Lipid-Assisted Microinjection: Introducing Material into the Cytosol and Membranes of Small Cells

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ABSTRACT   The microinjection of synthetic molecules, proteins, and nucleic acids into the cytosol of living cells is a powerful technique in cell biology. However, the insertion of a glass micropipette into the cell is a potentially damaging event, which presents significant problems, especially for small mammalian cells (spherical diameter = 2–15 μm), especially if they are only loosely adherent. The current technique is therefore limited to cells that are both sufficiently large or robust and firmly attached to a substrate. We describe here a modification of the standard technique that overcomes some of the problems associated with conventional microinjection but that does not involve the insertion of a micropipette deep into the cell cytoplasm. Instead, this method depends on lipid fusion at the micropipette tip to form a continuous but temporary conductance pathway between the interiors of the micropipette and cell. This technique thus also provides a novel method of transferring lipids and lipid-associated molecules to the plasma membrane of cells.

INTRODUCTION

Microinjection of agents such as synthetic molecules, peptides, proteins, and nucleic acids into the cytosol of living cells is a powerful technique in cell biology. However, microinjection of small mammalian cells (spherical diameter = 2–15 μm) or very flat cells (1–2-μm thickness) has always been difficult to achieve without damaging the cell. The penetration of the plasma membrane is often achieved by a rapid entry and exit "stab" and requires the cell to be firmly attached to a substrate. Thus "stab injection," where the micropipette is within the cell for short periods of time (in the order of 100 ms) requires high pressure (100–200 mbar) to introduce sufficient material from the micropipette into the cell during that time. This must be carefully controlled, as insufficient pressure results in too little material being injected and excessive pressure causes cell damage or even cell rupture. Apart from these problems, in small cells there is the additional potential for damage to intracellular organelles. In the absence of full 3D control of the micropipette tip (Kurat et al., 1997), its location within the cell is uncontrolled and is liable to cause damage to intracellular organelles. Whereas this may not be a problem for larger cells, small cells often have a large percentage of the cell cytoplasm occupied by organelles such as the nucleus, lysosomes, and endoplasmic reticulum. Microinjection at high speed may thus cause inadvertent intracellular damage to one of these organelles. The velocity at which the micropipette tip enters the cell may be on the order of 700 μm/s (Guse et al., 1997) and is likely to displace, damage, or enter the nucleus (which in small cells such as neutrophils and basophils accounts for up to 50% of the cell volume) rather than directly entering the cytosol.

Despite these potential problems, traditional pressure injection has been successfully used in a number of small cells, including hepatocytes (Cobbold and Rink, 1987), Jurkat cells (Guse et al., 1997), MCF-7 cells (Li et al., 1997), RBL-2H3 cells (Hoffman et al., 1997), and J774 cells (Hackman et al., 1997). However, even with these cells, the success rate is not great, with many cells failing to survive "stab injection." There have been various attempts to minimize the problem of cell damage, including the use of pharmacological Ca^{2+} channel blockers (Bartoli and Claycomb, 1997), or to avoid microinjection entirely by introducing agents into cells in which the plasma membrane has been permeabilized either physically by electroporation (e.g., Teruel and Meyer, 1997) or by the use of biological molecules, such as streptolysin O (e.g., Coppolini et al., 1995; Fitzsimmons et al., 1997). Although these approaches have been extremely useful, cell damage is inevitably significant, and the mechanisms by which the cell repairs this damage are poorly understood (Terasaki et al., 1997) but may involve either a zone of high cytosolic free Ca^{2+} and vesicle fusion (Steinhardt et al., 1994; Terasaki et al., 1997) or active signaling by the cell (Morgan and Campbell, 1985). Thus the recovered cells may not represent those in the state that was originally intended for study. With internal perfusion (e.g., Nüsse and Lindau, 1993), a glass micropipette is used that does not enter the cell. Instead, a portion of the plasma membrane is sucked into the mouth of the micropipette, causing local rupture of the cell membrane at that point. Damage to intracellular structures is thus avoided, but the process of "breaking into" the cell results in an irreversible seal, and the cell must remain attached to the pipette for its survival. This precludes studies of cell behavior that depend on the cell being free, such as chemotaxis, phagocytosis, and other forms of cell shape change. The negative pressure required to break in may also withdraw

cytosol if not carefully controlled, and in any case, during the period that the micropipette is attached, diffusion of material both from the micropipette into the cell and from the cytosol into the micropipette occurs. Thus all of these techniques, while providing important progress, have limitations.

Another approach that does not involve glass micropipettes uses lipid fusion, by the use of liposomes (e.g., Hallett and Campbell, 1980) or erythrocyte ghost fusion (e.g., Hallett and Campbell, 1982), as a means of introducing material into small cells. Although lipid fusion can be successful, the amount of material injected per cell can be very small. The technique is thus useful for introducing nucleic acid into cells, as with lipofection, where amplification of the effect of the injected molecule can occur but may be insufficient for experiments with no amplifying effect. The inefficiency of the method is seen by considering that to inject a small cell ($d = 10 \ \mu$m) with 1% of its volume by fusion of liposomes ($d = 50$ nm) requires thousands of fusion events/cell (Dormer et al., 1985). As this number of fusion events is unlikely, the use of liposomes for cell physiology has not been widely successful. The problem is overcome by the fusion of larger vesicles such as red cell ghosts (e.g., Hallett and Campbell, 1982), where one fusion event can introduce a large amount of material, but the method suffers because of the large amount of foreign plasma membrane that is also introduced.

In this paper we describe a "hybrid" technique involving both the glass micropipette and lipid fusion approaches (Laffafian and Hallett, 1998). The technique involves the use of a lipid-coated micropipette, where fusion between the lipid at the micropipette tip and the cell membrane results in a channel into the cell cytosol. Like internal perfusion, this avoids the possibility of organelle damage and as only contact, rather than penetration, is required for microinjection, the cell need not be firmly adherent. However, unlike internal perfusion, the micropipette can be withdrawn and the cell survival is good. Furthermore, the low pressure in the micropipette ensures that the amount of material injected is controlled and does not unduly damage the cell. We demonstrate the use of simple lipid-assisted microinjection ("slam") on human neutrophils (diameter 10 μm), when loosely adherent as spherical individual cells or spread on glass coverslips with a cell thickness of just 1–3 μm. These cells have been very difficult, if not impossible, to successfully microinject by conventional means, and to the authors' knowledge, no results on the microinjection of neutrophils have been published. Using this approach, we demonstrate the successful microinjection of neutrophils both loosely adherent (10 μm diameter) and thinly spread (1 μm thick) on glass, which remain viable and chemotactic.

MATERIALS AND METHODS

Materials

Micropipettes were premade (Eppendorf), and the micromanipulation was achieved with an Eppendorf manipulator (model 9525). Phosphatidylcho-line-oleyl-palmitoyl (POPC) (Sigma) was dissolved in chloroform (20 mg/ml) and stored below 0°C. Aliquots of this solution were diluted with chloroform before use (final POPC concentration ~1 mM). DiIC$_{18}$ (3) (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate) was obtained from Molecular Probes (Eugene, OR) and lucifer yellow CH from Sigma (Poole, England).

Neutrophil isolation

Neutrophils were isolated from the heparinized blood of healthy volunteers as described previously (Hallett et al., 1990). After dextran sedimentation, centrifugation through Ficoll-Paque (Pharmacia) and hypotonic lysis of red cells, neutrophils were washed and resuspended in Krebs buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 1.3 mM CaCl$_2$, 25 mM HEPES, and 0.1% bovine serum albumin, adjusted to pH 7.4 with NaOH).

Lipid coating of micropipette

The micropipette was back-loaded with sufficient volume of the injection medium, containing Lucifer yellow (10–50 mg/ml), to exert a pressure to offset capillary pressure. The tip of the micropipette was dipped in the lipid solution (POPC, 1 mM, dissolved in chloroform and kept on ice), or a drop (~10 μl) of the lipid solution was applied to the tip of the micropipette. Evaporation of the chloroform resulted in a coating of lipid on the glass. The micropipette was then connected to a control pressure device (Eppendorf, microinjector) with the pressure set to zero. The micropipette was then placed in the aqueous medium bathing the cells, and the dried lipid on the tip of the micropipette was swollen to form a bilayer. When the pressure in the micropipette was increased to 5–10 mbar, the lack of ejection or leakage of the dye from the micropipette as observed by epifluorescence microscopy provided evidence that an effective lipid seal had formed at the micropipette tip.

The "slam" procedure

Neutrophils were allowed to sediment onto a glass coverslip, maintained at 37°C by a stage heater mounted for viewing with an oil immersion objective (100×). The loaded lipid-coated micropipette was brought into the field of view with a motorized, microprocessor-controlled micromanipulator and placed in gentle contact with the surface of a neutrophil. This resulted in the transfer of lipid and the aqueous contents of the micropipette to the cell. The pressure within the micropipette was not increased during the microinjection but was held constant at 5–10 mbar. This low pressure was important to prevent rupture of the lipid seal at the micropipette tip and to prevent subsequent damage to the cell undergoing microinjection. The lipid-coated micropipette could be used more than once (see, for example, Fig. 4, in which three neutrophils were injected). The number of possible successful injections depended on the amount of lipid in the coating.

Image collection and analysis

For demonstration purposes, lucifer yellow was used as a fluorescent marker of the aqueous phase. This enabled quantification of the timing and amount of material microinjected. Images were acquired using either a standard CCD camera or, for low-level fluorescence detection, an intensified CCD camera (ISIS; Photonic Science) coupled to an inverted Zeiss IM35 microscope. Images were subsequently recorded from tape using a video printer. The intensity of signals from individual cells was quantified by setting an exclusion mask over the cell of interest for photometric recording with a photomultipler tube and Spex DM3000CM software (Spex) set for acquisition an integration time of at 100 ms. The intensity was linearly related to the concentration (and hence the amount) of lucifer yellow in the cell. The amount of injected material was estimated from the intensity of dye inside the cell relative to that in a micropipette (at equal

diameter, 10 μm) containing 100$\times$ dilution of the dye. For recording of the time course of the micropipette procedure, the micropipette was initially placed close to the neutrophil under brightfield illumination, and the position of the cell edge was established. With the white light switched off, the micropipette was advanced to touch the cell during fluorescence imaging. The tip of the micropipette was visualized under fluorescence illumination (see Figs. 5 and 6), enabling the time of contact between the micropipette and the cell to be established.

RESULTS

Lipid transfer

To demonstrate the coating of the tip of the micropipette with lipid, as described in Materials and Methods, the fluorochrome $DiIC_{18}$ (3) (1,1'-dioctadecyl-3,3,3',3'-tetra-methylindocarbo-cyanine perchlorate) was used. This probe is weakly fluorescent in water but fluoresces strongly in lipid bilayers (Tsein and Waggoner, 1995). It was thus used to visualize the lipid coating at the micropipette tip (Fig. 1) by addition (1 mg DiI/ml) to the outside of the lipid coating on the micropipette. This demonstrated that the procedure described in Materials and Methods resulted in lipid coating the tip of the micropipette (Fig. 1). On touching a loosely adherent neutrophil (diameter 10 μm) with the lipid-coated micropipette, the $DiIC_{18}$ (3) was transferred to the cell (Fig. 1 b). The dye did not transfer to the cell by release into the fluid phase, as contact between the micropipette and the cell

was required. Simply placing the micropipette near but not touching the cell did not result in DiI transfer (Fig. 1 a). On touching the cell, $DiIC_{18}$ (3) fluorescence was strongest at the point of contact, whereas later the fluorescence became more uniform, with significant fluorescence at the opposite pole of the cell (Fig. 1 b). This was consistent with the transfer of $DiIC_{18}$ (3) from the lipid-coated micropipette to the cell membrane by direct contact and the result of the formation of a lipid "bridge" between the micropipette and the plasma membrane of the cell.

Aqueous transfer

To determine whether an aqueous-filled lipid bridge formed on contact between the lipid-coated micropipette and the cell, lucifer yellow was loaded into the micropipette and used as a marker of aqueous phase transfer between the contents of the micropipette and the cell cytosol. Although "stab" injection by an untreated micropipette could transfer lucifer yellow to the cell, merely touching the cell with the micropipette failed to transfer any detectable lucifer yellow to the cell cytosol (Fig. 2). However, coating the micropipette tip with lipid produced significant transfer of lucifer yellow to the cell (Fig. 2). There was a sudden release of dye from the micropipette into the cell cytosol observable by fluorescence microscopy. As the pressure within the micropipette was not increased during microinjection, this was the result of opening a channel from the micropipette to the cell cytosol. This observation was therefore consistent with the formation of an aqueous-filled lipid bridge from the micropipette tip to the cell cytosol (Fig. 3) and formed the basis for introducing material into the neutrophil cytosol without penetration of the cell.

Cellular "damage"

It was important to determine whether the "slam" procedure, which effectively transferred material to the neutrophil cytosol, occurred without excessive cell damage. A crude way to assess this was by the use of trypan blue. By the addition of trypan blue to the incubation medium, gross damage to the cell was detectable as the dye accumulated within the cell. With "stab" injection, few (less than 5%) neutrophils survive the "stab" without becoming trypan blue positive either immediately or during the subsequent 10 min and were thus discounted as nonviable. In contrast, under optimal conditions, the "slam" procedure produced very good survival rates; in some experiments the trypan blue exclusion (for more than 10 min) after "slam" was 100%. A critical factor in determining the survival of the neutrophil after "slam" was the pressure exerted by the micropipette within the cell. This could be monitored by the amount of material injected in a unit of time. Fig. 4 shows a demonstration experiment in which three neutrophils have been slam-injected with different pressures to illustrate the problem of using retention of lucifer yellow alone as a criterion
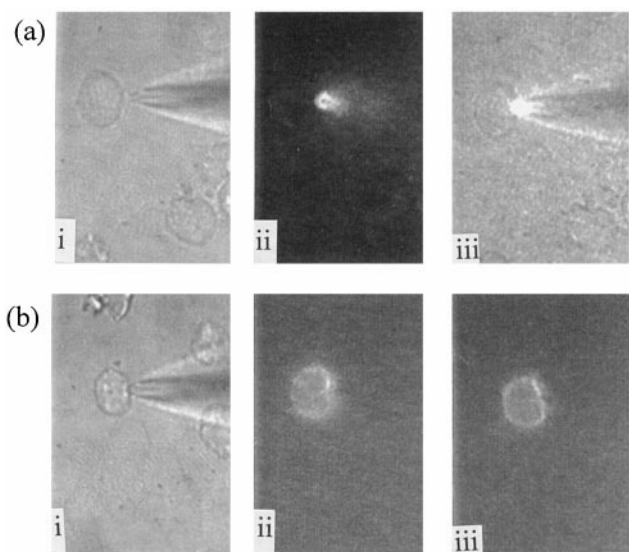

FIGURE 1 Lipid transfer from micropipette to cell membrane. (*a*) The series of images shows (*i*) the phase-contrast view of a single neutrophil with the lipid coated micropipette before contact; (*ii*) the fluorescent image of DiI staining marking the site of lipid at the micropipette tip; and (*iii*) the superimposition of the fluorescence and phase-contrast images. Note that there is no transfer of the DiI fluorescence to the cell. (*b*) The series of images shows (*i*) contact between the micropipette and the neutrophil under phase contrast, (*ii*) the fluorescent image of DiI transfer to the neutrophil membrane immediately after contact, where the fluorescence is brightest at the micropipette contact point; and (*iii*) the membrane 2 min later, when the fluorescence is more uniformly distributed around the cell membrane.
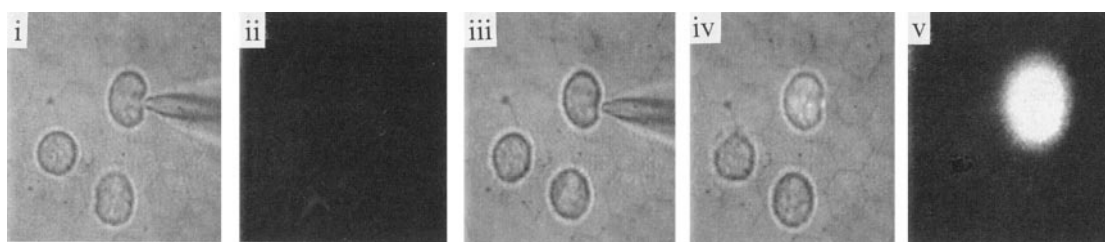
FIGURE 2 Transfer of aqueous contents to the cytosol. The series of images shows (*i*) the noncoated micropipette touching a neutrophil and the consequent lack of transfer of fluorescent Lucifer yellow seen in (*ii*) the corresponding fluorescent image. (*iii*) The procedure was repeated after the micropipette tip was coated with lipid, showing (*iv*) transfer of dye to the neutrophil. (*v*) Fluorescence microscopy, for comparison with image *ii*.

for successful microinjection. The middle cell was slam-injected at a pressure (100 mbar) that was sufficient to completely rupture the cell, so that it neither retained lucifer yellow nor excluded trypan blue. The upper cell was slam-injected at a pressure (10 mbar) that injected an appropriate amount into the cell (~1% of its volume), causing no increase in permeability to trypan blue, which was excluded for the cell. However, the lower cell was slam-injected with an intermediate pressure (50 mbar) that did not rupture the cell and permitted the retention of microinjected lucifer yellow. However, the permeability of the plasma membrane was increased and cellular damage had occurred, as is clear from the inclusion of trypan blue (Fig. 4). Neutrophils appropriately microinjected by the slam procedure retained low cytosolic free Ca^{2+} (as determined by fura2 ratio imaging) and remain responsive to f-met-leu-phe, as determined by an increase in cytosolic free Ca^{2+} and by oxidase activation (nitroblue tetrazolium reduction).

Characterization of lipid-assisted microinjection

Under an appropriately low pressure for microinjection (10 mbar), there was no apparent increase in cell volume, as determined by cell diameter or change in cell shape. To gain

insight into the mechanism by which the aqueous material entered the cell, the time course of the increase in cell fluorescence was determined. The were two characteristics of the entry of aqueous material. The first was that the release of dye from the lipid-sealed micropipette into the cytosol did not occur immediately on contact between the micropipette and the cell. The delay between contact and formation of the aqueous channel was often less than 1 s, but in some cases was several seconds. This may be consistent with the time required for formation of a lipid bridge or the low probability (per unit time) of fusion of the lipid at the micropipette tip with the cell membrane. The second characteristic was that in suitably spread neutrophils, a clear wave of fluorescent material was observed entering the cell during the initial stage of the "slam" procedure (Fig. 5). The apparent kinetics of the "wave" were consistent with diffusion from the micropipette tip, with the diffusion constant for lucifer yellow within the neutrophil cytosol being $D = 100 \ \mu m^2/s$. As this value is similar to that expected for a small molecule in cytosol (e.g., $D = 283 \ \mu m/s$ for IP_3; Allbritton et al., 1992), this suggested that the entry of material in the first second may be by diffusion. Thereafter, the intensity of fluorescence within the cell rose with a $t_{1/2}$ of
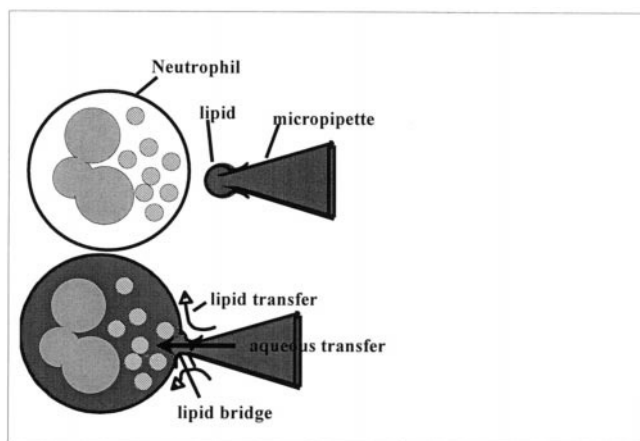

FIGURE 3 Mechanism of simple lipid-assisted microinjection (SLAM) technique. The diagram shows the location of the aqueous and lipid components of the micropipette before and after contact with the cell membrane.
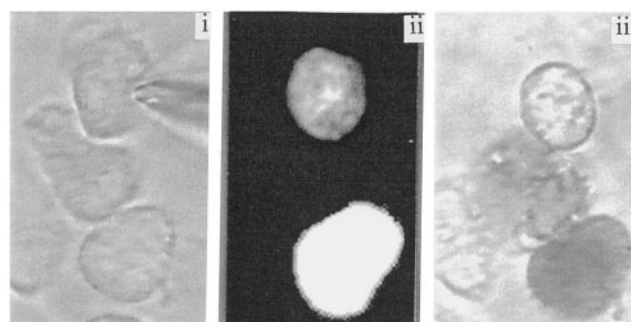

FIGURE 4 Cell "damage" associated with injection pressure. The series of images shows (*i*) neutrophils microinjected by the SLAM procedure at various injection pressures approximately as follows: upper cell, 10 mbar; lower cell, 50 mbar; middle cell, 100 mbar. Images *ii* and *iii* show the corresponding loading of Lucifer yellow and trypan blue, respectively, after all three cells were microinjected. As seen in image *ii*, the middle cell was lysed by excessive pressure and did not retain Lucifer yellow, whereas the lower cell initially retained Lucifer yellow but failed to exclude trypan blue (10 min). The upper cell represents an optimal microinjection in which Lucifer yellow was retained and trypan blue was excluded.

FIGURE 5　Initial entry of Lucifer yellow into cytosol. The lower series of images shows the fluorescent distribution of Lucifer yellow within the neutrophil shown in the upper panel after contact with the micropipette. The images are for the first second after entry of Lucifer yellow.
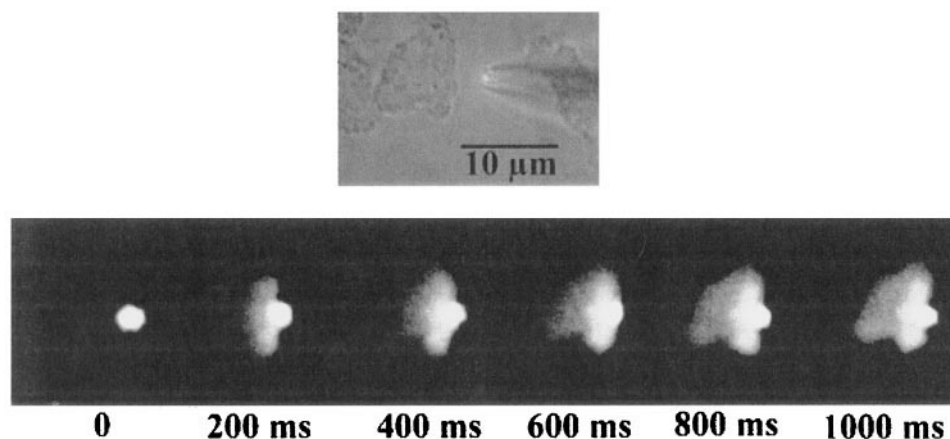
~10 s until a quasiequilibrium was formed (Fig. 6). It was unlikely that the plateau represented an equilibrium between the concentration of lucifer yellow inside the micropipette and the cytosol as the concentration of lucifer yellow was high (15 mg/ml) and self-quenching, whereas the concentration within the cell was below that for self-quenching. One possibility for the quasiequilibrium was that the rate of injection declined as the (low) pressures within the micropipette and the cell approximately equalized.

CONCLUSION

In this paper we have demonstrated the use of lipid-coated micropipettes as a means of introducing soluble agents into the cytosol of small and loosely adherent cells. With neutrophils, the technique successfully introduced material into both the cytosol of loosely adherent resting cells and cells that were spread and actively engaged in locomotion. The amount of material transferred to a single cell was considerably more than could be transferred by liposome fusion (equivalent to ~10^4 liposome fusion events/cell), and similar to that achieved by conventional "stab" microinjection. However, unlike conventional "stab" microinjection, lipid-assisted microinjection resulted in less cell damage (cell viability after injection being less than 5% for "stab" and up to 100% for "slam"), making the slam technique ~20 times more efficient than conventional microinjection for these cells. The fusion event at the micropipette tip between the lipid coating and the plasma membrane provided a "gentler" means of inserting water-soluble agents into the cytosol, minimizing damage to the cell and inadvertent activation of pathways within the cell. It will also provide a means of inserting lipids and lipid-soluble proteins into the cell plasma membrane. This latter feature represents a novel approach that may prove useful in cell physiology, especially in inserting molecules like GPI-linked protein recep-

FIGURE 6　Time course of transfer of material to cytosol. The graph shows the fluorescent intensity of Lucifer yellow within the neutrophil (shown in the *inset*) during the SLAM process. Contact between the lipid-coated micropipette containing Lucifer yellow and the cell is marked on the trace by the arrow. The bottom series of images were taken during the first 25 s after contact between the same cell and a micropipette.
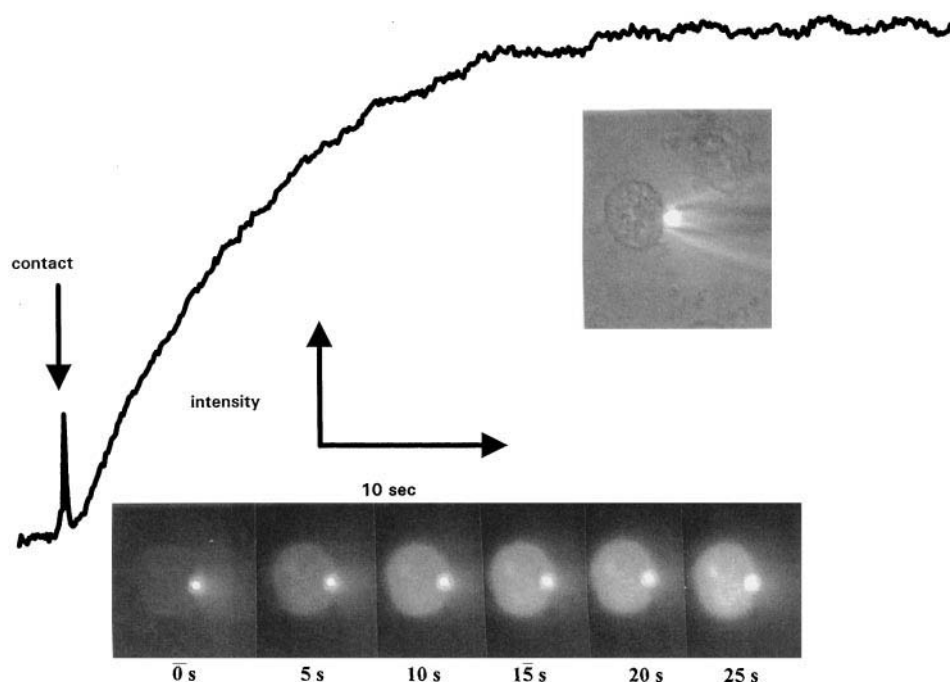
tors (van den Berg et al., 1995) into plasma membranes of individual cells on demand. At present, GPI-linked proteins can be incorporated into cell populations, but the microtechnique would allow loading of individual cells in a microscopic field to different levels to act as non-GPI-linked protein controls and to establish the relationship between the amount of GPI-linked protein/cell and response. The lipid we used here proved to be an effective fusion system, but others may also be successful and may vary with cell type. In particular, the charge on the phospholipid may influence the efficiency and kinetics of fusion. In this paper we have used the neutrophil as a demonstration cell type because it is notoriously difficult to microinject, with no reports, to our knowledge, of its successful microinjection. However, the technique should be applicable to other cell types, and we have demonstrated its use (without modification) on another cell type, MCF-7.

There are, of course, some potential problems with the technique we describe here. These include 1) the introduction of unwanted lipid and 2) the length of time (1–20 s) that may be required for the introduction of sufficient material. In this paper, we used a synthetic lipid that may produce minimal unwanted biological effects. However, this "problem" could also be viewed as a "benefit," as it provides a means of incorporating biologically active lipids, such as PIP_2, in cell membranes, with the intention of investigating their biological effects. The relatively long contact time raises a potential problem with the possibility that diffusion of mobile components of the neutrophil cytosol may enter the micropipette. However, with a patch pipette of a size similar to that used here, a diffusion time constant on the order of 5 min (Nüsse and Lindau, 1993) is required for small proteins (20–40 kDa) and is long compared with the contact times (1–20 s) required here. An insignificant loss of proteins would therefore be expected to occur. With smaller molecules, the possibility of diffusion into the micropipette tip becomes more of a problem, but the time required for equilibrium to be established by diffusion of fura2 from a patch pipette was 1–2 min (Nüsse and Lindau, 1993), and at 20 s there would be little loss of cytosolic molecules of this size.

As with any procedure involving microinjection, caution must be exercised in the interpretation of results, and controls must be performed to exclude the possibility of inadvertent effects of the microinjection procedure. However, it is hoped that the technique described here will make it possible to extend many of the microinjection approaches widely used in larger robust cells to smaller cells and thereby increase our understanding of their cell biology and physiology.

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