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Cocaine Potentiates the Switch between Latency and Replication of Epstein–Barr Virus in Raji Cells

Paolo Di Francesco,*[,1] Antonella Lisi,† Sabrina Rieti,† Vanessa Manni,† Settimio Grimaldi,† and Enrico Garaci*

**Department of Experimental Medicine and Biochemical Sciences, Microbiology, University of Rome Tor Vergata, Rome, Italy; and †Institute of Experimental Medicine, CNR, Rome, Italy*

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This paper shows that cocaine amplifies Epstein–Barr virus (EBV) reactivation in Raji cells. Its effect on early viral protein synthesis was maximal when it was added with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) plus n-butyrate, but nil when added alone. The enhancing effect of cocaine on early replicative stages of latent EBV was associated with an increase of Ca^{2+} mobilization induced by the drug and with an induction of cellular protein phosphorylation in chemicals and cocaine-treated Raji cells. Cocaine also acted synergistically with TPA and n-butyrate to induce Z Epstein–Barr replication activator (ZEBRA), a nuclear phosphoprotein responsible for the activation of early viral gene expression. These findings provide the first evidence that cocaine may represent an important cofactor in the reactivation of early stages of latent EBV infection.

Recent works have shown that cocaine has a direct effect on the susceptibility to viral infection of a pathogen's target cells. Peterson *et al.* (1, 2) found that cocaine potentiates human immunodeficiency virus type 1 (HIV-1) replication in mitogen- or cytomegalovirus-activated human peripheral blood mononuclear cells (PBMC). Bagasra and Pomerantz (3) also found that PBMC treated with cocaine had increased levels of HIV-1 replication after an acute infection *in vitro.* In addition, the rate of HIV-1 production in alveolar macrophages from crack abusers is increased two- to threefold when compared to infected cells from non-smoking subjects (4). We have recently demonstrated that cocaine increases the replication of a cytoplasmic RNA virus, i.e., parainfluenza-1 Sendai virus, and this change in susceptibility to infection is directly related to the alteration in intracellular redox status induced by cocaine (5). Whether cocaine can also affect the induction of the replicative cycle of viral latent infections is at the present unknown.

Epstein–Barr virus (EBV) is a human herpes virus etiologically linked to chronic active virus infection, infectious mononucleosis and closely associated with human malignancies, i.e., Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease (6, 7). EBV can infect human B lymphocytes: cells became immortalized and the viral genome persists in infected cells in a latent state (8). A variety of chemical and biological inducing agents added *in vitro* to the cells can trigger viral reactivation. These include phorbol esters, n-butyrate, calcium modulators, nitric oxide, anti-immunoglobulins and the superinfection with the lytic strain P3HR-1 of EBV (9–11). The capacity to activate replication results initially in the expression of viral gene BZLF1, which encodes a protein termed Z Epstein–Barr replication activator (ZEBRA). This protein is responsible for activation of the viral early gene expression and ultimately viral replication (12, 13).

To investigate the effect of cocaine on EBV reactivation, we utilized a virus-carrying nonproducer human Burkitt's lymphoma cell line (Raji cells). Our data showed that cocaine potentiates the early stages of the viral replicative cycle induced in Raji cells by treatment with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and n-butyrate. Its effect is associated with induction of intracellular Ca^{2+} mobilization and cellular protein phosphorylation and with enhanced expression of ZEBRA.

EXPERIMENTAL PROCEDURES

Cells line. Raji cells were grown in RPMI 1640 (Gibco Lab., Scotland) supplemented with 10% fetal calf serum (Gibco Lab.). These cells are EBV-genome positive non-producing human B-lymphoid cell line that expresses CD21.

In vitro phosphorylation. Raji cells (5×10^6/ml) were rinsed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM Glucose, 5 mM Hepes, pH 7.4) containing 10 mM $MgCl_2$. After washing cells were suspended in 100 (Locke's solution containing 0.4 mM $MgCl_2$ and 0.3 mCi/ml ^{32}P-orthophosphate (Am-

[1] To whom correspondence should be addressed. E-mail: difra@uniroma2.it.

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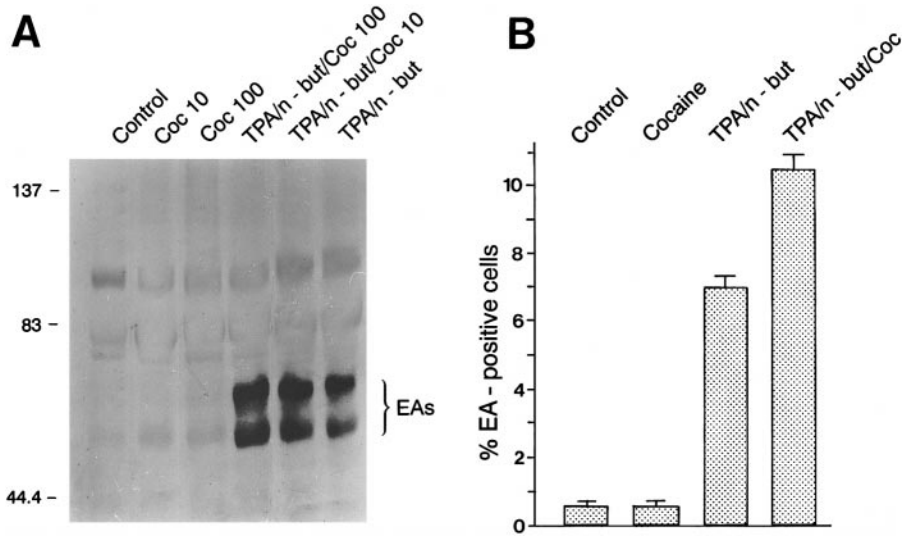

FIG. 1. (A) Effect of cocaine on EBV early antigens (EAs) expression detected by immunoblotting assay. Raji cells were treated for 72 h with cocaine (10 and 100 μg/ml) and chemicals (TPA, 30 ng/ml and n-butyrate, 3 mM). EAs were identified on the basis of their molecular weights and in relation to the position of marker proteins (left of the gel). (B) EBV-EAs detected by indirect immunofluorescence. Raji cells were treated for 72 h with cocaine (100 μg/ml) and chemicals, as in A. Data points represent means ± SE of three different experiments, in triplicate. TPA/n-but/Coc differed significantly from TPA/n-but ($P < 0.05$ using Student's *t* test).

ersham) 1 h on a rocker at room temperature. Cells were then treated with cocaine and chemicals and lysed with isoelectrofocusing buffer (ethylene glycol 33%, NP-40 2.5%, ampholine pH range 3-10 8%, SDS 0.05%, DTT 0.2 mM).

Isoelectric focusing. The first dimension, nonequilibrium isoelectric focusing gel (1.8 × 75 mm) containing a 3% total carrier ampholytes mixture composed of 75% pH 3.5-9.5 25%, pH 5.0-8.0, 9 M Urea, 0.5% Nonidet P-40, 1.6% 3-(3-cholamidopropyl)-dimethylammonio-1-propane sulphonate and 5% acrylamide were run for 1500 Vh. The second dimension was carried out on 6-14% SDS polyacrylamide gradient gel. Incorporation of ^{32}P-label was revealed by autoradiography and, in two-dimensional gels, quantitated with a phosphorimages densitometer.

Immunoblotting for detection of early antigens and ZEBRA. Cells, suspended in SDS sample buffer at a concentration of 1×10^6 cells/ml, were sonicated and boiled prior to electrophoresis through a 10% polyacrylamide gel. After electrophoresis, gel were transferred to nitrocellulose over night and blocked with 5% milk buffer (5% non fat dry milk, 0.03% sodium azide in PBS) for 2 h at 37°C. Then nitrocellulose was incubated with 1:50 dilution of anti-ZEBRA or 1:200 dilution of anti EA specific monoclonal antibodies in milk buffer for 18 h at 4°C, washed with Tween buffer (0.05 mM Tris-HCl, pH 7.4, 0.15M NaCl, 5 mM EDTA, 0.05% Ten Tween 20) and reacted with a 1:100 dilution of anti-mouse IgG biotynilated for 1 h at 4°C, washed with Ten Tween buffer and reacted with a 1:100 dilution streptavidin horseradish peroxidase coniugate, than developed with the peroxidase colorimetric methods.

Immunofluorescence assay. Raji cells were cultured for 72 h in complete medium, starting at a concentration of 5×10^5/ml. The cells were washed, air-dried, fixed in cold acetone for 10 min, and tested by indirected immunofluorescence for the presence of EBV early antigens. An EA+serum (titer 1:1280) from a nasopharyngeal carcinoma patients and an EA-serum from a healthy donor were used for the staining at a dilution of 1:20. After 45 min of incubation, the slides were washed three times with PBS, dried, and stained with fluorescein-isothiocyanate-conjugated goat antiserum to human immunoglobulin G (Cappel, West Chester, PA) at a dilution of 1:15. After further incubation for 45 min the slides were washed and examined by fluorescence microscopy at least 500 cells were counted in each assay.

Detection of intracellular calcium. Cells suspended in Ca^{2+}-free Krebs-Ringer solution were mixed with a stock solution of 10 mM Quin 2-AM in dimethyl sulfoxide to a final concentration of 100 mM, incubated for 20 min at 37°C, diluted tenfold, and further incubated for 40 min. Finally the cells were washed and resuspended in Krebs-Ringer (containing Ca^{2+}) or in Krebs-Ringer $[Ca]^{2+}$-free solution containing 1 mM EGTA. Fluorescence was determined with a recording spectrofluorimeter (Perkin-Elmer 650-40) (emission, 490 nm; excitation, 339 nm). The relation of fluorescence at 492 nm with the concentration of free calcium is determined from the equation: $[Ca]^{2+} = K_d (F - F_{min})/(F_{max} - F)$, where K_d is 1.15×10^{-7} M, F is the fluorescence of the intracellular indicator, F_{max} is designal 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 mM).

RESULTS

EBV reactivation is marked by renewed synthesis of early gene products such as the EBV early antigens (EAs). In order to study the effect of cocaine on EBV-EAs expression, Raji cells were treated for 72 h with different cocaine concentrations (10 and 100 μg/ml) alone or in combination with chemicals, such as TPA (30 ng/ml) and n-butyrate (3 mM). Figure 1 shows that cocaine alone is unable to induce the expression of early antigens tested both by immunoblotting assay (panel A) and by indirect immunofluorescence (panel B). On the contrary, the density of immunoblot lanes 4, 5 and 6 in Fig. 1A showed that the treatment of cocaine, in cells activated with TPA plus n-butyrate, increases the expression of EBV-EAs respect to chemical treatment alone. This enhancing effect was also confirmed by a significant induction of the percentage of EAs-positive Raji cells treated with the combination of drug and chemicals (Fig. 1B). Cell viability and cellular

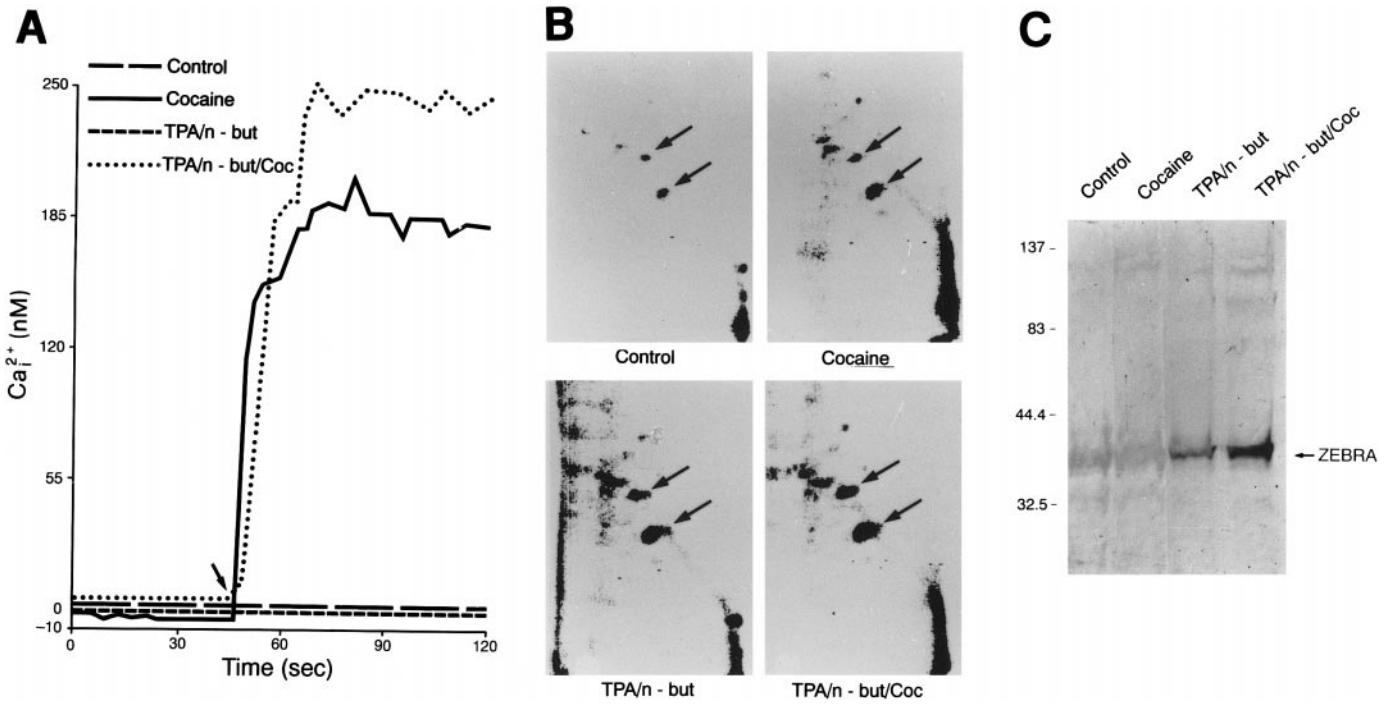

FIG. 2. (A) Effect of cocaine on rise of Ca^{2+} in quin 2-AM loaded Raji cells. Cells were suspended in Krebs-Ringer solution containing 1 mM Ca^{2+}. Arrow indicates the addition of cocaine (100 μg/ml) and chemicals, respectively. The tracings shown were obtained from an experiment representative of four. (B) Effect of cocaine on total protein phosphorylation. Raji cells were incubated with ^{32}P-orthophosphate for 1 h and then treated with cocaine (100 μg/ml) and chemicals for 5 min. Cell homogenates were separated by charge in the first dimension and in SDS-PAGE in the second dimension. Autoradiograph of the gels shows lebelled spots (arrows) corresponding to proteins with the major variation in ^{32}P incorporation. (C) Effect of cocaine on ZEBRA expression. Raji cells were treated with cocaine (100 μg/ml) and chemicals for 72 h. ZEBRA expression was detected with immunoblotting assay and identified on the basis of their molecular weights, in relation to the position of marker proteins (left of the gel).

protein synthesis were not affected by cocaine under the conditions used (data not shown).

Activation of latent EBV is a result of a variety of early intracellular signals (10). Our main objective was to determine whether cocaine was interfering with the mobilization of cytosolic free calcium, namely the most important component of the reactivation stimulus (9, 14). Raji cells were supplied with cocaine (100 μg/ml) and/or chemicals. As seen in Fig. 2A, the free calcium concentration increased upon exposure to the drug, while the treatment with TPA and n-butyrate had no effect on the Ca^{2+} mobilization. Additionally, incubation of the cells with cocaine plus TPA and n-butyrate resulted in the same induction when compared to phorbol ester alone. The results obtained monitoring the free intracellular calcium using the calcium fluorophore quin-2 in our system show that cocaine alone is inducing the influx of extracellular calcium, as also demonstrated in nervous and lymphatic systems (15, 16).

Since protein phosphorylation plays a key role in EBV reactivation (17), we also investigated the effect of cocaine on protein kinase activity. An autoradiograph from Raji cells of the phosphorylated species resolved in two-dimensional gels was shown in Fig. 2B (control panel). Cell exposure to cocaine (100 μg/ml) or chemicals for 5 min resulted in a modulation in the incorporation of ^{32}P in phosphoproteins: some of these proteins are more phosphorylated (see spots indicated by arrows in the panels). Interestingly, TPA and n-butyrate acted additionally with cocaine to induce phosphorylation in Raji cells. A densitometric analysis revealed that the expression of proteins was increased by from 30% to 70% respect to chemicals or drug alone.

EBV encodes a phosphoprotein, termed ZEBRA, which enables the virus to switch from a latent to a lytic life cycle (13). After activation by phosphorylation, ZEBRA binds to its own promoter, which is also the EBV promoter. The results in Fig. 2C clearly show that cocaine can trigger ZEBRA induction in Raji cells in synergism with TPA/n-butyrate treatment.

DISCUSSION

The results presented here indicated that treatment of Raji cells with a combination of cocaine and tumor promoters enhanced the reactivation of EBV. In this cell line the spontaneous induction of the EBV replicative cycle normally either does not occur (latency) or occurs at a low rate. The latency can be overcame *in vitro* by the combination of various chemical-inducing

agents such as TPA and n-butyrate (9, 10). However, our findings provide the first evidence that cocaine may represent a pharmacological stimulus in the reactivation of latent EBV infection.

Although our results support the conclusion that the combination of cocaine and chemicals is more effective than treatment with either cocaine alone, we found a variation in the Ca^{2+} concentration induced only by cocaine. Cocaine treatment of Raji cells induces in fact a rise in intracellular Ca^{2+} from basal level to 180–200 nM. We have previously shown that calcium ionophores (i.e., ionomycin) induce the replication of EBV in target cells, and this event is mediated by activation of cellular protein kinases (9). While the increase in Ca^{2+} induced by cocaine treatment may be correlated in Raji cells with the activation of calcium-dependent protein kinases, as shown by the increase of ^{32}P incorporation in some phosphoproteins, the same reported increase by cocaine treatment seems to be not sufficient to induce EBV-EAs. This is not surprising since it is known that the intracellular calcium must be at μM levels to have detectable production of viral antigens (9). On the other hand, TPA also induces protein kinase activities that play an important role in reactivating latent EBV (17, 18). Cocaine could thus cooperate with chemical treatment to modulate protein phosphorylation, leading to an additional effect on early viral antigen expression.

It is noted that cocaine generally by itself does not activate cellular responses but, rather, the effect of the drug is synergistic with other activating signals. For example, the drug was incapable of stimulating HIV-1 replication in nonactivated PBMC, whereas marked augmentation of viral replication was observed when cocaine-treated PBMC were stimulated with mitogens (1). Similarly, cocaine only potentiates HIV-1 replication in cytomegalovirus-activated PBMC (2). In our paper this combined effect is particularly evident in the induction of ZEBRA. In fact cocaine is unable per se to induce ZEBRA, but it acts synergistically with chemicals to upregulation of the EBV replication activator. There is however a need to know more about the evidently complex mechanism(s) through which cocaine influences viral replication. In this regard, drug's effect on early antigen expression in cells stimulated by ligation of the B cell receptor, i.e. the more likely scenario for *in vivo* activation, or in cells that are spontaneously entering the lytic cycle is currently the subject of further investigation.

In summary, our data suggested a potential physiological role for cocaine on EBV reactivation. In particular, cocaine can be considered an important co-factor in the activation of EBV latency. Cocaine seems to be necessary, but not sufficient to induce the early stages of EBV replication. The hypothesis that drugs of abuse act as co-factors in increasing the severity of infections is supported by several evidences from *in vivo* and *in vitro* investigations (4, 19–23). However, the study of co-factors already represents a promising line of research aimed at understanding the pathogenesis of viral diseases and hence their therapeutic approach.

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