt-Strübe and Resch, 1987) demonstrates that the *fluorescence polarisation value of a certain fluorescent* probe is greatly different in isolated plasma membranes from that measured in whole cells. In this way the differences of the literary data may arise from the uneven distribution of the hydrophobic fluorescent probe in the different membranes of whole cell systems.

Our results show that if we use the Ca-ionophore (A23187) itself as a "fluorescent probe" its fluorescence polarisation remains essentially unchanged (or slightly decreased) upon TPA treatment (Table 2). This finding shows a slightly increased rotational mobility of the Ca-ionophore. Figure 5 shows an unchanged average distance between A23187 and membrane proteins upon TPA incubation. These observations render explanations 3 and 4 (i.e., "Ca-ionophores are 'frozen' in the plasma membrane or bound to membrane proteins") unlikely.

In Figure 6 and Table 3 it is shown that the intracellular heavy metal chelator TPEN (reintroduced by Arslan et al., 1985) can restore the ionophoretic ability of A23187 to the original level which is observed without TPA or OAG treatment. This suggests that TPA or OAG can increase the availability of intracellular heavy metal ions and so the "excess" of heavy metals could block a part of the Ca-ionophores.

Heavy metals (Mn^{2+}, Zn^{2+}, etc.) have 100–1,000 times higher affinities for A23187 than Ca^{2+} (Pfeiffer and Lardy, 1976). Mn^{2+} ions block the A23187-induced influx of Ca^{2+} in lymphocytes (Resch et al., 1978). In the presence of extracellular Mn^{2+} ions A23187 raises Ca_i only at much higher concentrations than in the absence of Mn^{2+} (P. Csermely and J. Somogyi unpublished observations). Thus heavy metal ions are able to impair the Ca^{2+}–transporting properties of Ca-ionophores.

What is the heavy metal responsible for the effects observed? We have demonstrated earlier that the most abundant cytosolic heavy metals are (in the order of decreasing concentrations) Zn, Fe and Cu (Csermely et al., 1987b). Since the fluorescence of A23187 is unchanged upon TPA treatment (Fig. 4) and of these heavy metals only Zn does not quench the fluorescence of A23187 (Pfeiffer et al., 1974; and data not shown) Zn is the most likely candidate as a TPA-induced Ca-blocker.

Our present results are in good agreement with our findings that TPA induces a heavy metal (mainly Zn) translocation from the nucleus and mitochondria to the cytosol and microsomes of thymocytes measured by X-ray fluorescence (Csermely et al., 1987a) and plasma emission spectroscopy (Csermely et al., 1987b). Our results are supported by the earlier observations (Arslan et al., 1985 and references therein) that the Ca^{2+}–transporting ability of A23187 is impaired in ascites tumour cells which have an extraordinarily high availability of heavy metal ions in their cytosol (Arslan et al., 1985).

Our data show that not only TPA, but diacyl-glycerols can also attenuate the Ca^{2+}–transporting ability of Ca-ionophores (Fig. 6 and Table 3). Since TPEN can restore the original efficiency of Ca-ionophores after diacyl-glycerol treatment (Table 3) it seems plausible that diacylglycerols induce similar changes in the subcellular distribution of heavy metals like TPA. However to prove this hypothesis direct measurements are necessary which are in progress in our laboratory.

On the basis of our results presented it seems obvious, that TPA and diacyl-glycerols increase the availability (relative amount) of heavy metals in the cytosol and

these heavy metals are able to block Ca-ionophores. These findings have a number of *consequences:* (1) TPA-induced heavy metals may be partly responsible for the TPA-induced block of changes in intracellular calcium concentration; (2) Heavy metals exhibit a higher affinity for a number of calcium binding sites (see e.g., Haberman and Richardt, 1986). Therefore an excess of heavy metal ions can occupy some of the calcium binding sites of different proteins (enzymes, receptors, channels, etc.) influencing their functions; (3) The primary structure of several receptor proteins, e.g., that of cortisol, oestradiol, thyrotropic hormone (Weinberger et al., 1986) or protein kinase C itself (Parker et al., 1986) show the existence of putative Zn-binding sites. The increased availability of heavy metals (mainly Zn) in the cytosol might change the behaviour of these proteins; (4) Our analytical studies (Csermely et al., 1987a,b) have revealed that the *source* of heavy metals appearing in the cytosol (and microsomes) is not extracellular but a decrease in the heavy metal (mainly Zn) content of the nucleus and mitochondria accompanies the TPA-induced increase of heavy metals in the cytosol. Zinc seems to be essential for the function of several proteins, e.g., enzymes involved in RNA and DNA synthesis (Bertini et al., 1986). Therefore the TPA-induced depletion of zinc in the nucleus might be related to the antiproliferative activity of TPA, the arrest of $G_1 \rightarrow S$ phase transition (Gescher, 1985; Dröge, 1986). In this way the TPA-induced changes in the availability and distribution of heavy metals observed might show a new way of action of TPA. The fact that diacyl-glycerols have very similar effects like TPA probably reflects the physiological significance of this phenomenon.

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