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USE OF ELECTROPORATION TO STUDY THE CYTOTOXIC EFFECTS OF FLUORODEOXYURIDYLATE IN INTACT CELLS*

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Abstract—The introduction of 2′-deoxyuridine 5′-monophosphate and its analog, 5-fluoro-2′-deoxyuridine 5′-monophosphate, into intact CCRF-CEM and NIH3T3 cells was achieved by electroporation. Following electroporation, cells were shown to be fully functional as monitored by the incorporation of deoxyuridylate, after conversion to thymidylate, into DNA. Pretreatment of cells with fluorodeoxyuridine completely abolished this effect. In contrast, introduction of the fluoro analog into cells by electroporation markedly inhibited both DNA synthesis and cell growth in a time-dependent manner. Thus, electroporation offers a powerful tool to permeabilize cells to a variety of cellular metabolites and antimetabolites.

Several clinically useful cancer chemotherapeutic agents are active only upon conversion to their phosphorylated forms. However, the metabolic actions of these phosphorylated metabolites are difficult to assess directly, due to their lack of membrane transport. We demonstrated recently that deoxyribonucleoside triphosphates can be introduced into a variety of cells by electroporation [1], a procedure previously found useful as an alternative to calcium phosphate mediated transfection of cloned DNAs into cells [2–6]. In this report, we demonstrate the value of this method to produce detectable biological effects exerted by phosphorylated compounds introduced into functional cells. Fluorouracil and fluorodeoxyuridine are widely used in the treatment of certain human epithelial tumors [7]. The inhibition of DNA synthesis is believed to be the primary mechanism of 5-fluoro-2′-deoxyuridine (FdUrd**) induced cytotoxicity [7], resulting from conversion of FdUrd to the active thymidylate synthase inhibitor, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP). In the presence of the cofactor 5,10-methylenetetrahydrofolate, FdUMP forms a stable, covalent ternary complex with thymidylate synthase which effectively blocks further enzyme action [8]. Therefore, the phosphorylated metabolite, FdUMP, was used to study the cytotoxic effects, directly following its introduction into cells by electroporation. Deoxyuridine monophosphate (dUMP) introduced into intact cells by electroporation, after conversion to thymidylate, was detected in DNA, a process that was inhibited completely when cells were pretreated with FdUrd. We show that FdUMP, introduced into cells via electroporation, had a pronounced cytotoxic effect as a consequence of inhibition of thymidylate synthase activity.

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** Abbreviations: dUrd, 2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; Kb, kilobase pairs; and PCA, perchloric acid.

MATERIALS AND METHODS

Materials. dUMP and FdUMP were obtained from the Sigma Chemical Co. (St. Louis, MO). [2-^{14}C]dUMP (56 mCi/mmole), [5-^{3}H]dUMP (1.9 Ci/mmole) and [6-^{3}H]dUrd (21 Ci/mmole) were obtained from Moravek Biochemicals (City of Industry, CA). All other reagents were purchased from the Sigma Chemical Co.

Cell lines. The cell lines CCRF-CEM [9] and NIH3T3 [10] were grown in continuous culture. CCRF-CEM cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml of penicillin (base), and 100 μg/ml of streptomycin (base). NIH3T3 cells were maintained in DMEM medium containing 10% calf serum (heat-inactivated) and antibiotics as above. Media and sera were obtained from Grand Island Biological (Grand Island, NY).

Electroporation. Compounds were introduced into logarithmically growing cells by electroporation. The instrument for electroporation was similar to that described by Potter *et al.* [5], comprising a power source (ISCO 494 Electrophoresis Power Supply) and an electroporation chamber. The chamber was constructed using a disposable plastic cuvette (Sarstedt, Princeton, NJ) by mounting two aluminum foil electrodes (thickness 0.025 mm, length 50 mm, and width 9 mm) down the sides and held in place with epoxy. The distance separating the electrodes was approximately 4 mm.

Cells from exponentially growing cultures were collected by centrifugation, washed once with phosphate-buffered saline (PBS), and resuspended to a final concentration of $1–5 \times 10^7$ cells/ml in sterile PBS. The cells were chilled on ice for 10 min, the compounds to be studied were added, the cells were again chilled for 10 min, and 0.5 ml of the suspension was placed into a chamber that had been sterilized by successive washes with 70% ethanol and sterile PBS. Cells were then subjected to an electric pulse with the power supply preset to 2 kV and the current at the maximum of 0.9 mA. The current and the wattage were set to a value of 5 on a scale of 100. The cells and the compounds were kept on ice for 10 min, at room temperature for 10 min, diluted to 10 ml with complete medium, and incubated at 37° for various lengths of time.

Cell viability and cytotoxicity studies. The cells were counted using an electronic Coulter Counter (Coulter Electronics, model B). Cell viability following permeabilization was monitored by trypan blue exclusion. For the shorter incubation times, cells were incubated at 37° with gentle agitation. FdUMP cytotoxicity was measured by monitoring cell growth as compared to control, as well as by determining the percent inhibition of [6-^{3}H]2′-deoxyuridine ([6-^{3}H]dUrd) incorporation into PCA-insoluble material [11].

Assay of thymidylate synthase activity in cell extracts. Thymidylate synthase activity was determined by a modified method of Roberts [12]. The standard reaction mixture in a total volume of 40 μl contained: 1.8 nmoles [5-^{3}H]dUMP, 5.2 nmoles (±)-L-tetrahydrofolate, 0.1 μmole formaldehyde, 0.4 μmoles 2-mercaptoethanol, 2 μmoles NaF, 2 μmoles phosphate buffer, pH 7.5, and the enzyme, which in controls was substituted by adding buffer. The reaction was started by addition of the enzyme and terminated after 1 hr of incubation at 37° by addition of 200 μl of a suspension of charcoal (Norit, 100 mg/ml) in 2% trichloroacetic acid. After centrifugation, 100-μl samples of the supernatant fraction were counted in a Beckman LS230 liquid scintillation counter.

Analysis of DNA. DNA was purified as previously described [6]. The incorporation of [2-^{14}C]dUMP into DNA was detected after fractionation by electrophoresis on 1% agarose gels followed by Southern transfer [13] and autoradiography at −70°.

RESULTS

Incorporation of dUMP into DNA following electroporation. CCRF-CEM and NIH3T3 cell lines were chosen to investigate the effects of permeabilizing cells to take up [2-^{14}C]dUMP by electroporation. The cells (CCRF-CEM and NIH3T3) were subjected to a high voltage electrical pulse in an electroporation cuvette in the presence of 25 μCi of [2-^{14}C] dUMP. The viability of cells permeabilized by this technique was greater than 90% as monitored by trypan blue (data not shown). The maintenance of normal metabolic functions of the permeabilized cells was confirmed by monitoring the incorporation of radioactivity from [2-^{14}C]dUMP into the genomic DNA within 2 hr after electroporation (Fig. 1). The incorporation of radioactivity from [2-^{14}C]dUMP into the genomic DNA was observed only when the cells were subjected to electroporation (lanes a and c), whereas the autoradiography failed to reveal any detectable radioactivity when the cells were not permeabilized by electroporation (lanes b and d). The incorporation of [2-^{14}C]dUMP into cellular DNA following electroporation was inhibited completely by pretreatment of cells with 2 μM FdUrd, an inhibitor of DNA synthesis [7], for 5 min (data not shown). Thus, dUMP introduced into cells by electroporation was converted rapidly by thymidylate synthase to thymidylate and subsequently incorporated into DNA in a normal fashion.

Thymidylate synthase activity following electroporation. To ascertain that dUMP introduced into intact cells by electroporation is utilized by thymidylate synthase as a substrate, we measured this enzyme activity in CCRF-CEM and NIH3T3 cells permeabilized by electroporation in the presence of

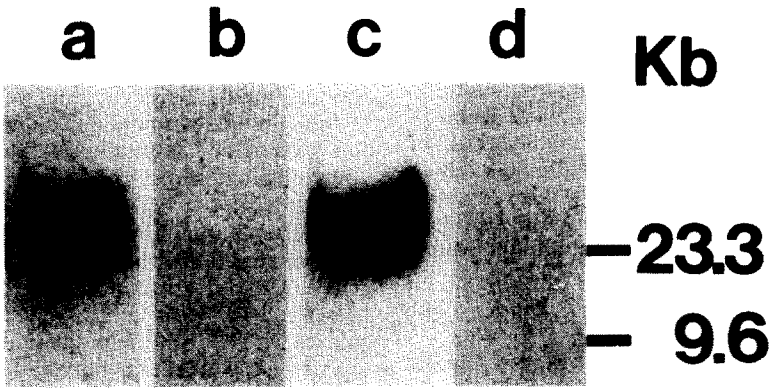

Fig. 1. Incorporation of [2-^{14}C]dUMP into genomic DNA of CCRF-CEM and NIH3T3 cells. [2-^{14}C]dUMP was introduced into CCRF-CEM and NIH3T3 cells by electroporation. After 2 hr of incubation, genomic DNA was extracted, analyzed by Southern blotting, and autoradiographed. Key: 1.0 μg of genomic DNA from electroporated (a) CCRF-CEM and (c) NIH3T3 cells and unelectroporated (b) CCRF-CEM and (d) NIH 3T3 cells. DNA digested with the restriction endonuclease HindIII served as a molecular weight marker.

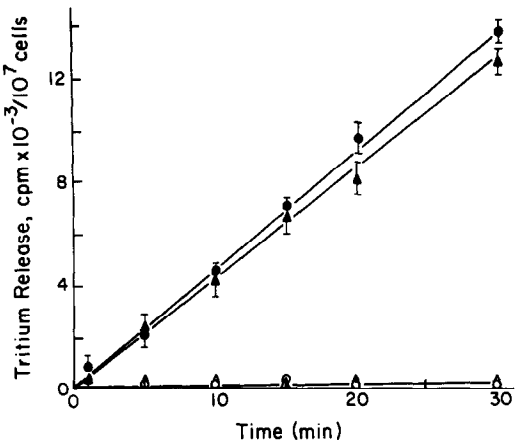

Fig. 2. Measurement of thymidylate synthase activity in CCRF-CEM and NIH3T3 cells following electroporation of [5-^{3}H]dUMP. [5-^{3}H]dUMP was introduced into the CCRF-CEM (●, ○) or NIH3T3 (▲, △) cells by electroporation, and thymidylate synthase activity was measured as described in Materials and Methods. Control cells (○, △) were pretreated with 2 μM FdUrd for 5 min before electroporation. Results are the mean ± SE of three experiments.

[5-^{3}H]dUMP (Fig. 2). A time-dependent release of tritium from [5-^{3}H]dUMP by thymidylate synthase was detected in both cell lines following electroporation and was inhibited completely when the cells were pretreated with 2 μM FdUrd.

Inhibition of [6-^{3}H]*dUrd incorporation into DNA.* The cytotoxic effect of FdUMP following electroporation into CCRF-CEM and NIH3T3 cells was investigated next. Various concentrations of FdUMP were introduced into CCRF-CEM and NIH3T3 cells by electroporation, and incorporation of [6-^{3}H]dUrd into acid-insoluble fraction was determined 4 hr after electroporation (Fig. 3). FdUMP caused a concentration-dependent inhibition of [6-^{3}H]dUrd incorporation into acid-insoluble fraction, an effect which was maintained for 24 hr (data not shown). In contrast, using the identical conditions of electroporation in nonpermeabilized control cells, FdUMP in a concentration as high as 5×10^{-6} M did not show inhibition of [6-^{3}H]dUrd incorporation into DNA (Fig. 3).

Cytotoxic effects of FdUMP. The number of cells present at different time intervals following electroporation of 5×10^{-9} M FdUMP into CCRF-CEM and NIH3T3 cells is shown in Fig. 4. Cell growth was arrested for at least 24 hr, and recovery of the FdUMP-induced cytotoxic effects was not observed until 48 hr. Furthermore, cell viability as measured by trypan blue exclusion showed that approximately 50% of cells were not viable at 24 hr after electroporation of the inhibitor, and the reduction in cell viability continued up to 72 hr (data not shown). Similar to the results of [6-^{3}H]dUrd incorporation into DNA, in unelectroporated control cells the cytotoxic effects were not observed when the nonpermeabilized cells were incubated with 5×10^{-9} M FdUMP (data not shown).

DISCUSSION

The relatively impermeable nature of the plasma membrane to a variety of nucleotides and phosphorylated cellular metabolites and antimetabolites often has presented constraints on our ability to investigate the biochemical pathways in which these compounds are implicated. Currently, these compounds are monitored indirectly, either by using the precursor molecules or by *in vitro* assays. Until recently, electroporation, a reversible permeabilization of the cell membrane by application of a high voltage electrical discharge, has been used mainly to transfect cloned DNA into cells [3] or to induce

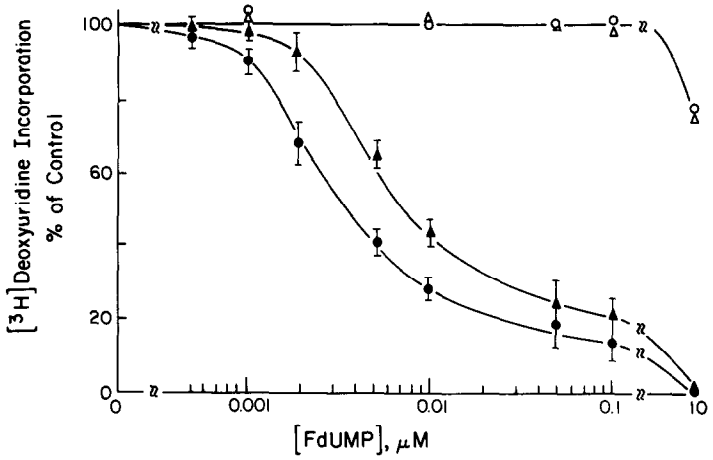

Fig. 3. Effect of FdUMP on DNA synthesis. Various concentrations of FdUMP were introduced into CCRF-CEM (●) and NIH3T3 (▲) cells by electroporation. Key: CCRF-CEM (○) and NIH 3T3 (△) control unelectroporated cells. Cells were incubated for 4 hr, and [6-^{3}H]dUrd incorporation into acid-insoluble fraction was determined by PCA precipitation. In the absence of the drug, the amounts of [6-^{3}H]dUrd incorporated into electroporated and unelectroporated cells were 3400 cpm/10^6 cells and 3700 cpm/10^6 cells respectively. Results are the mean ± SE of three experiments.

fusion of cells [4]. This method is based on the facts that (1) an external electric field applied to a cell suspension induces a position-dependent membrane potential change [14, 15] and (2) permeant structures (the *pores*) are induced in membranes when the membrane potential is larger than 200–250 mV [2]. In this paper we demonstrate that electroporation can be used to introduce biologically active levels of FdUMP, the phosphorylated antimetabolite, into intact cells. To monitor this effect we utilized a key enzyme, thymidylate synthase, as a model. Until now, intracellular thymidylate synthase activity could only be followed *in situ* by indirect assays involving the intracellular phosphorylation of nucleosides to the corresponding 5′-monophosphates [16–18].

The observation that cells (CCRF-CEM and NIH3T3) following permeabilization by electroporation could incorporate [2-^{14}C]dUMP into the genomic DNA (Fig. 1) demonstrated that the basic metabolic functions of the cells were maintained. In addition, the finding that thymidylate synthase activity was measurable following the electroporation of [5-^{3}H]dUMP into CCRF-CEM and NIH3T3 cells (Fig. 2) suggests the possibility that this mode of permeabilization of cells may provide a useful tool for investigating various enzyme substrate/inhibitor(s) interactions *in situ*. Furthermore, we have shown that it is possible to measure the cytotoxic effects of nucleotide analogs by introducing them into intact cells by electroporation. Subsequent to introduction of the antimetabolite, FdUMP, into CCRF-CEM and NIH3T3 cells, inhibition of DNA synthesis was apparent within 4 hr of permeabilization by electroporation (Fig. 3), and the cytotoxic effects of the inhibitor were maintained for at least 48–72 hr after electroporation (Fig. 4). Although the relatively impermeant nature of the phosphorylated nucleosides and their analogs is known, phosphorylated derivatives of deoxyuridine have been described as inhibitors of [^{14}C]formate incorporation into DNA of intact Ehrlich ascites cells [19]. Therefore, the effects of various concentrations of FdUMP on [6-^{3}H]dUrd incorporation into DNA of electroporated and unelectroporated cells were investigated. FdUMP at a concentration of 5×10^{-9} M inhibited [6-^{3}H]dUrd incorporation into the DNA of electroporated cells, whereas in unelectroporated control cells the same concentration of FdUMP had no effect. Furthermore, 5×10^{-9} M FdUMP was not cytotoxic in unelectroporated control cells. These results demonstrate that the phosphorylated metabolite FdUMP at the concentrations used is cytotoxic only when cells are permeabilized by electroporation.

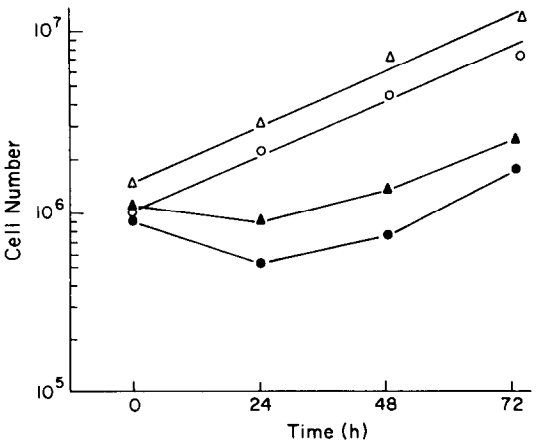

Fig. 4. Effect of FdUMP on cell growth. FdUMP (5×10^{-9} M) was introduced into CCRF-CEM (●) and NIH3T3 (▲) cells by electroporation, and the cell numbers were determined as indicated. Open symbols represent untreated control electroporated cells: CCRF-CEM (○) or NIH3T3 (△). Results are from one of two representative experiments.

The relatively high number of viable cells following permeabilization by high voltage electrical discharge, the ease of use, and the degree of versatility of this technique suggest that it is possible to investigate the metabolic pathways of a variety of membrane impermeable compounds directly inside the cell (*in situ*).

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