Quantitative Study of Molecular Transport Due to Electroporation: Uptake of Bovine Serum Albumin by Erythrocyte Ghosts

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ABSTRACT Electroporation is believed to involve the creation of aqueous pathways in lipid bilayer membranes by transient elevation of the transmembrane voltage to approximately 1 V. Here, results are presented for a quantitative study of the number of bovine serum albumin (BSA) molecules transported into erythrocyte ghosts caused by electroporation. 1) Uptake of BSA was found to plateau at high field strength. However, this was not necessarily an absolute maximum in transport. Instead, it represented the maximum effect of increasing field strength for a particular pulse protocol. 2) Maximum uptake under any conditions used in this study corresponded to approximately one-fourth of apparent equilibrium with the external solution. 3) Multiple and longer pulses each increased uptake of BSA, where the total time integral of field strength correlated with uptake, independent of inter-pulse spacing. 4) Pre-pulse adsorption of BSA to ghost membranes appears to have increased transport. 5) Most transport of BSA probably occurred by electrically driven transport during pulses; post-pulse uptake occurred, but to a much lesser extent. Finally, approaches to increasing transport are discussed.

INTRODUCTION

Electroporation involves the creation of transient aqueous pathways in lipid bilayer membranes by the application of a short (μs, ms) electric field pulse (Neumann et al., 1989; Chang et al., 1992; Orlowski and Mir, 1993; Weaver, 1993). When the voltage across a lipid bilayer reaches ~1 V, a dramatic membrane reorganization takes place, usually termed electroporation or electropermeabilization. Although its exact nature is unclear, this reorganization can allow extensive molecular transport across the membrane, presumably due to creation of aqueous pathways, or electropores. These pores are believed to subsequently shrink and disappear. Although most pores close rapidly, some may remain open for hours. Typical pulses used to cause electroporation of cells are 1–20 kV/cm (depending on cell size and orientation) and have durations of 10 μs to 10 ms (Neumann et al., 1989; Chang et al., 1992).

Because of its ability to greatly increase transport across lipid bilayer membranes, electroporation has found application as a method of introducing macromolecules into cells, particularly for gene transfection (Neumann et al., 1989; Chang et al., 1992). Other possible applications exist, including introduction of foreign proteins into cells (Zimmermann et al., 1975; Mir et al., 1988; Hashimoto et al., 1989; Lambert et al., 1990; Berglund and Starkey, 1991; Graziadei et al., 1991; Prausnitz et al., 1993a), release of cellular contents for intracellular assays (Suprynowicz and Mazia, 1985; Knight and Scrutton, 1986; Swezey and Epel, 1988), cell killing for sterilization (Sale and Hamilton, 1967; Hulsheger et al., 1981; Jarayam et al., 1992), and tissue electroporation for gene therapy, cancer chemotherapy, and transdermal drug delivery (Okino and Mohri, 1987; Mir et al., 1991; Titomirov et al., 1991; Prausnitz et al., 1993b; Salford et al., 1993).

Most applications have the common goal of transporting useful numbers of molecules across cell membranes. However, few studies have measured the actual number of molecules transported, making efforts to advance applications and basic modeling difficult. For example, the majority of published electroporation studies emphasize transfection and generally assess gene expression, not molecular transport into cells. Although transport across the cell membrane is a necessary part of transfection, it is not sufficient, because successful gene expression involves many other processes, including cell survival and correct incorporation of DNA into the cell's genetic material. Thus, determinations of gene expression are of limited use for characterizing molecular transport due to electroporation.

Some studies have directly measured indicators of molecular transport. However, most have not actually measured transport itself, but instead assess "percent of cells electroporated" or unitless "relative transport" (Hashimoto et al., 1989; Berglund and Starkey, 1991; Graziadei et al., 1991; Kinosita and Tsong, 1977a; Mishra and Singh, 1986; Sowers and Lieber, 1986; Escande-Geraud et al., 1988; Weaver et al., 1988; Dimitrov and Sowers, 1990; Kwee et al., 1990; Rosemberg and Korenstein, 1990; Sixou and Teissié, 1990; Brown et al., 1992). A few studies have provided quantitative determinations (i.e., numbers of molecules transported) for one or a few electroporation conditions (Michel et al., 1988; Bartoletti, et al., 1989; Chakrabarti et al., 1989; Lambert et al., 1990; Casabiance-Pignéde et al., 1991); others have systematically measured the number of molecules transported over a range of conditions (Mir et al., 1988; Bazile et al., 1989; Rols and Teissié, 1990; Poddevin et al., 1991; Wilson et al., 1991; Glogauer and McCulloch, 1992; Prausnitz et al., 1993a).

Received for publication 13 December 1993 and in final form 1 February 1994.

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0006-3495/94/05/1522/09 $2.00

To further assist applications and aid in understanding mechanisms, more studies that quantitatively measure molecular transport over a range of conditions are essential. In a previous study, we measured the number of molecules transported into erythrocyte ghosts as a function of applied field strength (Prausnitz et al., 1993a). We found that net molecular transport increased with field strength, but reached an apparently nonequilibrium plateau at higher field strengths, i.e., uptake saturated at higher field strengths, even though the internal concentration was less than the external concentration. This finding could not have been made without measurements of the numbers of molecules transported per cell. Here we examine the dependence of molecular transport on other electroporation conditions.

EXPERIMENTAL METHODS

The experimental methods used in this study have been described previously (Prausnitz et al., 1993a). Briefly, human erythrocytes were washed and converted into ghosts by hypotonic lysis. Approximately 10^7 ghosts/ml were then suspended in a solution containing 10^{-5} M fluorescein-labeled bovine serum albumin (BSA) (Molecular Probes, Eugene, OR and Sigma Chemical Co., St. Louis, MO) in diluted Dulbecco's phosphate-buffered saline (20 mM total salts, pH 8.2 ± 3; Gibco, Grand Island, NY). Pulsing was performed at 0–4°C with an Electro Cell Manipulator 600 (BTX, San Diego, CA) and 2-mm-gap cuvettes. Exponential electric field pulses were used (decay time constant, $\tau = 1.1 \pm 0.1$ ms). Five min after pulsing, cells were washed, co-suspended with fluorescent latex microspheres as an internal volumetric standard, and analyzed by flow cytometry. Exceptions to this protocol are noted below and in the text.

Samples pulsed at 0°C were kept on ice, whereas samples pulsed at 37°C were kept in a 37°C water bath (VWR Scientific, Cleveland, OH) before and after pulsing. Samples pulsed at 23°C were kept at room temperature (23 ± 1°C) throughout. In multiple pulse experiments with inter-pulse spacing, $t_{inter} = 60$ s, samples were kept on ice between pulses, whereas for $t_{inter} = 5$ s, samples were on ice only before and after all pulses were applied. Sample temperature was not controlled for the 5–10 s during which samples were placed in the pulsing unit. We have measured additional temperature rises due to the pulse itself (data not shown), but we have not included these effects in this analysis. Although not usually discussed explicitly, such temperature rises are often present in transfection and other electroporation protocols.

In experiments where the time before pulsing during which BSA could adsorb to ghosts was controlled, two solutions were prepared: one contained twice the desired ghost concentration and no BSA, whereas the other contained twice the desired BSA concentration and no ghosts. At the appropriate time these solutions were mixed. For example, to expose ghosts to BSA 5 s post-pulse, a sample containing the concentrated ghost suspension was first pulsed. Five seconds later, the concentrated BSA solution was added and shaken by hand, yielding a preparation with the desired final ghost and BSA concentrations.

Reported electric field values are nominal electric fields (applied voltage divided by electrode spacing), as commonly used in the literature. However, we have determined that, under the conditions of this study, the actual electric field within the cuvette was up to 10% less than the nominal electric field, perhaps due to voltage drops at the electrode interface (data not shown). Although only nominal electric fields are generally reported in the literature, differences between nominal and actual electric fields are probably present in many electroporation protocols.

For each sample, light scatter and fluorescence measurements were made on 2×10^4 individual ghosts by flow cytometry (Shapiro, 1988; Melamed et al., 1990; Prausnitz et al., 1993a) to measure the molecular transport caused by electroporation. The use of flow cytometry allowed discrimination between ghosts and debris or other objects, as well as quantitative measurement of ghost fluorescence at the single ghost level. Using calibration beads (Flow Cytometry Standards, Research Triangle Park, NC), ghost fluorescence was converted into numbers of molecules associated with each ghost (Bartoletti et al., 1989; Prausnitz et al., 1993a). Within a population of ghosts exposed to the same conditions, the distributions of net molecular transport per ghost showed no significant subpopulations, as discussed by Prausnitz et al. (1993a). Thus, the average number of BSA molecules taken up by ghosts represents the mean response of a single population of ghosts.

RESULTS AND DISCUSSION

Multiple pulses

Erythrocyte ghosts were exposed to different numbers of electric field pulses in the presence of fluorescein-labeled bovine serum albumin. Fig. 1 *A* shows BSA uptake as a function of field strength, E, for different numbers of pulses, N_{pulse}, with inter-pulse spacing, $t_{inter} = 5$ s. Uptake increased with both N_{pulse} and E, but appeared to plateau at large E, as reported previously (Prausnitz et al., 1993a) and discussed further below. A related result is shown in Fig. 1 *B*, for which multiple pulses were applied with $t_{inter} = 60$ s.

Fig. 1, *C* and *D* present the data from Fig. 1, *A* and *B*, respectively, in another form to show the enhancement of uptake caused by additional pulses relative to the uptake due to one pulse at each E. For smaller E, multiple pulses resulted in up to 7 times greater uptake than a single pulse. However, the relative enhancement of multiple pulses at larger E was progressively less. Finally, to assess the effect of $t_{inter} = 5$ s versus 60 s, Fig. 1 *E* shows ratios of uptake with $t_{inter} = 5$ s to $t_{inter} = 60$ s. In each case the ratio was approximately 1.

This result shows that application of multiple pulses increased uptake, as reported previously (Dekeyser et al., 1990; Rols and Teissié, 1990). However, the relationship between N_{pulse} and uptake is not linear, i.e., the net effects of a series of pulses were not additive. The relative enhancement caused by additional pulses at small E was greater than at large E. This and other data (see below) suggest the existence of a transport maximum beyond which additional pulses could not increase uptake under the conditions of this study. If so, for applications this suggests that more pulses at moderate E lead to the same uptake as fewer pulses at larger E. However, pulses at larger E are generally associated with lower cell viability (Chang et al., 1992). Multiple pulses at moderate E may maximize transport and cell viability. Finally, multiple pulses with $t_{inter} = 5$ and 60 s caused approximately the same uptake. Rols and Teissié (1990) have also reported that pulse rate (≤0.1 Hz) did not affect net transport.

Longer pulses

The uptake of BSA by ghosts due to single pulses having different time constants, τ, was also assessed. Fig. 2 *A* shows uptake as a function of E for $\tau \leq 2$ ms, whereas Fig. 2 *B* shows uptake due to longer pulses, but only at small E. Longer pulses at large E were not used, because under those conditions, large (>50%) fractions of ghosts were destroyed (i.e., appear as many small particles in the flow cytometer). Overall, uptake generally increased when longer pulses were used.

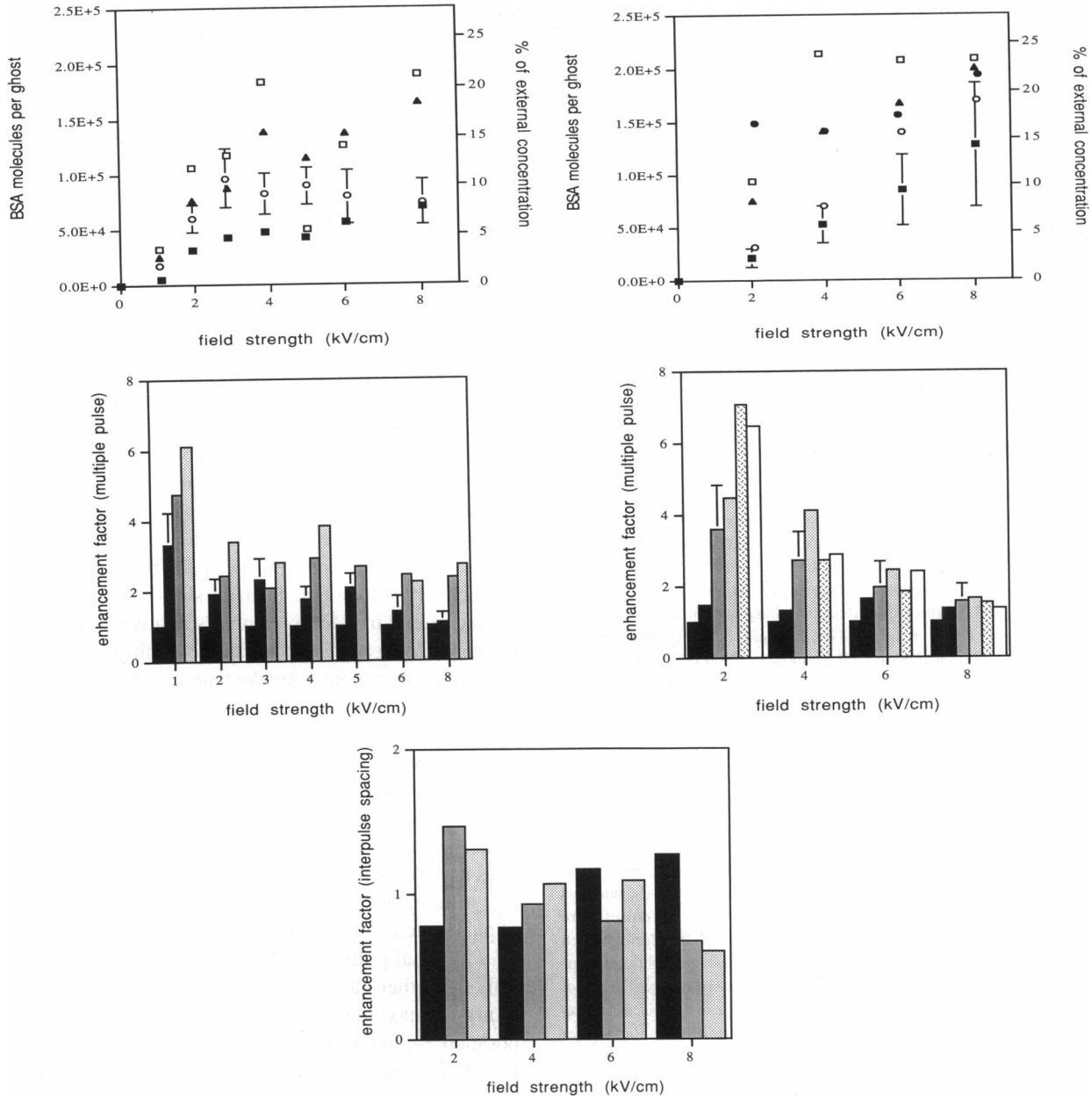

FIGURE 1 The effects of multiple pulses on uptake of fluorescein-labeled bovine serum albumin by erythrocyte ghosts. (*A*) Uptake of BSA (68 kDa, ~−25 charge) due to different numbers of exponential-decay pulses is plotted versus field strength. N_{pulse} = (■) 1, (○) 2, (▲) 3, (□) 5. Inter-pulse spacing, t_{inter} = 5 s. (*B*) A related result is shown, for t_{inter} = 60 s. N_{pulse} = (■) 1, (○) 2, (▲) 3, (⊠) 5, (●) 10. (*C*) The increase in uptake relative to that of a single pulse is expressed as an enhancement factor for multiple pulses for t_{inter} = 5 s. N_{pulse} = (■) 1, (■) 2, (▨) 3, (▤) 5. (*D*) A related result is shown, for t_{inter} = 60 s. N_{pulse} = (■) 1, (■) 2, (▨) 3, (▤) 5, (▨) 10, (□) 15. (*E*) Enhancement of uptake due to pulses with t_{inter} = 5 s relative to t_{inter} = 60 s is shown: N_{pulse} = (■) 2, (▨) 3, (▤) 5. These graphs suggest that multiple pulsing enhanced uptake strongly at lower field strengths, but only weakly at higher field strengths. Moreover, no difference in effects on uptake were evident for t_{inter} = 5 vs. 60 s. Figs. 1–6 each include data from 10^6-10^7 individual ghosts; each point represents the average of 3–12 samples collected during 2–6 different experiments. Representative standard error bars are shown throughout.

It has been widely reported that increasing pulse length increases transport (Kinosita and Tsong, 1977a; Dekeyser et al., 1990; Rols and Teissié, 1990; Rosemberg and Korenstein, 1990; Wilson et al., 1991), where longer pulses may be associated with larger pores (Kinosita and Tsong, 1977a; Serpersu et al., 1985; Rosemberg and Korenstein, 1990). However, we found that our ghost preparation appeared to be destroyed when longer pulses with E > 2 kV/cm were used. For cell electroporation, this suggests that longer pulses are less effective than multiple pulses for maximizing transport while minimizing damage.

Multiple versus longer pulses

A comparison is made of the effects of multiple pulses and single pulses having the same time integral of field strength

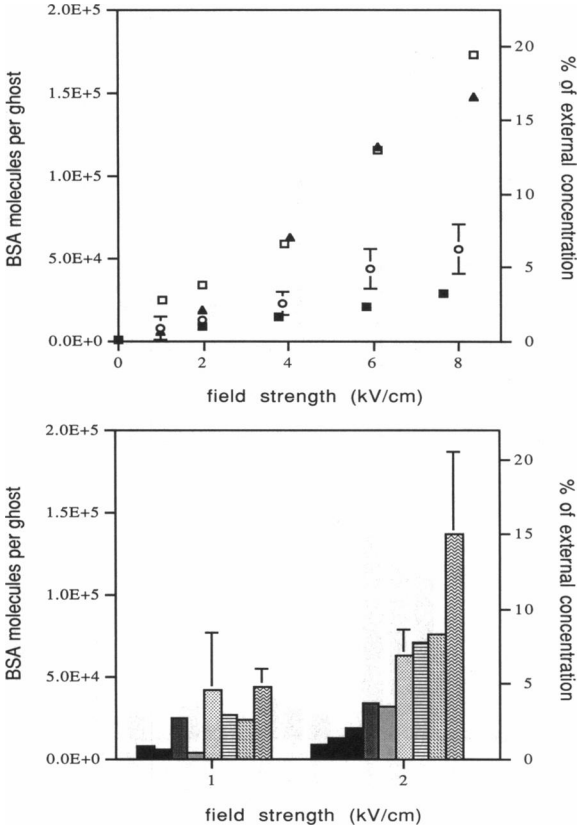

FIGURE 2 The effects of pulse time constant, τ, on uptake. Uptake of BSA is plotted versus field strength for pulses of different length. (*A*) τ = (■) 0.5 ms, (○) 1 ms, (▲) 1.5 ms, (■) 2 ms. (*B*) τ = (■) 0.5 ms, (▨) 1 ms, (■) 1.5 ms, (□) 2 ms, (▨) 3 ms, (▤) 4 ms, (■) 6 ms, (▧) 8 ms, (▩) 14 ms. These data suggest that uptake increased with τ.

(*INT*), defined as

$$INT = \int_0^\infty E_o e^{-t/\tau}\,dt = E_o\tau$$

where E_o is the peak field strength, t is time, and τ is the decay time constant. In Fig. 3, uptake is plotted versus *INT*, where $INT = E_o\,\tau$ for a single pulse and $INT = \sum E_o\,\tau$ for multiple 1-ms pulses. Proportionality between transport and *INT* has been previously proposed (Schwister and Deuticke, 1985; Liang et al., 1988; Jayaram et al., 1992) and, therefore, was investigated here. Linear regressions are shown.

Fig. 3 addresses two issues: (a) whether *INT* correlates well with uptake and (b) whether multiple pulses and longer pulses with the same *INT* were equivalent. Addressing the first point, there is a relationship between *INT* and uptake, which seems to be roughly linear. Thus, the linear regressions for the multiple pulse data and the longer pulse data, each treated separately, may be useful for application-oriented estimations. However, for mechanistic understanding, a linear fit seems inappropriate. First, the r^2 correlation constants are approximately 0.6, indicating only a mediocre fit. Second, the y-axis intercepts (corresponding to no pulse) are approximately 3×10^4 molecules per ghost. However, this value

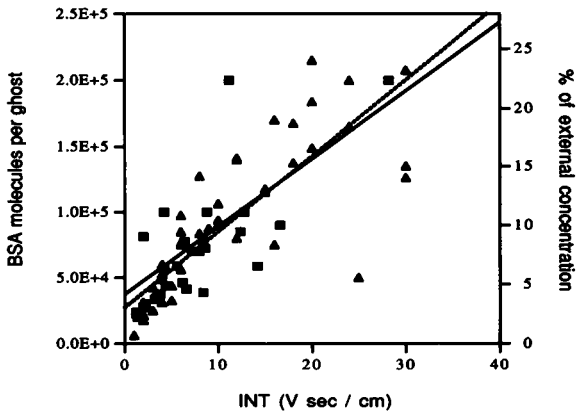

FIGURE 3 Uptake as a function of the time integral of field strength, *INT* (see text). Uptake of BSA is plotted versus *INT*, where (■) $INT = E_o\tau$ for a single pulse (data from Fig. 2) or (▲) $INT = \sum E_o\,\tau$ for multiple 1-ms pulses (data from Fig. 1). Least-squares linear regressions are shown: multiple pulse data (*solid line*, $y = 5160x + 36400$, $r^2 = 0.60$) and single pulse data (*dashed line*, $y = 5810x + 25200$, $r^2 = 0.56$). This graph suggests that (a) uptake can be approximately described by a linear function of *INT*, and (b) multiple short pulses and single long pulses having the same *INT* result in similar uptake, over the range of conditions used.

should be zero, because "background" fluorescence of unpulsed controls was subtracted from all pulsed samples. Finally, the correlation consistently overpredicts the measured uptake for $INT < 10$ V s/cm and generally underpredicts at higher values. Such systematic deviations suggest that a nonlinear model is appropriate.

The second issue concerns equivalency of multiple and single pulses of the same *INT*. The points corresponding to multiple and single pulses in Fig. 3 have approximately the same distribution. Moreover, their linear regressions are very similar. This suggests that different pulsing protocols having the same *INT* caused approximately the same uptake, over the range of conditions used. This has important mechanistic implications. First, it suggests that transport occurred primarily during pulses, because different t_{inter}—whether 60, 5, or 0 s (for longer single pulses)—did not cause differences in transport. This point is discussed further below. Second, the nonlinear dependence of uptake on *INT* is probably a result of the complex, time-dependent behavior of the pore population distribution and transmembrane voltage. Nevertheless, given the complexity of the phenomenon, it is remarkable that uptake can be approximated as a linear function of *INT* under the conditions used.

BSA adsorption to ghosts

To assess the possible role of BSA adsorption to ghost membranes, BSA was added to ghost suspensions at different times before pulsing. Fig. 4 shows that ~50% more uptake resulted when BSA was added to ghost suspensions 1 h before pulsing than when added only 5 s before. This suggests that an interaction between BSA and ghosts occurred that affected uptake. It is reasonable to expect that an interaction

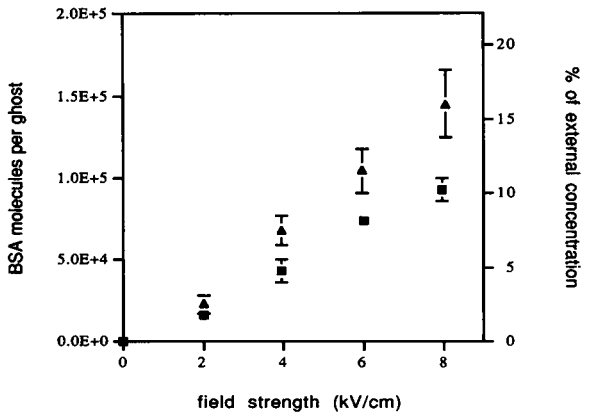

FIGURE 4 The effects of BSA adsorption to ghosts before pulsing. Uptake is plotted versus field strength for cells exposed to BSA for different amounts of time before pulsing: (■) 5 s, (▲) 1 h. This result suggests that increased BSA adsorption to ghost membranes increased uptake.

might have occurred, such as nonspecific binding (adsorption) of BSA to ghost membranes. Microscopy supports this, showing weak fluorescence apparently associated with the ghost membrane of unpulsed control samples (data not shown). However, no quantitative difference between fluorescence of unpulsed ghosts exposed to BSA for 5 s vs. 1 h could be detected. Note that such "background" fluorescence has been subtracted from all data presented in this paper, because it does not represent uptake. Thus, higher fluorescence associated with longer co-incubation of ghosts and BSA before pulsing did not represent additional adsorption to the external surface, but represented increased uptake upon pulsing, probably due to BSA adsorption.

Similar results have been reported, where DNA expression after electroporation was enhanced up to threefold by longer pre-pulse exposure of cells to DNA (Dekeyser et al., 1990; Klenchin et al., 1991). Moreover, Xie et al. (1990) have demonstrated that enhanced DNA binding to cell membranes, caused by elevated divalent cation concentrations, increased DNA expression by up to almost two orders of magnitude. Finally, membrane interactions with molecules having surfactant properties have been shown to alter electropore growth kinetics (Klotz et al., 1993). Although there are differences in experimental protocols, there appears to be evidence from a number of investigators that adsorption of a molecule to a cell membrane can increase transport of that molecule across the membrane.

Time scale of transport

To distinguish between the relative importance of uptake during a pulse and that after a pulse through long-lived electropores, BSA was added to ghost suspensions either before pulsing or at various times after pulsing. Different waiting times (i.e., the time between pulsing and washing the ghosts) were also used. Fig. 5 shows these results and suggests that: (a) although some uptake occurred when BSA was added after a pulse, much more occurred when BSA was added

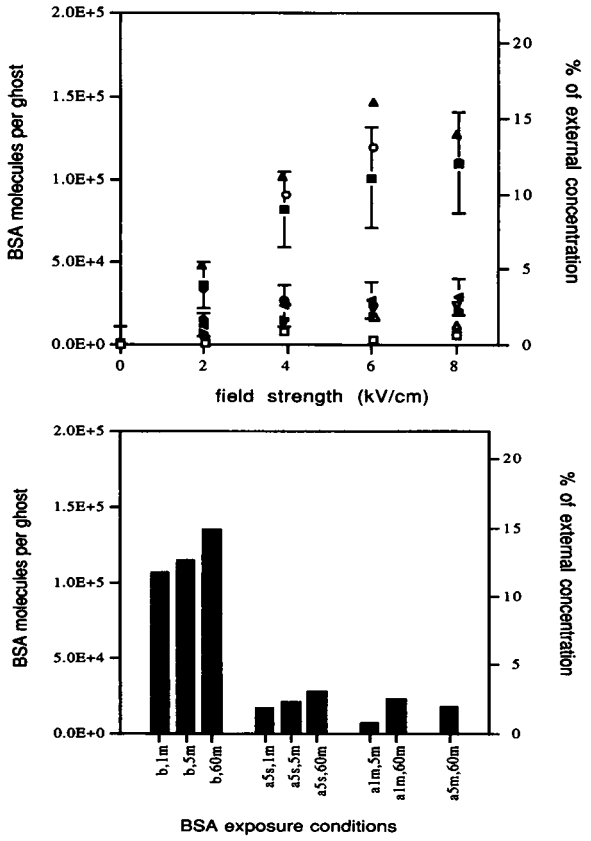

FIGURE 5 Uptake for pre-pulse and post-pulse addition of BSA. (*A*) Uptake is presented as a function of field strength for ghosts exposed to BSA at different times. BSA was added to ghost suspensions either before pulsing or at various times after pulsing. Different waiting times (i.e., the time between pulsing and washing the ghosts) were also used. Conditions were as follows (see key below): (■) b, 1m; (○) b, 5m; (▲) b, 60m; (▶) a5s, 1m; (▽) a5s, 5m; (◀) a5s, 60m; (□) a1m, 5m; (●) a1m, 60m; (△) a5m, 60m. (*B*) Average uptake in the plateau region (4–8 kV/cm, see text) is shown for each condition. This figure suggests that most uptake occurred during and/or within 5 s after a pulse, although some uptake occurred well after the pulse too.

Key: b = before pulsing; a = after pulsing; s = seconds; m = minutes. The first code represents when BSA was added, whereas the second code represents when ghosts were washed. For example, "a5s, 1m" indicates that BSA was added to the ghost suspension 5 s after the pulse and that the ghosts were washed 1 min after the pulse.

before; (b) the waiting time allowed after the pulse had only small effects on uptake; and (c) the time of BSA addition, when added after the pulse, also had only small effects on uptake. This constrains possible mechanisms of transport.

Uptake is expected to occur by different mechanisms at different times: (a) before, or in the absence of, a pulse, transport could in principle occur by diffusion through the intact lipid bilayer membrane; (b) during a pulse, transport could occur by diffusion and/or electrically driven transport (e.g., electrical drift and electro-osmosis) through electropores; and (c) after a pulse, transport could occur through electropores, as long as pores exist, by diffusion and/or low voltage, electrically driven transport due to a small transmembrane diffusion potential (Weaver and Barnett, 1992). Because no uptake was observed in unpulsed controls, it

appears that significant diffusion of BSA across an intact membrane did not occur. Given the unfavorable energy cost to insert a charged species into the membrane (Parsegian, 1969), this result is expected. That uptake was observed even when BSA was added 5 min post-pulse suggests that long-lived electropores capable of allowing penetration of BSA by diffusion and/or low-voltage electrically driven transport existed for at least minutes after a pulse. Finally, because uptake was much greater when BSA was added before a pulse suggests that: (a) a larger total effective pore area existed during and within 5 s after a pulse and/or (b) electrically driven transport present during a pulse was significantly greater than post-pulse transport.

Additional results from the present study further suggest that the important events that affect transport occur during a pulse. First, Fig. 6 shows BSA uptake at three different temperatures (0, 23, and 37°C). The results suggest that uptake was independent of temperature from 0–37°C. Temperature has previously been shown to have little effect on pore formation, but to strongly affect pore lifetime (Kinosita and Tsong, 1977b; Serpersu et al., 1985; Escande-Geraud et al., 1988; Michel et al., 1988). If significant transport occurred post-pulse through long-lived pores, then elevated temperature would be expected to decrease pore lifetime and thereby decrease uptake. This was not the case, indicating that pore lifetime did not affect uptake. In this analysis, we have neglected changes in post-pulse transport due to increased diffusion and diffusion potentials at elevated temperature. However, these parameters are expected to vary less than an order of magnitude over the temperature range considered (Bockris and Reddy, 1970), whereas pores are believed to close orders of magnitude more quickly at 37°C than at 0°C.

Second, Fig. 1 *E* suggests that multiple pulses applied every 5 or 60 s result in the same uptake. Moreover, comparisons between multiple and single pulses suggests that transport correlates with total *INT*, independent of N_{pulse} (Fig. 3). If significant transport occurred between pulses, then, for the same *INT*, single long pulses should have resulted in the least uptake, whereas pulsing every 60 s should have caused the most. However, if most transport occurred during pulses, then t_{inter} (0, 5, or 60 s) should not have affected uptake, as was observed.

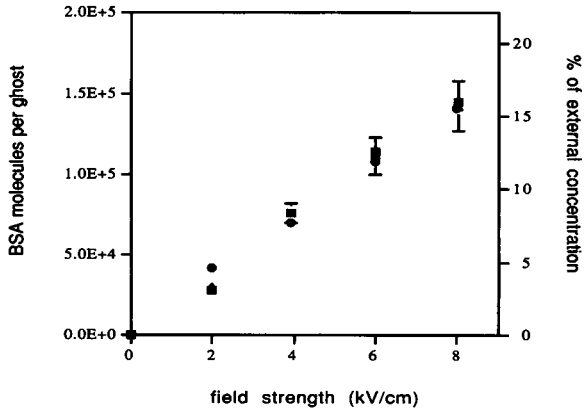

FIGURE 6 The effects of temperature on uptake. BSA uptake versus field strength of a single pulse is shown at three different temperatures: (■) 0°C, (▲) 25°C, (●) 37°C. Samples were maintained at these temperatures before and after pulsing. Additional temperature rises due to the pulse itself have been shown to occur, but are not included in this analysis. However, this graph suggests that temperature had little effect on uptake.

Additional evidence from the literature supports these findings. Although extensive transport of ions or molecules smaller than ~1 kDa has been shown to occur seconds to hours after electroporation (Kinosita and Tsong, 1977a; Schwister and Deuticke, 1985; Serpersu et al., 1985; Rols and Teissié, 1990), uptake of larger molecules, such as BSA, is not expected to occur extensively through long-lived electropores (Orlowski and Mir, 1993). Many investigators have demonstrated this for DNA, where essentially no expression was observed in unpulsed controls, orders of magnitude more was seen when DNA was added within minutes post-pulse, and still orders of magnitude more expression was found when DNA was added before the pulse (Taketo 1988; Xie et al., 1990; Klenchin et al., 1991; Chang et al., 1992). Also, a number of investigators have argued that macromolecular uptake by electroporation occurs primarily by electrically driven transport through electropores (Dimitrov and Sowers, 1990; Klenchin et al., 1991; Weaver and Barnett, 1992). It is presently unclear whether transport is driven predominantly by electrical drift or electro-osmosis, although both appear to be important. An exception to this is found in the early electroporation literature, where release from erythrocytes of hemoglobin (67 kDa) occurred for hours post-pulse (Kinosita and Tsong, 1977a; Kinosita and Tsong 1977b; Schwister and Deuticke, 1985). However, in this case, hemoglobin release was a result of cell rupture caused by water uptake due to osmostic imbalances. This was not an example of hemoglobin transport through long-lived electropores, but represented a secondary effect of electroporation.

Thus, the present study, in combination with previous work, suggests that most uptake of macromolecules occurs by electrically driven transport during a pulse, although transport, at a much slower rate, can also occur for at least minutes after a pulse.

Transport plateau at high field strength

It was previously shown, with four molecules of different physical characteristics, that uptake into erythrocyte ghosts first increased with *E*, but reached an apparently nonequilibrium plateau at large *E* (Prausnitz et al., 1993a). The onset of the plateau was different for each molecule, ranging from 2–5 kV/cm. To explain this result, it was proposed that transport is controlled by *E*-dependent processes at small *E*, but could be controlled by *E*-independent processes at large *E*. The existence of a nonequilibrium large-*E* plateau has also recently been demonstrated in yeast cells for transport of calcein and BSA (Hui, 1994).

The present study provides additional examples of transport plateaus for BSA. Moreover, it gives further evidence that the amount of uptake at the plateau is not an absolute

maximum in transport, but represents the maximum effect of increasing E, for a particular pulse protocol. Fig. 1 demonstrates this point most clearly. For a given N_{pulse}, a plateau was observed at large E, but the plateau amount of transport for each N_{pulse} was different. For example, in Fig. 1 *A*, application of a single pulse for E > 3–4 kV/cm did little to further increase uptake. In contrast, application of additional pulses increased uptake up to fourfold above the single-pulse, large-E plateau. Thus, our interpretation is that the plateau represents the maximum effect of increasing E for a particular pulse protocol, rather than the maximum uptake possible under any condition.

In some experiments, a plateau was not clearly observed (e.g., Figs. 4 and 6). Instead, uptake appeared to continue to increase for larger E over the range investigated. Although sometimes the large error bars associated with this inherently heterogeneous biological preparation allow a plateau, in other cases a plateau is excluded. The most notable exceptions are seen in Figs. 4 and 6. However, the data in these two figures come from the same set of experiments, suggesting that they represent an isolated result. Nevertheless, it is evident that, although plateaus are usually observed, it is not always the case.

Plateaus are probably a consequence of the interactive behavior of a dynamic pore population, the transmembrane voltage, and one or more molecular transport mechanisms. A number of potential explanations have been proposed. (a) We previously suggested that transport may be controlled by E-independent processes at large E. (b) Wang et al. (1993) recently demonstrated that a detailed computer simulation of electroporation predicted transmembrane voltage and molecular transport that featured an approximate plateau in transport at large E. This result was based on a dynamic, heterogeneous pore population and local electrophoretic transport through the transient pores. (c) Nonequilibrium steady-state transport could be achieved at large E, because molecules that have entered a cell can exit at the other side, by electrophoresis and/or electro-osmosis. This could also result in a plateau in net uptake.

Transport maximum

Although the maximum uptake which was achieved by increasing E alone was apparently not an absolute maximum, there does appear to be a maximum amount of BSA that could be transported into a ghost in this study. Under any condition used, uptake never exceeded approximately 2.5×10^5 BSA molecules per ghost. Assuming a spherical ghost with an internal volume of 150 μm^3 (Sowers and Lieber, 1986), this corresponds to an internal BSA concentration of approximately 2.8×10^{-6} M. Given that the supplied external BSA concentration was 10^{-5} M, then the maximum BSA internal concentration corresponds to only one-fourth of the external concentration, an apparently nonequilibrium state, assuming no partition coefficients or binding sites.

Determination of apparent equilibrium requires correct assessment of ghost volume. We have used the volume determined by Sowers and Lieber (1986) for ghosts prepared and used under conditions similar to those of the present study. Examination by microscopy showed that our ghost preparation appeared spherical and had average radii of 6–7 μm, which is in good agreement with Sowers and Lieber. To explain the apparent nonequilibrium presented here, our estimate of ghost volume would have to be a factor of four too large, which we believe is unlikely.

Apparent nonequilibrium uptake has precedent in the literature. Uptake of molecules ranging from antibodies to oligonucleotides to simple carbohydrates has corresponded to internal concentrations from 0.1 to 60% of external concentration (Serpersu et al., 1985; Mir et al., 1988; Bartoletti et al., 1989; Bazile et al., 1989; Casabiance-Pignéde et al., 1991; Poddevin et al., 1991). In contrast, other studies have reported uptake of proteins where internal concentrations appear to correspond to as much as one to two orders of magnitude above external concentrations (Wilson et al., 1991; Glogauer and McCulloch, 1992), according to our calculations. Although direct assessment of relative concentrations was not discussed by these authors, our calculations were made using cell dimensions and molecular uptakes given in the papers. These higher internal concentrations suggest that protein binding within cells occurred. In contrast to studies with macromolecules, uptake corresponding to equilibrium or Donnan equilibrium has been reported for Lucifer Yellow (450 Da) (Mir et al., 1988) and small ions (Kinosita and Tsong, 1977a; Schwister and Deuticke, 1985; Serpersu et al., 1985).

To summarize, the literature reports equilibrium uptake only for small molecules; macromolecules were transported at sub-equilibrium levels, except when internal binding appeared to occur. However, experiments in the literature differ significantly from the present study; nonequilibrium uptake did not represent the maximum possible uptake, but simply represented uptake observed under the particular conditions used. Nevertheless, to our knowledge, uptake of a macromolecule corresponding to apparant equilibrium has not been reported under any experimental conditions, including ours. This poses an intriguing problem relevant to mechanistic understanding. Explanations may involve the mechanisms proposed above to explain plateaus and/or other mechanisms, such as an electric field-altered equilibrium or a Donnan equilibrium.

Approaches to increase transport

The findings of this study may aid approaches to increase transport of molecules across lipid bilayer membranes in cells and in tissues, which is relevant to many existing electroporation protocols. Note that issues of cell viability are beyond the scope of this study and, therefore, are not considered.

- Increasing E may not always increase transport. The E above which transport plateaus is expected to be a function of cell size and orientation, as well as the molecule being transported.

- Using longer and/or multiple pulses can increase transport, even beyond the large-E plateau. *INT* appears to be the most important parameter, where t_{inter} is less important. The added constraint of cell viability issues may determine the best pulsing protocol.
- It may not be possible to transport macromolecules to apparent equilibrium, although transport to well within an order of magnitude of apparent equilibrium has been demonstrated with macromolecules here and previously. Binding within cells may affect maximum possible transport.
- Because molecule-membrane interaction may be important, longer pre-pulse exposure times could increase transport.
- Most transport appears to occur primarily during pulses and is electrically driven. Transport after pulsing may be limited by lack of a powerful driving force to transport molecules through long-lived pores. Additional driving forces, such as pressure gradients or weak electric fields (e.g., electrophoresis), could be applied after pulsing to enhance post-pulse transport.
- Temperature does not appear to have substantial effects on transport.

CONCLUSIONS

The results of this quantitative study of molecular transport into erythrocyte ghosts due to electroporation give insight into mechanisms of transport and suggest approaches to maximizing transport for improved electroporation protocols. Plateauing, nonlinear, and nonequilibrium relationships between transport and a number of experimental parameters indicate the complexity of electroporative phenomena. Quantitative determination of the number of molecules transported for a range of conditions is vital to advancing applications and understanding mechanisms.

We thank S. Conner and G. A. Paradis for assistance with flow cytometry, U. Pliquett for temperature measurements and electric field validation, Y. A. Chizmadzhev and D. A. Edwards for helpful discussions, and M. Llabres for help with statistical analysis. This work has been supported in part by a fellowship provided by Cygnus Therapeutic Systems (M. R. Prausnitz), by the M.I.T. Undergraduate Research Opportunities Program (C. D. Milano, J. A. Gimm), by National Institutes of Health grants GM44884 (R. Langer) and GM34077 (J. C. Weaver), and by Army Research Office grant DAAL03-90-G-0218 (J. C. Weaver).

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