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Calcium dependence of glucocorticoid-induced lymphocytolysis

(ionophore/thymus/lymphocytes/cytolysis)

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ABSTRACT **A potent glucocorticoid, triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide) and a divalent cation ionophore (A23187) had similar effects *in vitro* on [^{3}H]uridine uptake and on lysis of thymocytes of adrenalectomized rats. Removal of Ca^{2+} from the medium blunted the cytolytic action of triamcinolone acetonide and virtually eliminated that of A23187. In Ca^{2+}-free media, treatment of the thymocytes for 15 hr with triamcinolone acetonide or A23187 followed by re-introduction of Ca^{2+} resulted in a rapid decrease in cell survival. Based on the time courses of the responses, triamcinolone acetonide and A23187 evoked proportionate increases in ^{45}Ca uptake and lysis of the thymocytes. These findings implicate enhanced Ca^{2+} uptake in glucocorticoid-dependent lymphocytolysis.**

Glucocorticoid-induced lymphocytolysis was first described in the 1940s (1), but attempts to define the key biochemical event in this process are still in progress. The effects of glucocorticoids on lymphocytes include diminution of glucose, amino acid, uridine, and Rb^+ uptake (2–4), of incorporation of precursors into RNA, DNA, and protein (3, 5, 6), of RNA polymerase activity (3), of oxidation of free fatty acids (7), and of ATP levels (4, 8). The diversity of these effects and the complexity of their interrelationships have frustrated efforts to assign a key role to any one process in the lymphocytolytic response (9, 10).

Morphological studies show that nuclear disruption is an early consequence of glucocorticoid action (10–12), implying that cytolysis may result from changes in the intracellular milieu. We propose that one such change could be an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). The findings of Makman *et al.* (3) of glucocorticoid inhibition of Rb^+ uptake and of incorporation of orthophosphate into ATP in thymocytes lend some credence to this hypothesis. Evidence has been provided that Ca^{2+} inhibits the activity of the transport enzyme (Na^++K^+)-ATPase, the transducing system for K^+ (or Rb^+) uptake (13, 14), and competes with oxidative phosphorylation for electron transport energy (15).

MATERIALS AND METHODS

Male Sprague-Dawley rats (120–160 g) were adrenalectomized and maintained on saline drinking water for 4–14 days before use. Triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide) Squibb; TA was obtained as a gift from Dr. F. Rosen and A23187 (Eli Lilly Co.) from Dr. R. L. Hamill. Cortisol was purchased from Calbiochem, progesterone from Sigma, and cortexolone (17α,21-dihydroxy-4-pregnene-3,20-dione) from Steraloids. The steroids and A23187 were stored in absolute ethanol at −20°. The complete protein-free RPMI 1640 culture medium (16) was obtained from Grand Island Biological Co. and $Ca(NO_3)_2$–$MgSO_4$-deficient RPMI 1640 (Ca^{2+}–Mg^{2+}-free RPMI 1640) from Associated Biomedic Systems. Ca^{2+}-free RPMI 1640 was prepared from Ca^{2+}–Mg^{2+}-free RPMI 1640 by addition of $MgSO_4$ (final concentration = 1 mM) and ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA; final concentration = 0.5 mM). Mg^{2+}-free RPMI 1640 was prepared from Ca^{2+}-Mg^{2+}-free RPMI 1640 by addition of $Ca(NO_3)_2$ (final concentration = 0.4 mM) and sodium pyrophosphate (final concentration = 0.5 mM). The concentrations of Ca^{2+} and Mg^{2+} in the modified RPMI 1640 incubation media, as determined by atomic absorption spectrometry, were: 0.4 mM Ca^{2+} and 0.4 mM Mg^{2+} in complete medium, traces of Ca^{2+} and 1 mM Mg^{2+} in Ca^{2+}-free medium, and 0.3 mM Ca^{2+} and <0.1 mM Mg^{2+} in Mg^{2+}-free medium. The pH of the incubation media was 7.25 ± 0.05 at 25°, when gassed with 95% O_2:5% CO_2. [5-^{3}H]Uridine (25–30 Ci/mmol), [*G*-^{3}H]inulin (136.72 mCi/g), and $^{45}CaCl_2$ (24.5 mCi/mg) were obtained from New England Nuclear.

Preparation and Incubation of Cell Suspensions. All of the experimental procedures were done in siliconized glassware at 4°, unless otherwise specified. Suspensions of thymocytes were prepared from three to seven pooled thymuses in complete RPMI 1640 medium or in Ca^{2+}- or Mg^{2+}-free RPMI 1640, as described (17, 18). Five milliliters of cell suspensions (3.5×10^7–4.5×10^7 cells per ml) were incubated in Erlenmeyer flasks for up to 20 hr at 37° in a 95% O_2:5% CO_2 atmosphere with gentle shaking. Incubations for 4 hr or longer contained penicillin (100 units/ml) and streptomycin (100 μg/ml).

Cell Viability Studies. At the end of the incubation, the flasks were placed on ice, gassed gently with 95% O_2:5% CO_2, sealed with Parafilm, and stored for an hour or less. Viability was determined by transferring an aliquot of the cells to ice-cold RPMI 1640 medium with 1/10 volume of 4% trypan blue solution (19). The unstained cells were counted in a hemocytometer within 5–10 min of exposure to the dye and were taken as the viable cell population.

Uptake and Incorporation of Uridine. Five milliliters of thymocyte suspensions were incubated for 3 hr at 37° with diluent, TA (10^{-7} M), or A23187 (5×10^{-7} M) in complete, Ca^{2+}-free, or Mg^{2+}-free medium. At the end of 2.5 hr of incubation, 1-ml triplicate aliquots of the cells were labeled with [^{3}H]uridine (0.5 μCi/ml) for 30 min. The cells were cooled in ice, separated from the medium by centrifugation ($100 \times g$ for 5 min), and washed twice with 3 ml of the same fresh medium by resuspension and recentrifugation. The pellets were treated with 1 ml of 5% trichloroacetic acid (TCA). The TCA-soluble (uptake) and the TCA-insoluble (incorporation into RNA) fractions were assayed for radioactivity as described (17). [^{3}H]Uridine uptake was calculated from the ^{3}H content of the TCA-soluble fraction. The washing procedure eliminated the need for correction for extracellular contamination. [^{3}H]Uridine

Abbreviations: TA, triamcinolone acetonide (9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione-16,17-acetonide); cortexolone, 17α,21-dihydroxy-4-pregnene-3,20-dione; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; TCA, trichloroacetic acid.

Table 1. Effects of TA on [^{3}H]uridine metabolism

Incubation medium	Control	TA	Difference
	(cpm/10^7 cells)		
Complete			
TCA-soluble (7)	6357 ± 688	3914 ± 600	2443 ± 113*
TCA-insoluble (6)	742 ± 70	407 ± 37	335 ± 60*
Ca^{2+}-free			
TCA-soluble (6)	4833 ± 675	3240 ± 366	1593 ± 364†
TCA-insoluble (5)	536 ± 74	335 ± 36	201 ± 43†
Mg^{2+}-free			
TCA-soluble (5)	7941 ± 1094	4865 ± 627	3076 ± 606†
TCA-insoluble (4)	793 ± 91	427 ± 42	366 ± 73†

The effect of TA on uridine uptake and incorporation into RNA was assayed as described in *Materials and Methods*. The number of separate experiments is in parentheses. Results are mean ± SEM.
* Denotes $P < 0.005$.
† Denotes $P < 0.02$.

Table 2. Effects of A23187 on [^{3}H]uridine metabolism

Incubation medium	Control	A23187	Difference
	(cpm/10^7 cells)		
Complete			
TCA-soluble (7)	6357 ± 688	3850 ± 485	2507 ± 279*
TCA-insoluble (6)	742 ± 90	497 ± 69	245 ± 30*
Ca^{2+}-free			
TCA-soluble (6)	4833 ± 675	4384 ± 742	449 ± 353
TCA-insoluble (5)	536 ± 74	405 ± 18	131 ± 57
Mg^{2+}-free			
TCA-soluble (5)	7941 ± 1094	1491 ± 369	6450 ± 1217†
TCA-insoluble (4)	793 ± 91	98 ± 23	695 ± 113†

The effect of A23187 on uridine uptake and incorporation into RNA was assayed as described in *Materials and Methods*. The number of separate experiments is in parentheses. Results are mean ±SEM.
* Denotes $P < 0.001$.
† Denotes $P < 0.01$.

incorporation was estimated from the ^{3}H content of the TCA-insoluble fraction. The cells were counted at the time of labeling with [^{3}H]uridine, and results were calculated per 10^7 viable cells.

Calcium Uptake Studies. Five milliliters of cell suspensions were incubated in complete RPMI 1640 medium at 37° for 5–14 hr. Parallel incubations contained the diluent, TA (10^{-7} M), or A23187 (5×10^{-7} M). ^{45}Ca (1 μCi/5 ml) and [^{3}H]inulin (1 μCi/5ml) were added to the media 1 hr before termination of the incubation. The flasks were then cooled on ice for 5 min and the cells collected by centrifugation at $100 \times g$ for 5 min. The pellets were dissolved in 0.3 M NaOH at 37° overnight, and aliquots assayed for ^{45}Ca and ^{3}H contents. Aliquots of the media were also assayed, and ^{45}Ca content in the cell pellet was corrected for extracellular ^{45}Ca contamination using [^{3}H]inulin as a marker. Corrections for counting efficiencies and spillover were made by the external standard ratio method. Aliquots of the suspensions were counted for viable cell content at the time of addition of the ^{45}Ca, and uptake was expressed as fmol per 10^7 viable cells.

Statistics. All results were analyzed by a two-tailed paired sample Student's *t* test (20), because each experiment made use of aliquots from a single pool of cells from three to seven rats.

RESULTS

Ca^{2+} and Mg^{2+} Dependence of [^{3}H]Uridine Metabolism. Glucocorticoid inhibition of uridine uptake and incorporation into RNA precedes cell death (2). In complete RPMI 1640 medium, however, A23187, unlike TA, kills significant numbers of thymocytes in the first 2.5 hr of exposure. At 0 time, 95% or more of the cells were viable in all preparations. At 2.5 hr in the complete medium, viable cell counts (normalized to those at zero time) were 90.3 ± 3.4%, 84.7 ± 4.8%, and 55.0 ± 6.1% in the control, TA-treated, and A23187-treated preparations, respectively. Accordingly, results obtained on [^{3}H]uridine metabolism were expressed per 10^7 viable cells.

Removal of Ca^{2+} from the medium tended to impair [^{3}H]-uridine uptake and incorporation into RNA, and Mg^{2+} deficiency tended to increase these rates, but none of these changes was statistically significant (Table 1). TA and A23187 had similar effects on uridine metabolism in the complete medium; both agents inhibited [^{3}H]uridine uptake by about 40% and incorporation into RNA by 45% (TA) and 33% (A23187) (Tables 1 and 2). In Ca^{2+}-free media, TA had lesser but significant effects on both [^{3}H]uridine uptake and incorporation into RNA, and those of A23187 were almost nil. Under Mg^{2+}-free conditions, the response to TA was preserved (Table 1), and that to A23187 was significantly enhanced (Table 2).

Thymocyte Viability. In complete RPMI 1640, there was little change in the trypan blue-impermeable cell counts after 12–13 hr of incubation (Table 3). Steroid specificity was explored with analogues, including cortisol (agonist) and cortexolone and progesterone (partial agonists-antagonists). This classification is based on assays in rat thymocytes (21–23). Maximal inhibitory concentrations of TA and cortisol reduced the viable cell count about 50%. Cortexolone and progesterone at even higher concentrations than the agonists were minimally cytolytic. Thus, the incubation conditions and the dye exclusion

Table 3. Steroid specificity of lymphocytolysis (12- to 13-hr incubations)

Treatment	Cell counts (%)*	Δ%†
Control	97.1 ± 4.2 (8)	—
TA (10^{-7} M)	52.1 ± 6.3 (8)	−46.3
Cortisol (10^{-6} M)	46.7 (1)	−51.9
Cortexolone (10^{-5} M)	81.2 ± 1.0 (4)	−16.4
Progesterone (10^{-5} M)	81.4 ± 1.2 (4)	−16.2

Rat thymocytes (3.5–4.5 × 10^7 cells per ml) were incubated in complete RPMI 1640 medium with steroids as indicated. Incubations were for 12–13 hr at 37°. At the end of the incubation period the dye-impermeable cell counts were measured. Each experiment included a control, an agonist, and a partial agonist-antagonist with the same population of thymocytes. The number of experiments is in parentheses.
* Compared to untreated cells at 0 time (mean ±SEM).
† Compared to control cells at 12–13 hr.

Table 4. Effects of TA and A23187 on viable cell counts of thymocytes (15- to 16-hr assay)

Incubation medium	Treatment	Cell counts (%)*	Δ%†
Complete	Control	84.9 ± 4.7 (13)	—
	TA (10^{-7} M)	27.3 ± 3.1 (13)	−67.8
	A23187 (5×10^{-7} M)	20.3 ± 2.3 (13)	−76.1
Ca^{2+}-free	Control	68.1 ± 3.9 (13)	—
	TA (10^{-7} M)	49.1 ± 3.7 (13)	−27.9
	A23187 (5×10^{-7} M)	56.7 ± 2.9 (13)	−16.7
Mg^{2+}-free	Control	95.2 ± 10.6 (4)	—
	TA (10^{-7} M)	52.4 ± 5.9 (4)	−45.0
	A23187 (5×10^{-7} M)	34.5 ± 9.5 (4)	−63.8

Rat thymocytes were incubated for 15–16 hr as described in the *text*. The number of experiments is in parentheses.

* Compared to untreated cells in the same medium at 0 time by dye exclusion assays (mean ±SEM).

† Compared to control cells in the same medium for 15–16 hr.

test are suitable for assays of glucocorticoid-specific cytolysis of thymocytes *in vitro*.

Ca^{2+} and Mg^{2+} Dependence of the Cytolytic Response. TA- and A23187-induced cytolysis was assayed in complete, Ca^{2+}-free and Mg^{2+}-free RPMI 1640 media. Removal of Ca^{2+} and addition of EGTA impaired cell survival but protected against the cytolytic action of TA and A23187 (Table 4). In the complete medium TA and A23187 reduced cell survival by about 70%. In Ca^{2+}-free medium, the comparable cell losses were 28% and 17% with TA and A23187, respectively. The effectiveness of the incubation media in supporting the cytolytic activity of TA or A23187 was: complete > Mg^{2+}-free > Ca^{2+}-free.

EGTA (0.5 mM) was added to the Ca^{2+}-free media to minimize the amount of free Ca^{2+}. To ensure that EGTA did not in itself impair the cytolytic response, 0.5 mM EGTA and added Ca^{2+} sufficient to maintain the usual Ca^{2+} concentration (0.4 mM) were included in the RPMI 1640 medium and the response to both agents was assessed. In the EGTA-modified media, TA (10^{-7} M) lysed 70% and A23187 (5×10^{-7} M) 57% of the thymocytes; these responses were indistinguishable from those obtained in the conventional RPMI 1640 medium.

If glucocorticoid action requires the presence of extracellular Ca^{2+}, addition of Ca^{2+} to cells that had been exposed to glucocorticoids in Ca^{2+}-free medium should evoke cytolysis with minimal latency. As shown in Fig. 1, incubation for 15 hr in complete medium supplemented with TA or A23187 decreased the number of viable cells by about 73%. In the absence of Ca^{2+}, there was a 29% decrease in viable cells with TA and a 12% decrease with A23187. Addition of Ca^{2+} to the Ca^{2+}-free medium and incubation for an additional 3 hr had no effect on the viable cell content of control samples. In the presence of TA or A23187, however, addition of Ca^{2+} accelerated the loss of cells almost 2-fold ($P < 0.02$) as compared to those maintained in the Ca^{2+}-free media (middle and right-hand panels, Fig. 1).

There were considerable differences in the sensitivity of various preparations of thymocytes to glucocorticoid-induced cytolysis in the complete medium. Thus, we analyzed the results based on the criterion of either greater than or less than 60% cell survival after 15 hr of incubation with TA. The former were classified as resistant populations and the latter as sensitive. Fewer than 30% of the cells in the sensitive populations survived after 15 hr of incubation in TA (middle panel, Fig. 1). In contrast, more than 65% of the cells in the resistant populations survived under the same conditions (middle panel, Fig. 2). In

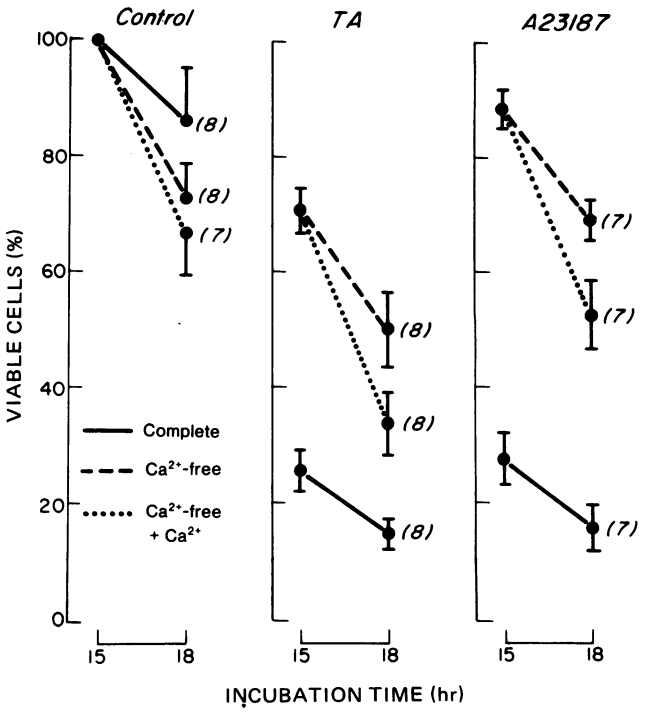

FIG. 1. Ca^{2+}-dependence of glucocorticoid- and ionophore-induced lymphocytolysis in sensitive cells. Thymocyte suspensions (3.5 to 4.5 × 10^7 cells per ml) in either complete or Ca^{2+}-free media were supplemented with the diluent (control), TA (10^{-7} M), or A23187 (5×10^{-7} M), and incubated at 37° for 15–18 hr. Viability was determined by the dye exclusion method at 15 and 18 hr. The number of viable cells in the complete or Ca^{2+}-free media of control flasks at 15 hr was used as the reference quantity (100%) for the effects of TA and A23187 in comparable media. In the controls, the 15-hr cell survival in complete medium compared to zero time was 74.3 ± 5.7% (n = 8 incubations); in Ca^{2+}-free medium, it was 62.6 ± 3.5% (n = 15 incubations). At 15 hr, $CaCl_2$ (final concentration = 1.0 mM) was added to one of each pair of incubations in Ca^{2+}-free media. All flasks were then incubated for an additional 3 hr. The circles and the lengths of the vertical lines indicate the mean ±SEM. The number of separate experiments is given in parentheses.

Ca^{2+}-free medium, addition of either TA or A23187 decreased the number of viable cells by about 10% and addition of Ca^{2+} had little further effect in the 15- to 18-hr interval (Fig. 2). Addition of Ca^{2+} to the Ca^{2+}-free controls maintained the viable cell counts at the same level as in complete medium (left-hand panel, Fig. 2). These results indicate that addition of Ca^{2+} to cells that had been treated with TA or A23187 in Ca^{2+}-free media did not elicit the cytolytic response in populations resistant to TA.

Time-Course of Cytolysis and ^{45}Ca Uptake. The dependence of glucocorticoid- and ionophore-induced lymphocytolysis on extracellular Ca^{2+} suggests that changes in Ca^{2+} transport may be involved in the cytolytic response. To assess this prediction we incubated thymocytes in ^{45}Ca for 1 hr at various times after continuous exposure to TA or A23187. The uptake of ^{45}Ca was corrected for contamination with extracellular fluid, based on the assumption that the lysed cells are permeable to the extracellular marker, [^{3}H]inulin, and expressed per 10^7 viable cells. TA and A23187 elicited the usual decline in thymocyte viability (Fig. 3A). A23187 lysed 54% of the cells in 13 hr, as compared to 38% lysis with TA, and evoked the response sooner. A similar pattern on ^{45}Ca uptake was obtained. In the controls, cellular uptake of ^{45}Ca was almost nil (Fig. 3B). Both agents produced a curvilinear increase in ^{45}Ca uptake; A23187 acted faster and augmented ^{45}Ca uptake to a

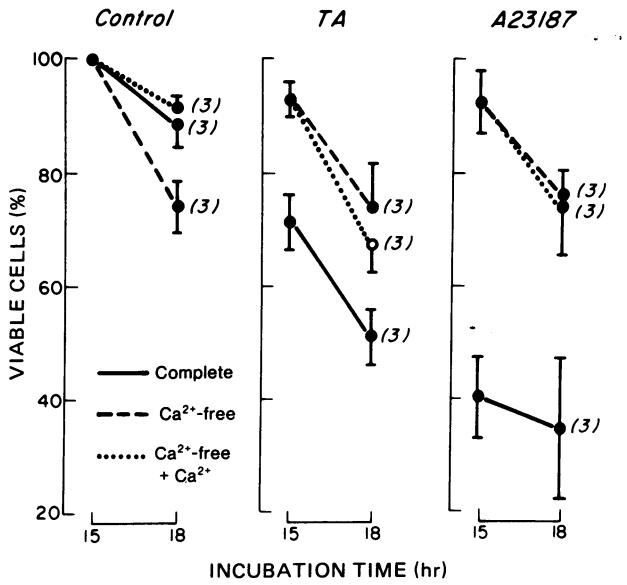

FIG. 2. Ca^{2+}-dependence of glucocorticoid- and ionophore-induced lymphocytolysis in resistant cells. The conventions used in this figure and the experimental details are outlined in the legend of Fig. 1. In the controls, the 15-hr cell survival in complete medium, compared to zero time, was 101.6 ± 1.2% (n = 3 incubations); in Ca^{2+}-free medium it was 74.7 ± 4.5% (n = 6 incubations).

greater extent than TA. Regression analysis of these responses indicates a linear correlation of viability and ^{45}Ca uptake (Fig. 4).

DISCUSSION

A23187, a divalent cation ionophore (24, 25), mimicked two of the characteristic actions of glucocorticoids on thymocytes, inhibition of uridine metabolism (uptake and incorporation) and cytolysis (Tables 1, 2, and 4). The inhibitory effect of A23187 on uridine metabolism was markedly dependent on Ca^{2+} in the medium. The inhibitory effect of TA on uridine metabolism was only partially sensitive to the presence of Ca^{2+} in the medium. The interpretation of these results is complicated by the failure of Ca^{2+}-free media to maintain normal levels of [^{3}H]uridine uptake and incorporation into RNA in the absence of these agents.

Thymus lymphocytes consist of large blast cells and nonproliferating small lymphocytes (26, 27). The small lymphocytes are lysed by glucocorticoids, both *in vivo* and *in vitro* (27, 28). By isolating cells from adrenalectomized rats, we obtained cell suspensions composed mostly of glucocorticoid-sensitive cells. On occasion, however, glucocorticoid-resistant cell populations were obtained, perhaps because of higher than usual proportions of thymic blast cells. To explore the role of Ca^{2+} in glucocorticoid-induced lymphocytolysis, we analyzed the results on the basis of two populations of cells, those with marked sensitivity compared to those with resistance to the cytolytic action of TA (10^{-7} M, 15-hr incubation).

Maximal inhibition of [^{3}H]uridine uptake and of occupancy of the cytoplasmic receptors is achieved with concentrations of TA of 10^{-7} M or higher*. In the present studies the actions of TA at 10^{-7} M were compared to those of A23187 at a concentration (5×10^{-7} M) that lysed about the same proportion of thymocytes, in complete RPMI 1640 media (Table 4 and Fig.

* N. Kaiser, A. J. Solo, M. Mayer, R. J. Milholland, and F. Rosen, unpublished data.

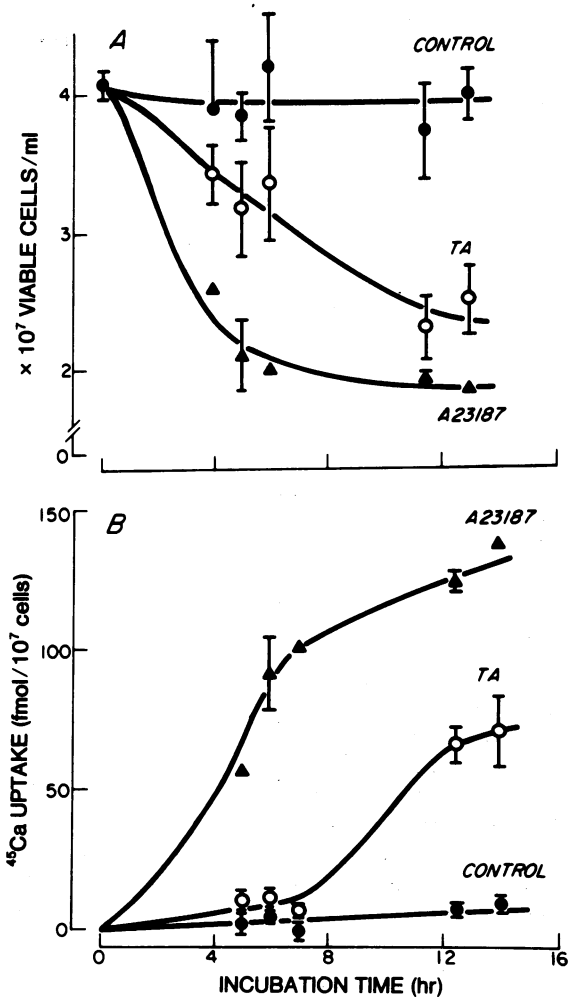

FIG. 3. Effects of TA and A23187 on survival of thymocytes and ^{45}Ca uptake. Suspensions of thymocytes (3.5 to 4.5 × 10^7 cells per ml) were incubated in diluent, TA (10^{-7} M), or A23187 (5×10^{-7} M) for varying periods of time and labeled with ^{45}Ca (1 μCi/5 ml) for the last 60 min of incubation. Cell survival is represented in panel *A* and ^{45}Ca uptake (expressed per 10^7 viable cells) in panel *B*. The points and vertical lines represent the mean ±SEM.

1). In the sensitive populations, removal of Ca^{2+} impaired the lytic actions of both agents; the Ca^{2+}-dependence of the effect of A23187, however, was somewhat more pronounced. In the resistant thymocyte populations, removal of Ca^{2+} from the medium virtually eliminated the lytic actions of both TA and A23187 (Fig. 2). Addition of Ca^{2+} to the Ca^{2+}-free medium augmented cell lysis in the presence of TA or of A23187 in the sensitive but not in the resistant populations (compare Figs. 1 and 2). Moreover, in the absence of TA or A23187 (control incubations) addition of Ca^{2+} to Ca^{2+}-free media either had no effect on cell survival (Fig. 1) or promoted cell survival (Fig. 2).

The similarities in the responses to TA and A23187 raise the possibility that altered distributions of divalent ions, in particular accumulation of Ca^{2+}, may contribute significantly to glucocorticoid-induced lymphocytolysis. A considerable body of evidence indicates that the glucocorticoids induce the synthesis of specific proteins that mediate lymphocytolysis (2, 4). The parallel effects obtained with A23187 and the significant dependence of the action of TA on Ca^{2+} in the medium suggest that the TA-induced proteins may facilitate Ca^{2+} entry into the cell or inhibit Ca^{2+} extrusion from the cell (e.g., by impairing a Ca^{2+}-dependent ATPase). The differences in the time-courses

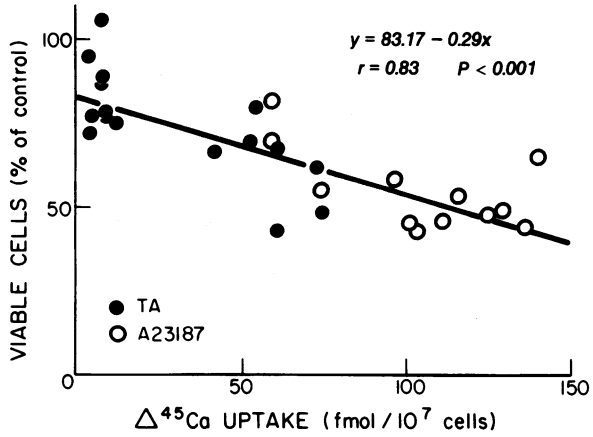

FIG. 4. Analysis of the correlation in TA and A23187 effects on thymocyte survival and ^{45}Ca uptake. Thymocytes were treated as described in the legend of Fig. 3. The viable cell counts, expressed as % of the control, and the absolute increment in ^{45}Ca uptake from that of the controls were computed for each time point from the data shown in Fig. 3.

of action of TA and the ionophore, A23187, presumably reflect the time required for the synthesis of significant quantities of the glucocorticoid-induced proteins. The ionophore, A23187, is expected to act rapidly, simply by binding to the plasma cell membrane. If the glucocorticoid-induced proteins function by either of the mechanisms described above, there should be an increase in the intracellular acquisition of Ca^{2+} from the medium. This prediction was tested by measuring the effects of TA and A23187 on ^{45}Ca uptake. As shown in Fig. 3, these agents augmented ^{45}Ca uptake considerably. Moreover, the magnitude of lymphocytolysis was linearly proportional to ^{45}Ca uptake; with both agents, the results fit a single regression relationship ($P < 0.001$) (Fig. 4). Although these results, in conjunction with those on Ca^{2+}-dependence of lymphocytolysis, imply the participation of enhanced Ca^{2+} uptake in the lytic effect, the results shown in Fig. 1 and Table 4 indicate that this effect is not abolished even in the virtual absence of Ca^{2+}. An important contributory role of Ca^{2+}, however, deserves consideration.

Among the known actions of intracellular Ca^{2+}, two may play key roles in destroying thymocytes, inhibition of the Na^+ pump and impairment of mitochondrial oxidative phosphorylation. Ca^{2+} is a powerful inhibitor of $(Na^+ + K^+)$-dependent ATPase, the enzymatic equivalent of the Na^+ pump (13, 14). This proposal is supported by the findings that cortisol depressed the intracellular K^+:Na^+ ratio of the thymus *in vivo* and inhibited the uptake of ^{86}Rb (a K^+ tracer) *in vitro* (4, 29). In addition, Ca^{2+} transport into isolated mitochondria competes with phosphorylation of ADP for electron transport energy (15). It is noteworthy that Makman *et al.* (4) found that cortisol impaired phosphorylation of ADP in intact thymocytes prior to significant cytolysis.

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