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VERAPAMIL ENHANCES THE EFFICIENCY OF DNA-MEDIATED GENE TRANSFER IN MAMMALIAN CELLS

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SUMMARY: Treatment of Ltk$^-$ cells with the calcium antagonists, verapamil and diltiazem, but not nifedipin, causes a 3-fold enhancement of the frequency of transfer of the cloned gene for herpes simplex virus thymidine kinase (HSV-tk). The frequency of phenotypic expression of the HSV-tk DNA was 20 to 34 times higher than that of genotypic transformation. Phenotypic expression was also 2.3 to 2.6 times increased when 20 μg/ml of verapamil was present during calcium phosphate-mediated DNA transfection.

The use of $CaPO_4$-DNA precipitates has allowed the successful transfer of DNA into some mammalian cells such as mouse L cells (1, 2, 3). Gene transfer is now becoming a widely applied technique, and it has become necessary to understand the mechanisms limiting the efficiency of this process in order to improve the reproducibility and frequency of transfer for many cell types as well as for L cells.

The calcium antagonist, verapamil, appears to inhibit the secretion of many peptide hormones (4, 5, 6) and increases the intracellular concentration of anticancer agents in leukemia cells by inhibiting the efflux of these agents from cells (7).

Because of the known capacity of Ca^{++} antagonists to promote accumulation of agents and hormones in mammalian cells, we have tested verapamil and other calcium antagonists to affect the frequency of DNA-mediated gene transfer. We find that these agents enhance the frequency of DNA-mediated transformation in L cells.

MATERIALS AND METHODS

Cell culture and cell lines: Mouse Ltk$^-$ cells were obtained from L. Siminovitch. They were grown at 37°C in minimal essential medium (MEM) (Nissui Seiyaku Co., Tokyo) containing 10% newborn calf serum (Flow Lab.,

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Stanmore, N.S.W. Australia), 1 mg/ml of bactopeptone, 0.292 mg/ml of glutamine, and 100 units/ml of penicillin-G (Meiji Seiyaku Co., Tokyo).

Isolation of plasmid DNA and Ltk$^-$ DNA: The E. coli strain 1106 (803 rk$^-$ mk$^-$ supE, supF), which contains the herpes simplex virus (HSV-1) gene coding for thymidine kinase in the PvuII site of pBR 322 (pAGO), was provided to us by A.-C. Garapin (8). The plasmid, pAGO, was isolated by the method of Clarke and Carbon (9). Ltk$^-$ DNA was isolated from Ltk$^-$ cells by the method of Pellicer et al. (10), and used as carrier DNA.

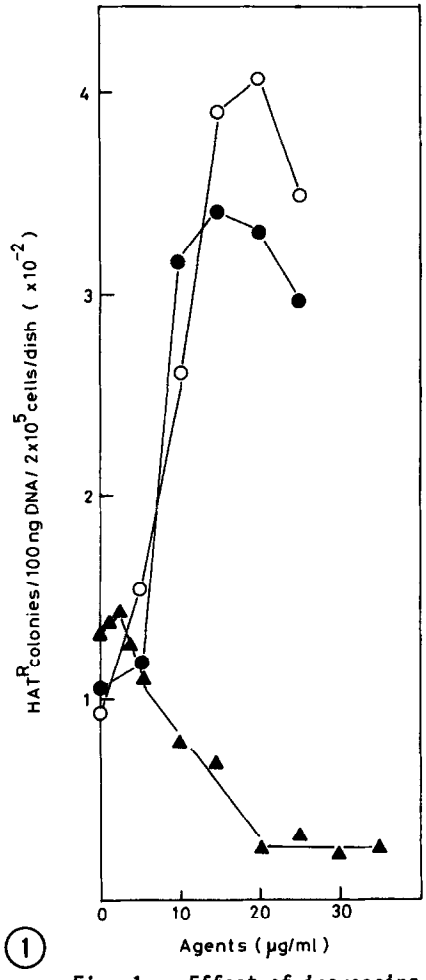
Plasmid DNA transformation: DNA transformations were performed by a slight modification of the procedure described by Wigler et al. (11). Cells were plated at 2 x 10^5 cells/100 mm dish in 10 ml of growth medium. Twenty-four hours later, the calcium phosphate precipitates of DNA were prepared by the method of Wigler et al., and 1 ml of precipitate was added with or without each of calcium antagonists to each dish. Calcium phosphate-containing medium was left on 24 h, then removed and replaced with normal medium. Forty-eight hours after the calcium phosphate precipitate addition, normal medium was replaced with selective HAT medium. HAT medium contained hypoxanthine (1 x 10^{-4} M), aminopterine (4 x 10^{-7} M), and thymidine (1.6 x 10^{-5} M). Colonies were allowed to grow for 10-14 days and then were stained with Giemsa.

Measurement of phenotypic expression: Phenotypic expression was measured by a method of Linsley and Siminovitch (12). Ltk$^-$ cells (6 x 10^4 cells/well) in two-well Lab-tek chambers were incubated for 24 h and then DNA precipitates with 100 ng pAGO DNA per 2 x 10^5 cells were added to cells with or without verapamil (20 µg/ml). After 24 h, the precipitate was removed, fresh medium was added and incubated for an additional 48 h. The cells were then incubated for 24 h with 25 µCi/ml [^{3}H]thymidine (New England Nuclear: 20 Ci/mmol). Alpha minimal essential medium supplemented with 10% dialyzed fetal bovine serum was used for labeling. After a 24 h incubation period at 37°C, the monolayers were washed with phosphate-buffered saline, fixed with 10% formalin in phosphate-buffered saline, and washed again with phosphate-buffered saline. Slides were then placed briefly in cold 5% trichloroacetic acid and water, and finally dried from methanol. Autoradiography was performed with NR-M2 emulsion (Konishiroku, Tokyo) and an exposure time of 7 days. The number of isotopically labeled cells per well was determined as described by Linsley and Siminovitch and expressed as a fraction of the 6 x 10^4 cells inoculated per well at the start of the experiment.

Chemicals: Verapamil was kindly supplied by the Eisai Co., Ltd., Tokyo. Nifedipin was purchased from the Bayer A.G., Leverkusen-Bayerwerk, W. Germany. Diltiazem was purchased from Tanabe Pharmaceutical Co., Tokyo.

RESULTS AND DISCUSSION

The calcium antagonists, verapamil, diltiazem and nifedipin, were tested to see if they would enhance transformation frequencies of HSV-tk in Ltk$^-$ cells. Verapamil and diltiazem, at concentrations between 10 and 30 µg/ml, produced a 3- to 4-fold increase in the transformation frequency. On the other hand, the frequency of HAT^R colonies was the same or less when nifedipin was added to the medium during DNA uptake (Fig. 1).



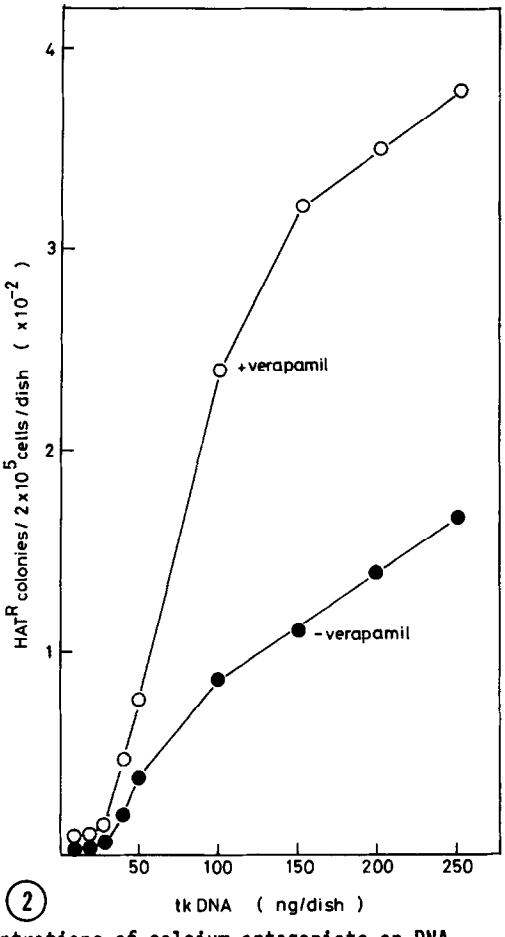


Fig. 1. Effect of increasing concentrations of calcium antagonists on DNA-mediated transformation with Ltk⁻ cells. Ltk⁻ cells were treated with DNA precipitates containing 100 ng of pAGO in the presence of the indicated concentrations of the calcium antagonists, verapamil (○), diltiazem (●) and nifedipin (▲).

Fig. 2. Effect of increasing amounts of pAGO DNA on transformation of Ltk⁻ cells in the presence or absence of 20 μg/ml verapamil.

We next determined the relationship between DNA dosage and the frequency of transfer, and at the same time, the effect of verapamil (20 μg/ml) on transformation frequencies. The number of transformants observed increased in proportion to the concentration of DNA. As can be seen in Fig. 2, with verapamil treatment, the number of transformants was increased 2- to 3-fold over the number of transformants without verapamil.

We also investigated transformation frequencies as a function of the number of cells exposed to DNA (Fig. 3). An increase in the number of plated cells

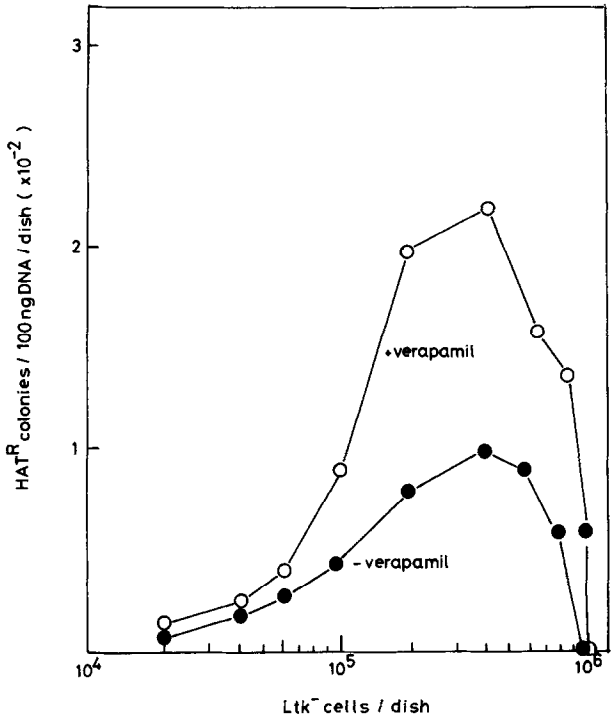


Fig. 3. Effect of increasing numbers of cells exposed to DNA on transformation of Ltk$^-$ cells in the presence or absence of 20 μg/ml verapamil.

to 4 x 10^5 cells/100 mm dish produced a maximal increase in transformation frequency. When the number of cells plated was increased to 10^6 cells/dish, no HAT^R colony was seen with our assay system. Verapamil increased the number of transformants at least 2-fold in the range of cell numbers from 10^5

TABLE I

The Effect of Verapamil on Phenotypic Expression of tk after DNA Transformation[a]

Agents	Frequency of phenotypic expression (x 10^2)			
	Without plasmid		With plasmid	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
---	0.003	0.003	0.95	1.63
20 μg/ml verapamil	0.002	0.003	2.48	3.76

[a]DNA precipitates with or without 100 ng of pAGO per 2 x 10^5 cells were added to Ltk$^-$ cells in the presence or absence of 20 μg/ml verapamil. After 24 h, the precipitate was removed, fresh medium was added and incubated for an additional 48 h, then [^{3}H]thymidine was added and incubated for 24 h. The frequency of phenotypic expression of tk gene was determined as described in Materials and Methods.

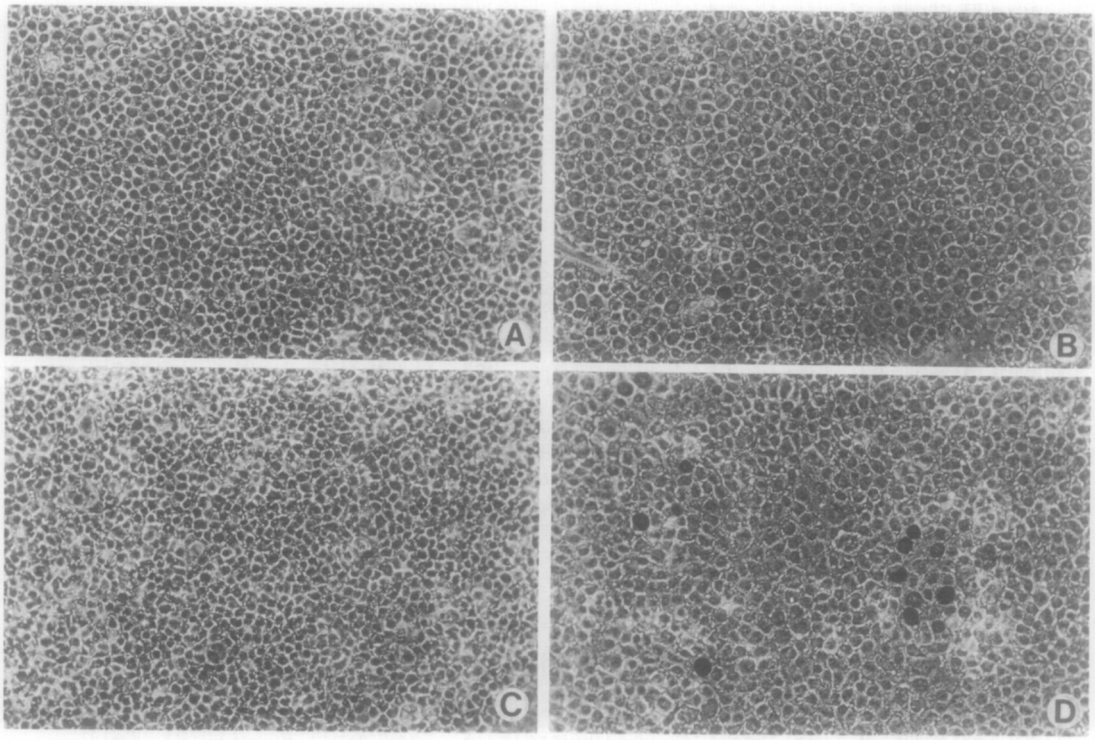


Fig. 4. Phenotypic expression of tk genes. The tk^- line, Ltk^- was treated with DNA precipitates containing 100 ng of pAGO DNA per 2 x 10^5 cells. Phenotypic expression of tk gene was detected as described in Materials and Methods. After autoradiography, the cells were photographed unstained.

Ltk^- without both pAGO and verapamil (A).
Ltk^- with pAGO in the absence of verapamil (B).
Ltk^- without pAGO in the presence of 20 μg/ml verapamil (C).
Ltk^- with pAGO and 20 μg/ml verapamil (D).

to 6 x 10^5 cells/100 mm dish. The frequency of phenotypic expression in Ltk^- cells at a given concentration of plasmid DNA increased 2.3 to 2.6 times when verapamil was added with calcium phosphate precipitates to the medium (Table I and Fig. 4). There was a large difference (ca. 20- to 34-fold) between phenotypic expression and genotypic transformation. These data are in agreement with those of Linsley and Siminovitch (11).

Our data suggest that verapamil enhances either the uptake and expression of exogenous DNA or inhibits the efflux of exogenous DNA from the cells. The mechanism of the action of verapamil and diltiazem on DNA-mediated transformation is still unknown in detail and deserves further study, since one of the calcium antagonists tested, nifedipin, failed to affect DNA-mediated transformation.

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