Calcium Modulation Activates Epstein-Barr Virus Genome in Latently Infected Cells

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In many viral infections the host cell carries the viral genome without producing viral particles, a phenomenon known as viral latency. The cellular mechanisms by which viral latency is maintained or viral replication is induced are not known. The modulation of intracellular calcium concentrations by calcium ionophores induced Epstein-Barr viral antigens in lymphoblastoid cell lines that carry the virus. When calcium ionophores were used in conjunction with direct activators of protein kinase C (12-*O*-tetradecanoyl phorbol-13-acetate and a synthetic diacylglycerol), a greater induction of viral antigens was observed than with either agent alone. Activation of protein kinase C may be required for the expression of the viral genome.

LYMPHOID CELL LINES INFECTED with Epstein-Barr virus (EBV) contain multiple copies of the viral genome and express the EBV-specific nuclear antigen EBNA (*1*). In these cells the spontaneous induction of the viral replicative cycle and particle synthesis normally either does not occur (latency) or occurs at a low rate (*2*). The latency can be overcome in vitro by superinfection with the lytic strain P_3HR_1 of EBV. The mechanism of induction of viral antigen expression after superinfection is unknown. Several chemically unrelated agents also induce such expression (*3–7*). Tumor promoters are among the inducers of the EB viral replicative cycle, whereas chemically related compounds that are devoid of tumor-promoting activity are not (*8*). We have noticed that tumor promoters that induce EBV genome activation also activate protein kinase C (*9, 10*). Since this enzyme depends on intracellular Ca^{2+} (*11*), and since changes of free cytosolic $[Ca^{2+}]$ have been implicated in the regulation of many cellular events (*12*), we examined the effect of modifying intracellular $[Ca^{2+}]$ in lymphoblastoid cell lines that carry the EBV genome.

After 3 days of culture in the presence of the calcium ionophore A23187, there was a significant induction of early antigens (EA's) in the Raji cell line (Table 1). Both viral capsid antigens (VCA's) and EA's increased dramatically in the two EBV-producer cell lines B95-8 and P_3HR_1. Using a newly established cell line obtained by in vitro immortalization of human peripheral blood lymphocytes by the transforming strain B95-8 of EBV led to similar induction. The dose responses of EA induction in Raji cells by the two ionophores A23187 and ionomycin are shown in Fig. 1A. The time course of induction is similar to that described for 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (*4*) (Fig. 1B). The maximum number of induced cells was obtained at 72 hours of treatment; thereafter a slow decline (probably due to cell death) was observed. The continuous presence of the calcium ionophores was not required; 3 hours of treatment was sufficient to elicit the maximum response. Since TPA both induces viral antigens and activates protein kinase C (*4, 9*), we tested the effects of the synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG), which also activates the enzyme (*13*). When Raji cells were cultured in the presence of this compound at a concentration of 50 μg/ml, a low induction of EA was observed (Fig. 1B).

To establish whether the effect of the calcium ionophores was due to their role as Ca^{2+} channel activators, we performed three experiments in Ca^{2+}-free medium and in the presence of EGTA (Fig. 2A). Treatment of Raji cells with the calcium ionophore in the absence of extracellular Ca^{2+} inhibited induction of EA; in contrast, the effect of TPA was fully expressed. A full response was observed when the cells that were incubated in the Ca^{2+}-free medium were washed, resuspended in medium containing Ca^{2+}, and restimulated with the ionophore.

To further determine whether induction of antigen expression by the calcium ionophores could be correlated with changes in the intracellular $[Ca^{2+}]$, we loaded Raji cells with Quin 2-AM (Calbiochem) (*14*). The basal free intracellular $[Ca^{2+}]$ in Raji cells was $47 \times 10^{-9} M$ (Fig. 2B). After the addition of ionomycin, $[Ca^{2+}]$ increased within 2 minutes to $12 \times 10^{-6} M$. If ionomycin was added to cells resuspended in Krebs-Ringer solution in the absence of Ca^{2+}, intracellular Ca^{2+} did not increase.

In conclusion, the modulation of intracellular Ca^{2+} seems to be of primary importance in activating the EBV genome. The Ca^{2+} is a second messenger for various ligand-receptor interactions (*12*), and it is important in activating several cellular functions; this activation is believed to depend on the action of at least two protein kinases: calmodulin-dependent protein kinase and protein kinase C. A role of calcium in regulating the infection of human B lymphocytes by EBV has been suggested by Nemerow and Cooper (*15*), who showed that calmodulin inhibitors blocked EBV transformation. Kaibuchi *et al.* (*16*) in a study of a platelet system demonstrated that activation of protein kinase C and mobilization of calcium were both essential for the expression of the biological activity (serotonin

Table 1. Effect of the calcium ionophore A23187 on viral antigen expression. Cells were cultured for 72 hours in RPMI 1640 medium with 10% fetal calf serum and antibiotics in the presence or absence of the calcium ionophore A23187 at a concentration of $1 \times 10^{-6} M$. The cells were washed, air-dried in multiwells on Teflon-coated slides, fixed in cold acetone for 10 minutes, and tested by indirect immunofluorescence for the presence of viral capsid antigen (VCA) and early antigen (EA) (*27*). A VCA^+ EA^+ serum (titer 1:1280) from a nasopharyngeal carcinoma patient and a VCA^+ EA^- serum (VCA titer 1:320) at a dilution of 1:20 were used for staining. Untreated cultures were used as controls. After 45 minutes of incubation, the slides were washed three times with phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}, dried, and stained with fluorescein isothiocyanate–conjugated goat antiserum to human immunoglobulin G (Cappel) at a dilution of 1:15. After an additional 45 minutes of incubation, the slides were washed and examined by fluorescence microscopy. At least 500 cells were counted in each assay. Results are expressed as the mean of triplicates ±SEM and are representative of several experiments.

Cell line	Experiments (no.)	Positive cells after 72 hours of culture (%)			
		No treatment		A23187	
		VCA	EA	VCA	EA
Raji*	15	0.1	0.1	0.1	2.4 ± 0.8
P_3HR_1†	5	3.4 ± 1.2	4.6 ± 0.5	25.3 ± 6.1	13.1 ± 3.5
B95-8‡	5	2.5 ± 0.6	5.6 ± 1.4	41.2 ± 7.0	24.5 ± 5.2
PG-2§	3	1.2 ± 0.4	1.4 ± 0.5	8.4 ± 1.2	11.3 ± 2.7

*EBV nonproducer. †Producing lytic EBV. ‡Producing transforming EBV. §Human peripheral blood lymphocytes immortalized in vitro by B95-8 EBV.

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release). This observation has been extended to *N*-acetylglucosaminidase (*17*) and superoxide (*18*) release by neutrophils and insulin release from pancreatic islets (*19*). A synergistic effect between TPA and calcium ionophores can also be demonstrated for the expression of EBV antigens (Fig. 3). Adding noninducing doses of ionophore or TPA simultaneously to Raji cells led to marked expression of EA (Fig. 3A). Moreover, a combination of their optimal doses led to greater expression than was obtained by the sum of the two separate effects (Fig. 3B).

Synergism between OAG and ionomycin has also been demonstrated. Noninducing concentrations of OAG (10 μg/ml) and ionomycin ($5 \times 10^{-8} M$) added together induced levels of EA expression similar to those observed with the optimal concentration of OAG (*20*). The possible role of protein kinase C in the induction of EA was investigated through the use of a highly specific inhibitor of this enzyme, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) (*21*). Raji cells exposed to this inhibitor at 50 μM [1 hour before the addition of TPA (30 ng/ml)] failed to induce detectable EA 72 hours after treatment. On the other hand, trifluoperazine (a calmodulin inhibitor), had no effect on the EA induction by TPA (*22*). The described effects indirectly implicate activation of protein kinase C. Since in many receptor-linked processes activation of this kinase is a prerequisite for the final expression of a cellular phenotype, the interaction of a permissive cell with an ap-

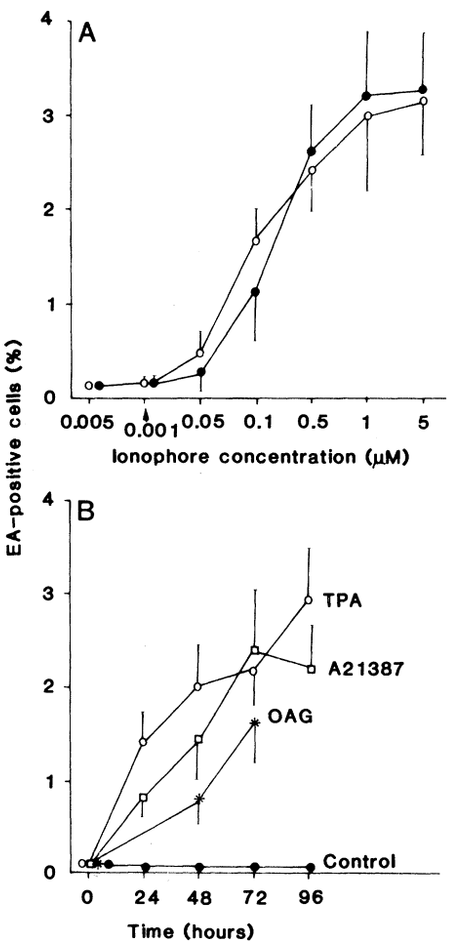

Fig. 1. (A) Dose response of EA induction in Raji cells exposed to different concentrations of calcium ionophores. Raji cells were resuspended in culture medium [RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Flow), 2 m*M* glutamine, penicillin, and streptomycin] in 5-ml portions of 3×10^5 cells per milliliter. Calcium ionophores A23187 (Sigma) and ionomycin (Calbiochem) were diluted from a 5-m*M* stock solution in dimethyl sulfoxide and added to give the desired final concentration. After 72 hours of culture at 37°C in a humidified CO_2 atmosphere the cells were analyzed for EA. (B) Time course for the EA induction by A23187 ($1 \times 10^{-6} M$), TPA (30 ng/ml) (Sigma), and the synthetic diacylglycerol OAG (50 μg/ml) (Avanti Polar Lipids). Means of triplicates ±SE from representative experiments are shown.

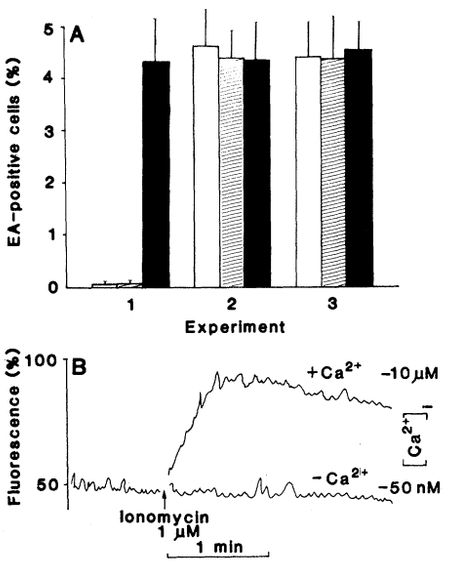

Fig. 2. (A) Inhibition of the calcium ionophore–induced EA expression by depletion of extracellular Ca^{2+}. Inducers: open bars, A23187 (1 μM); hatched bars, ionomycin (1 μM); and filled bars, TPA (30 ng/ml). (Experiment 1) Raji cells were resuspended (3×10^5 cell/ml) in Krebs-Ringer solution in the absence of Ca^{2+}: 125 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* KH_2PO_4, 2.4 m*M* $MgSO_4$, 25 m*M* Hepes (*p*H 7.4), 6 m*M* glucose, supplemented with 1 m*M* EGTA. (Experiment 2) Raji cells were resuspended in Krebs-Ringer solution with 2 m*M* $CaCl_2$ and lacking EGTA; the inducers were added. Cells in experiments 1 and 2 were incubated at 37°C for 4 hours, washed three times with phosphate-buffered saline, resuspended in complete tissue culture medium and cultured for an additional 72 hours without further addition of inducers. (Experiment 3) Cells were incubated in Ca^{2+}-free Krebs-Ringer solution with inducers for 4 hours, washed, resuspended in complete medium containing the inducers, and cultured for 72 hours. Bars represent the mean ±SEM of triplicates. (B) Effect of ionomycin on intracellular concentrations of Ca^{2+}. Cells suspended in Ca^{2+}-free Krebs-Ringer solution to a density of 10^7 cell/ml were mixed with a stock solution of 10 m*M* Quin 2-AM in dimethyl sulfoxide to a final concentration of 100 m*M*, incubated for 20 minutes at 37°C, diluted tenfold, and further incubated for 40 minutes. Finally the cells were washed and resuspended in Krebs-Ringer (containing Ca^{2+}) or in Krebs-Ringer Ca^{2+}-free solution containing 1 m*M* EGTA. Fluorescence was determined with a recording spectrofluorimeter (Perkin-Elmer 650-40) (emission, 492 nm; excitation, 339 nm).

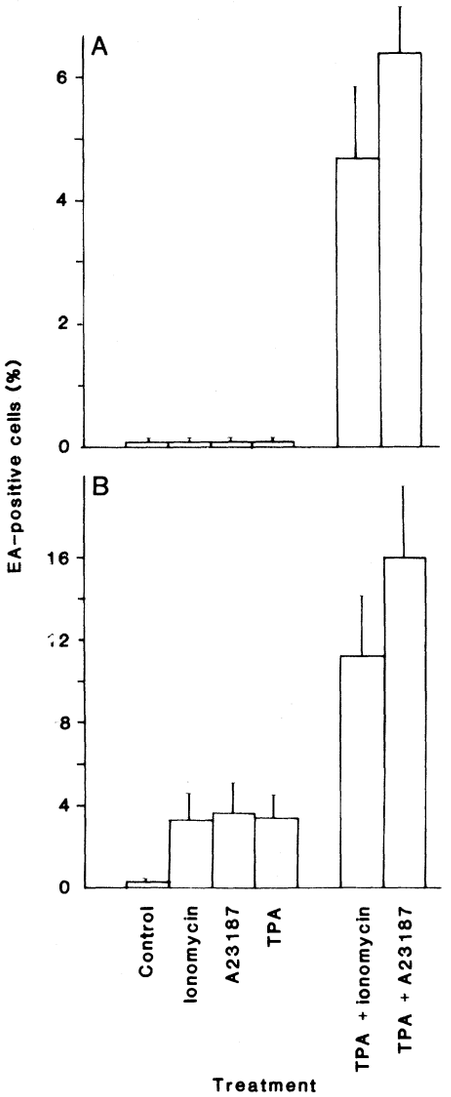

Fig. 3. Cooperative action of TPA and calcium ionophores in the induction of EA in Raji cells. The Raji cell suspension (5 ml, 3×10^5 cell/ml) in complete tissue culture medium was stimulated by the simultaneous addition of (A) noninducing concentrations of TPA (1 ng/ml) and ionomycin ($1 \times 10^{-8} M$) or A23187 ($1 \times 10^{-8} M$). The cells were cultured for 72 hours and the EA determined. (B) Optimal doses of TPA (30 ng/ml), ionomycin ($10^{-6} M$), or A23187 ($10^{-6} M$) were used alone or added simultaneously. Control cells had no additions. Bars represent the mean of triplicates +SEM.

propriate membrane-perturbing agent [such as a virus, an agent that cross-links immunoglobulins (*23, 24*), or a membrane fluidizer (*5*)] may trigger a similar metabolic activation, with the final consequence of transcribing the viral genome. Recently, we and others have documented changes in protein kinase C activity (*25*) and intracellular [Ca^{2+}] (*26*) that depend on the proliferative state of the cells. Certain inhibitors of cell division, such as bromodeoxyuridine, iododeoxyuridine, or mitomycin C, which do not directly activate protein kinase C or modulate Ca^{2+}, also induce EBV antigen expression (*3*). They could act by arresting the cells in a phase of the cell cycle in which the changes of intracellular [Ca^{2+}] or protein kinase C activity would favor the spontaneous expression of the viral genome. At present, we have no evidence to support this hypothesis nor to exclude other possible mechanisms by which these compounds could interfere with the regulation of viral latency.

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$$[Ca] = K_d (F - F_{min})/(F_{max} - F)$$

where K_d is $1.15 \times 10^{-7} M$, F_{max} is the signal detected after lysis of the cells with Triton X-100 (0.05%), and F_{min} is the signal after lysis with Triton X-100 and addition of EGTA (4 m*M*).
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