In Situ Electroporation of Radioactive Compounds Into Adherent Cells

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ABSTRACT

We previously developed a technique, termed *in situ* electroporation, where nonpermeant molecules are introduced through an electrical pulse into adherent cells, while they grow on electrically conductive, optically transparent, indium-tin oxide (ITO). Careful control of the electric field intensity results in essentially 100% of the cells taking up the introduced material, without any detectable effect upon the physiology of the cell, presumably because the pores reseal rapidly so that the cellular interior is restored to its original state. Electroporation of radioactive material is faced with two important considerations: (1) potential for exposure of personnel to irradiation, and (2) the requirement for electroporation of a large number of cells. In this report, we describe a modification in the geometry of the slides and electrodes which permits the use of inexpensive ITO-coated glass of lower conductivity that can be discarded after use, to electroporate large numbers of cells using a minimum volume of radioactive nucleotide solution. The results demonstrate that, using this assembly, the determination of the Ras-bound GTP/GTP+GDP ratios through electroporation of [$\alpha^{32}\text{P}$]GTP can be conducted using approximately five times lower amounts of isotope than in previous designs. Moreover, this assembly permits efficient upscaling, which makes the determination of Ras-GTP binding in cells which are deficient in Ras activity possible. In addition, we demonstrate the labeling of two viral phosphoproteins—the Simian Virus 40 Large Tumor antigen, and Adenovirus E1A—through [$\gamma^{32}\text{P}$]ATP electroporation using this setup. In both cases, electroporation of the nucleotide can achieve a great increase in the efficiency and specificity of labeling compared to the addition of [^{32}P]-orthophosphate to the culture medium, presumably because the immediate phosphate donor nucleotide itself is introduced, which can directly bind to the target proteins.

INTRODUCTION

BINDING TO NUCLEOTIDE(S) can determine a protein's state of activation; a number of GTP-binding proteins such as Ras exist in two distinct guanine nucleotide-bound conformations—the active Ras.GTP state and the inactive Ras.GDP form—so that the fraction of Ras bound to GTP (% Ras.GTP/GTP+GDP) can determine its state of activation (Lowy and Willumsen, 1993). Several indirect assays are in existence for the measurement of Ras activity (Scheele *et al.*, 1995; Taylor and Shalloway, 1996; Taylor *et al.*, 2001). However, in a number of instances a direct measurement of Ras-GTP binding is necessary

(Egawa *et al.*, 1999), and is commonly performed through the addition of ^{32}P -orthophosphate to the growth medium followed by Ras immunoprecipitation and guanine nucleotide elution (Downward, 1995). This approach is relatively inefficient due to the fact that the isotope is incorporated into all phosphate-containing cellular components. To circumvent this problem, [$\alpha^{32}\text{P}$]GTP has been introduced into the cell and its breakdown into [$\alpha^{32}\text{P}$]GDP after Ras binding monitored as above (Downward, 1995). Because, contrary to free bases or nucleosides, most nucleotides do not cross the cell membrane, [$\alpha^{32}\text{P}$]GTP has to be introduced into intact cells after cell membrane permeabilization.

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Protein phosphorylation is a ubiquitous regulator of a large variety of cellular functions. The *in vivo* radiolabeling and detection of phosphoproteins is usually conducted through the addition of [32 P]-orthophosphate to the culture medium, followed by immunoprecipitation and electrophoretic separation of precipitated proteins. Just as in the case of Ras activity measurement, this method is relatively inefficient; hence, ATP, the common immediate phosphate donor-nucleotide, may be used for *in vivo* protein labeling, which must be introduced into intact cells through membrane permeabilization (Tardif *et al.*, 1999).

We have previously developed a technique for the introduction of nonpermeant molecules into adherent cells, termed *in situ* electroporation (Raptis *et al.*, 1998). Cells are grown on a glass surface coated with electrically conductive, optically

transparent indium-tin oxide (ITO), a coating that promotes excellent cell adhesion and growth. Following addition of the material to be introduced to the cells, an electric pulse of a precisely controlled intensity and duration is applied, which opens transient pores on the cell membrane through which the material can enter. The pulse is delivered through an electrode placed on top of the cells, and a positive counterelectrode, which is in contact with the conductive coating (Fig. 1A and B). Unlike other techniques of cell membrane permeabilization such as Streptolysin O (SLO) treatment, *in situ* electroporation was previously shown not to detectably affect cellular metabolism, presumably because the pores reseal rapidly so that the cellular interior is restored to its original state (Brownell *et al.*, 1997, 1998).

FIG. 1. Electroporation electrode assembly, (A,B) Original design; (C,D) double positive contact bar design. Cells are grown on ITO-coated glass slides ([1], Applied films Corp., Boulder, CO), placed in a Petri dish to maintain sterility. The cell growth area [9] is defined by a "window" formed with an electrically insulating frame cut from adhesive-backed Teflon sheet [8]. Following addition of the radioactive nucleotide solution to the cells, electric pulses are applied, which open pores on the cell membrane. The pulses are transmitted through a circuit comprised of a stainless steel negative electrode [2], the cells and electroporation fluid, the conductive ITO coating on which the cells grow, and the positive contact bar [5], which contacts the conductive surface outside the area surrounded by the Teflon frame. (A) Side view: a Delrin™ carrier [7] holds the negative electrode [2] and the positive contact bar [5] so that they form one unit that can be placed on top of the slide [1] with its frame [8] and cells [4] in place. The negative (−) and positive (+) signs indicate the electrical connecting points via which the pulse of electricity is delivered to the electrodes from the pulse generator. The underside [3] of the negative electrode [2] is machined to a slight angle that compensates for the surface resistivity of the conductive slide to provide uniform electroporation of the whole cell growth area. The negative electrode rests on part of the Teflon frame [8], which insulates it from the conductive surface. The solution containing the radioactive material just fills the cavity below the negative electrode. The negative electrode is slightly larger than the cell growth area, so that when placed on top of the cells it rests on the insulating Teflon frame. Note that the angle of the negative electrode has been exaggerated to better illustrate the meniscus of the radioactive electroporation solution [6]. (B) Top view: the outline of the conductive slide [1] with a Teflon frame [8] in place to define the area of cell growth and electroporation are indicated [from Brownell *et al.*, 1997]. (C) Side view: the cells to be electroporated [4] are grown on the ITO-coated surface [1a]. The negative electrode [2a, 2b] is a relatively narrow steel bar mounted across the width of the slide, with the ends resting on the Teflon frame [8]. The underside of the negative electrode is inclined in both directions, such as to optimize the uniformity of electrical field. The solution containing the radioactive nucleotide is added and the electric pulses applied, which pass through a circuit formed by the negative electrode [2a, 2b], the electroporation solution [3] and the cells [4], the conductive surface of the slide [1a] and the two positive contact bars [5]. The electrode is translocated laterally and the procedure repeated, until the whole cell growth area is electroporated. Note that only the area of cells immediately below the electrode is electroporated by a given pulse. (D) Top view: the outline of the conductive slide with a Teflon frame [8] in place to define the area of cell growth and electroporation [9] are indicated.

Problems in electroporating radioactive material

Electroporation of nucleotides such as [$\alpha^{32}\text{P}$]GTP or γS -GTP (Haruta *et al.*, 1998), was previously conducted using the setup in Figure 1A and B (Brownell *et al.*, 1997). However, an important consideration in the case of electroporation of radioactive material is the exposure of personnel to irradiation. In previous experiments involving electroporation of nonradioactive molecules, the slides were washed and used repeatedly (Raptis *et al.*, 1998). However, removing ^{32}P -labeled nucleotides is difficult because the phosphate group is attracted to the ITO coating (Tomai *et al.*, 2000); hence, the use of inexpensive slides and electrodes that can be discarded after use is highly desirable. This is even more important when the cells have been lysed directly on the slides using strong detergents that are difficult to remove, while traces could inhibit cell attachment and spreading. To obtain a uniform electric field intensity over the entire cell growth area, despite the fact that the conductive coating exhibits a significant amount of electrical resistance (surface resistivity of 2–100 Ω per square), the bottom surface of the negative electrode (Fig. 1A, [2]) must be inclined relative to the glass surface, rising in the direction of the positive contact bar, at an angle proportional to the resistance of the coating [3]. Extensive previous experience (Raptis *et al.*, 1998) indicated that uniform electroporation using slides with a cell growth area of 32×10 mm and a surface resistivity of 2 Ω/sq requires the negative electrode to have an angle of ~ 1.5 degrees which, for a Teflon frame with a thickness of 0.279 mm, translates into approximately 140 μl of radioactive solution being held in place by surface tension (Fig. 1A [4]). In this case, the cost of ITO-coated slides with a surface resistivity of 2 Ω/sq is prohibitive for single experiments, while the availability of the material is often limited, as it may not be a regular production item. Gold-coated, transparent material can also be employed (Wegener *et al.*, 2002), but the cost is considerably greater. ITO-coated glass of lower conductivity such as 20 Ω/sq is commercially available and inexpensive; however, due to the higher surface resistivity, the angle of the negative electrode has to be greater, ~ 4.4 degrees, which translates into a volume of $\sim 280 \mu\text{l}$, with a proportional increase in the cost of purchase and disposal of the isotope.

A large number of important signal transducers are present in very small amounts in the cell, so that a large number of cells may be required to obtain a strong signal. The cell growth area of the assembly previously employed, 32×10 mm (Fig. 1A and B), is insufficient for a number of applications (Raptis *et al.*, 1998). Simple scale-up of this setup is faced with the problem of burning of the ITO coating that occurs at the higher voltage and capacitance settings required to electroporate a larger area, because of the resistance generated by the greater distance that the current has to travel. This is evidenced by a flash of light and a brown color of the coating under the positive contact bar. This problem cannot be relieved by using multiple pulses of lower voltage instead of one stronger pulse (Brownell *et al.*, 1997) and, aside from the cost of the isotope, this volume cannot be held in place by surface tension. In addition, the volume of solution required for an area of 50×30 mm, which can be accommodated in a standard, 10-cm Petri dish, is approximately 1.7 ml for an incline of 4.4 degrees, required for the glass of surface resistivity of 20 Ω/sq . Furthermore, posi-

tioning this large electrode on the cells and removing air bubbles that prevent current flow is exceedingly difficult.

In this communication, a novel electrode and slide configuration is described that can solve both of the above problems. Using a novel electrode that electroporates a strip of cells at a time, effective introduction of radioactive nucleotides such as [$\alpha^{32}\text{P}$]GTP or [$\gamma^{32}\text{P}$]ATP into a large number of adherent cells can be achieved with low-grade, inexpensive and disposable ITO-coated glass of low conductivity, and a minimal volume of solution. The results show that Ras activity measurement through electroporation of [$\alpha^{32}\text{P}$]GTP can be performed using approximately 100 times lower amounts of radioactivity than through the addition of ^{32}P -orthophosphate to the culture medium. At the same time, due to the fact that the ^{32}P is in the form of [$\alpha^{32}\text{P}$]GTP exclusively, this technique offers higher specificity compared to metabolic labeling. In addition, labeling of two viral phosphoproteins, the large tumor antigen of simian virus 40 (SVLT) and adenovirus E1A through [$\gamma^{32}\text{P}$]ATP electroporation requires a fraction of the amount of radioactive phosphorus, while offering enhanced specificity compared to labeling through the addition of [^{32}P]orthophosphate to the culture medium. For both of the above applications, at least four to five times lower amounts of radioactive nucleotide were sufficient than in the previously employed assembly, and the ability to electroporate larger numbers of cells made the determination of Ras.GTP binding in cells which are deficient in Ras activity possible.

MATERIALS AND METHODS

Cell lines and culture techniques

The $10\text{T}^{1/2}$ murine fibroblast line and its SVLT-transformed counterpart, 10SV2b have been previously described (Grammatikakis *et al.*, 2001). Rev3 is a $10\text{T}^{1/2}$ derivative expressing $\sim 30\%$ the cRas levels of the parental line, due to expression of a ras-antisense construct (Raptis *et al.*, 1991). All cells were grown in a 5% CO_2 incubator in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Analysis of Ras-bound, GTP/GDP+GTP ratios

Cells were grown to confluence on ITO-coated slides (cell growth area, 50×30 mm, Fig. 1C and D) and serum-starved for 48 h. Prior to labeling, cells were starved from phosphates by incubating in phosphate-free DMEM for 2 h. Following electroporation of 20 μCi [$\alpha^{32}\text{P}$]GTP in 200 μl phosphate-free DMEM from a 10 μF capacitor (30–100 V, 6 pulses), 1-ml phosphate-free DMEM was added, cells placed in a humidified chamber and incubated for 2 h at 37°C. Proteins were subsequently extracted with lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM MgCl_2 , 1% Triton, 0.5% Deoxycholate, 0.05% SDS, 1 mM EGTA, 1 mM PMSF, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 mM Benzamidine, and 1 mM Vanadate), and Ras precipitated using the panRas-Ab2 antibody (Oncogene Science, Uniondale, NY) or control, normal mouse IgG, and Staph. A-Sepharose beads (Pharmacia, Piscataway, NJ). Following washing, Ras-bound GTP and GDP were eluted with 2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, 0.5

mM GDP, and separated by thin-layer chromatography in 66% isopropanol, 1% concentrated ammonia (Brownell *et al.*, 1997).

Phosphorylation of the simian virus 40 large tumor antigen (SVLT) or adenovirus E1A

10SV2b cells were grown on ITO-coated slides (cell growth area 50 × 30 mm), starved from phosphates by incubating in phosphate-free DMEM supplemented with 5% dialyzed calf serum for 2 h and electroporated using 50 μCi [γ³²P]ATP in serum- and phosphate-free DMEM as above. Following the addition of 1 ml phosphate-free DMEM, cells were incubated at 37°C for 30 min and proteins extracted with lysis buffer (1% Triton X-100, 50 mM HEPES pH7.4, 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 2 mM sodium orthovanadate, and 0.5 mM phenylmethyl sulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Extracts were subsequently incubated with the p108, anti-SVLT antibody (Pharmingen, San Diego, CA) or control normal mouse IgG, and Staph. A-sepharose beads (Grammatikakis *et al.*, 2001). Precipitated proteins were separated through polyacrylamide gel electrophoresis and dried gels exposed to X-ray film. Adenovirus E1A was immunoprecipitated from similarly labeled, human 293 cells with the M73 antibody (Pharmingen), following preadsorption with normal mouse IgG and Staph. A-sepharose beads, which were run in parallel lanes.

Metabolic ³²P labeling

For the determination of Ras-GTP binding, cells were grown to confluence on ITO-coated slides (cell growth area, 50 × 30 mm) and serum-starved for 48 h. Prior to labeling, cells were placed in phosphate-free DMEM for 2 h. Labeling was performed through the addition of 50–500 μCi ³²P-orthophosphate added in 1 ml phosphate-free DMEM for 2 h. Ras-bound, GTP/GTP+GDP ratios were determined as above. For protein phosphorylation experiments, cells were treated in the same way, but incubated in phosphate-free DMEM supplemented with 5% dialyzed calf serum for 1 h. SVLT or E1A labeling was subsequently determined as above.

RESULTS

Electrode design

Previous results indicated that a very small proportion of the material present in the electroporation medium is taken up by the cells, so that the solution can be reused a number of times (Raptis *et al.*, 2003). Therefore, to enable the use of a minimal volume of solution with glass of a lower conductivity, a novel electrode arrangement was designed with a narrow, moveable electrode that electroporates a “strip” of cells at a time (Fig. 1C and D); in this configuration, only the cells immediately below the negative electrode are electroporated by a given pulse of electricity. After electroporation of the first strip of cells [2a], the electrode is translocated laterally [2b], dragging the solution under it by surface tension, so that a new strip of cells is electroporated using mostly the same solution. The electric circuit formed during pulse delivery starts at the negative electrode [2a or 2b], passes through the electroporation fluid [3],

the cells [4] and the conductive slide surface [1a], to the two positive contact bars [5], one on each side of the slide. The two positive contact bars form parallel circuit paths, both carrying current from the conductive surface. To compensate for the resistance of the coating, the bottom surface of the negative electrode was given a shape with an incline towards each of the positive contact bars. To determine the optimal contour, extensive experimentation using the fluorescent dye Lucifer yellow as an indicator of cell permeation was conducted, and it was found that a 25 mm radius on the bottom of the electrode can produce successive strips of even electroporation over the entire cell growth area. In this configuration, due to the small width of the electrode, the increase in volume of solution required due to the greater incline is minimal. Using this assembly, an area of 32 × 10 mm can be electroporated using less than 50 μl of solution with a 2.5 mm wide electrode and glass of a surface resistivity of 20 Ω/sq. This glass is readily available, at an approximately four times lower cost than the glass of 2 Ω/sq used before (Brownell *et al.*, 1997), hence, it can be discarded after use to limit exposure to radioactivity. Similarly, the electrode can be made out of inexpensive aluminum for a single use. This setup (Fig. 1C and D) also lends itself to effective upscaling; extensive testing using a cell growth area of 50 × 30 mm, revealed that the amount of energy required for

FIG. 2. Measurement of Ras-bound GTP/GTP+GDP ratio through *in situ* electroporation of [α³²P]GTP. cRas-deficient, Rev-3 cells (lane 1), or their counterparts expressing activated Ras^{leu61} (line Rev3ras, lanes 2–5) were grown on ITO-coated slides (growth area 50 × 30 mm), starved from serum and phosphates and labeled by *in situ* electroporation of 20 μCi [α³²P]GTP (6 pulses of 10 μF, 60 V). Ras-bound GTP and GDP were eluted from washed anti-Ras immunoprecipitates (lanes 1, 2, 4, and 5) and separated by thin layer chromatography (see Materials and Methods). The plate was exposed for 2 h to Kodak XAR-5 film with an intensifying screen. Lane 3: control immunoprecipitation of a labeled Rev3ras cell lysate using normal rat IgG. Lane 5: Rev3ras cells were labeled *in vivo* with 500 μCi ³²P-orthophosphate, and Ras-bound GTP and GDP determined as above. Numbers refer to % Ras-bound, GTP/GTP+GDP values obtained by phosphorimager scanning of the plate. Arrows refer to the position of cold standards, as indicated.

each 8 mm-wide strip of cells is well within the range of voltage and capacitance settings easily tolerated by the coating, even after repeated use, while the solution can be held in place by surface tension. Testing using Lucifer yellow as an indicator of cell permeation demonstrated that this area could be effectively electroporated in four strips, with a total of 200 μ l of solution using glass with a surface resistivity of 20 Ω /sq.

[α^{32} P]GTP electroporation for Ras activity measurement

In a previous report (Brownell *et al.*, 1997) we examined Ras.GTP binding in mouse 10T $^{1/2}$ cells containing normal cRas

levels using the setup in Fig. 1A and B. This setup, however, cannot provide enough material for Ras-GTP binding studies in Ras-deficient cells, such as the Rev3 line where cRas levels have been reduced through expression of an antisense message (Raptis *et al.*, 1997, Grammatikakis *et al.*, 2002). Therefore, we now examined Ras.GTP binding and degradation using the setup described in Fig. 1C and D, with a cell growth area of 50 $\times$ 30 mm. Following a 2-h phosphate starvation, 20 μ Ci [α^{32} P]GTP were added to 200 μ l phosphate-free DMEM and introduced by electroporation into Rev3 cells or their counterparts transformed through Ras^{leu61} expression (line Rev3ras; Raptis *et al.*, 1997), grown on 50 $\times$ 30 mm slides, as described above. The electrode was then removed, 1 ml phosphate-free

FIG. 3. Labeling of the simian virus 40 Large tumor antigen or adenovirus E1A through *in situ* electroporation of [γ^{32} P]ATP. (A) Mouse 10T $^{1/2}$ cells (lane 1) or their SVLT-transformed derivatives (line 10SV2b, lanes 2–8) were grown on 50 $\times$ 30 mm conductive areas [Fig. 1, (C) and (D)]. A solution containing 50 μ Ci [γ^{32} P]ATP in phosphate-free DMEM was added to the cells and six capacitor-discharge pulses of 10 μ F, 40–80 V applied as indicated. Cells were placed in a humidified incubator for 30 min. For a comparison (lanes 9–10), the same SVLT-transformed cells were labeled *in vivo* with 200 μ Ci of 32 P-orthophosphate. SVLT was precipitated from detergent extracts with the pAb108, anti-SVLT antibody (lanes 1, 3–7, and 10), or normal mouse IgG (lanes 2, 8, and 9) and labeled proteins resolved by acrylamide gel electrophoresis. Dried gels were exposed for 1 h to Kodak XAR-5 film with an intensifying screen. Note the intense and specific labeling of SVLT and the associated phosphoprotein, p53, by *in situ* electroporation (lanes 5 and 6), compared to cells labelled *in vivo* with 200 μ Ci [32 P]-orthophosphate (lanes 10, 12, and 13). (B) Human 293 cells transformed with Adenovirus DNA were labeled as above and extracts preadsorbed with normal mouse IgG (lanes 2, 4, 6, 8, and 10) or immunoprecipitated using the M73, anti-E1A antibody (lanes 1, 3, 5, 7, and 9). Bracket points to the position of the phosphorylated E1A bands. M: Molecular weight marker lanes.

DMEM prewarmed to 37°C added to the cells for 2 h and Ras-bound, GTP/GTP+GDP ratios determined (see Materials and Methods). As shown in Figure 2, this ratio for the Ras-deficient, Rev3 line was $52 \pm 5\%$ (mean $\pm$ SEM of three treatments, lane 1), as determined by phosphorimager analysis of the TLC plate, while for the same line transformed by the constitutively active Ras^{leu61} mutant it was $90 \pm 6\%$ (mean $\pm$ SEM of four treatments, lanes 2 and 4), respectively. These ratios are essentially the same as previously shown for 10T^{1/2} cells and their Ras^{leu61}-expressing counterpart, 2H1, after *in vivo* labeling with ³²P-orthophosphate (Krook *et al.*, 1993) and, as previously demonstrated, these ratios did not change with the voltage used, even when a significant proportion of the cells were killed by the pulse (Brownell *et al.*, 1997).

[γ ³²P]ATP electroporation for phosphoprotein labeling

For labeling of the simian virus 40 large tumor antigen (SVLT), SVLT-transformed mouse 10T^{1/2} fibroblasts (line 10SV2b) were plated on slides as described in Figure 1C and D (cell growth area 50 $\times$ 30 mm) and starved from phosphates for 2 h. [γ ³²P]ATP (50 μ Ci) in 200 μ l phosphate-free DMEM were added and introduced by electroporation using six pulses of 10 μ F, 40–80 V, as indicated. Following incubation with 1 ml phosphate-free DMEM for 30 min at 37°C, total cellular protein was extracted and SVLT immunoprecipitated as described in Materials and Methods. In a parallel control experiment, cells were grown on ITO-coated slides, starved from phosphates as above and labeled with 50–500 μ Ci ³²P-orthophosphate added in 1 ml phosphate-free DMEM supplemented with 5% dialysed calf serum for 1 h. As shown in Fig-

ure 3A (lanes 1–8), there was a dramatic increase in radioactivity incorporated into SVLT and the associated p53 phosphoproteins with increasing voltage up to 55 V. The small amount of incorporation observed in mock-electroporated cells (lane 3) could be due to uptake of free ³²P, produced through breakdown of [γ ³²P]ATP. This background increased substantially with time of incubation (not shown). As also shown in Figure 3A, labeling of SVLT by [γ ³²P]ATP electroporation was more efficient and specific than through the addition of ³²P-orthophosphate to the culture medium (lanes 4–7 versus 9 and 10). Similar results were obtained with the labeling of the Adenovirus E1A phosphoprotein in 293 cells (Fig. 3B). A careful examination of electrical field intensity revealed that slightly lower voltages were required for electroporation of ATP compared to GTP in all lines tested, although both molecules have similar size and charges (Brownell *et al.*, 1997). This could be due to the effect of ATP⁴⁻ produced by electroporation upon purinergic P_{2z} receptors, which may facilitate the formation of pores on the cell membrane (Brake and Julius, 1996) (Fig. 4).

DISCUSSION

The results presented clearly demonstrate that *in situ* electroporation of nucleotides can be employed very effectively for the study of Ras-GTP binding and breakdown in adherent cells, or for protein phosphorylation *in vivo*. As previously demonstrated (Brownell *et al.*, 1997), contrary to SLO-mediated permeabilization, electroporation does not activate nonspecific nucleotidases, which cause the degradation of the introduced [α ³²P]GTP prior to Ras binding and offers greater specificity

FIG. 4. Effect of field strength upon the introduction of nucleotides. Pulses of increasing voltage were applied from a 10 μ F capacitor to serum-starved 10T^{1/2} cells growing on a conductive surface of 50 $\times$ 30 mm, in the presence of 10 μ Ci [α ³²P]GTP (●), or 10 μ Ci [γ ³²P]ATP (▲). Total protein labeling was quantitated using a nitrocellulose filter-binding assay (Buday and Downward, 1993). Numbers refer to cpm per 100 μ g of protein in clarified extracts. As a control, cells were electroporated with 5 mg/ml Lucifer yellow (○) and its introduction was assessed by fluorescence measurement of cell lysates using a Perkin-Elmer model 204A fluorescence spectrophotometer. Cell killing (□) was calculated from the plating efficiency of the cells 2 h after the pulse.

compared to metabolic labeling. The modification of electrode geometry described can achieve the use of substantially lower amounts of radioactive isotope and inexpensive, hence disposable, low conductivity ITO-coated glass, while it lends itself to upscaling when electroporation of larger numbers of cells is required. Under current pricing of the isotopes and slides, the cost using this approach is at least 20 times lower compared to *in vivo* labeling and four to five times less compared to the original design (Fig. 1A and B).

The electroporation of radioactive nucleotides can be combined with the introduction of other nonpermeant molecules such as peptides (Raptis *et al.*, 2000) or drugs (Brownell *et al.*, 1998) for the study of protein phosphorylation or activation. In this case, because the cost of the slides may not be as important as the cost of custom-made peptides, glass of higher conductivity may be used. Extensive testing revealed that ITO-coated glass of 2 Ω /sq requires a contour of the underside of the electrode of a 35-mm radius to obtain a uniform electrical field intensity, with $\sim 150 \mu\text{l}$ of solution being sufficient to electroporate an area of $50 \times 30 \text{ mm}$ (not shown). On the other hand, the use of ITO-coated glass of very low conductivity (100 Ω /sq) would further reduce the cost of the slides. However, in our experience, the conductivity of this grade of glass is not sufficiently reliable for electroporation experiments, due to problems related to uniformity of thickness encountered with the thinner coating of the less conductive, commercially available surface. Future experiments will examine the phosphorylation of specific sites of these and other proteins by *in situ* electroporation of [$\gamma^{32}\text{P}$]ATP.

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