Phospholipase C activation by anesthetics decreases membrane-cytoskeleton adhesion

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SUMMARY

Many different amphiphilic compounds cause an increase in the fluid-phase endocytosis rates of cells in parallel with a decrease in membrane-cytoskeleton adhesion. These compounds, however, do not share a common chemical structure, which leaves the mechanism and even site of action unknown. One possible mechanism of action is through an alteration of inositol lipid metabolism by modifying the cytoplasmic surface of the plasma membrane bilayer. By comparing permeable amphiphilic amines used as local anesthetics with their impermeable analogs, we find that access to the cytoplasmic surface is necessary to increase endocytosis rate and decrease membrane-cytoskeleton adhesion. In parallel, we find that the level of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane is decreased and cytoplasmic Ca^{2+} is increased only by permeable amines. The time course of both the decrease in plasma membrane PIP_2 and the rise in Ca^{2+} parallels the decrease in cytoskeleton-membrane adhesion. Inositol labeling shows that phosphatidylinositol-4-phosphate levels are increased by the permeable anesthetics, indicating that lipid turnover is increased. Consistent with previous observations, phospholipase C (PLC) inhibitors block anesthetic effects on the PIP_2 and cytoplasmic Ca^{2+} levels, as well as the drop in adhesion. Therefore, we suggest that PLC activity is increased by amine anesthetics at the cytoplasmic surface of the plasma membrane, which results in a decrease in membrane-cytoskeleton adhesion.

Key words: Local anesthetic, Phospholipase C, Membrane-cytoskeleton adhesion, Calcium release

INTRODUCTION

Properties of local anesthetics

The addition of amphiphilic organic molecules to cells often produces dramatic changes in cell functions including an increase in fluid-phase endocytosis rate (Dai et al., 1997; Raucher and Sheetz, 1999). An important class of amphiphilic compounds is the local anesthetics, which typically have a positively charged group in the hydrophilic portion of the molecule. Many different physical chemical techniques have been used to provide evidence that anesthetic agents and similar drugs have a biophysical effect on cell membranes, which can often be described as a fluidizing or disordering action. For example, it has been shown that local anesthetics, such as tetracaine, destabilize membrane structure by interaction with polar headgroups of phospholipids (Shimooka et al., 1992). However, these drugs do not interact exclusively with membrane lipids, but they can affect the intracellular microtubules, microfilaments and membrane-associated enzymes (Butterworth and Strichartz, 1990). Some changes are due to specific effects of those molecules as enzyme inhibitors or substrates but there are other changes that occur with many amphiphilic compounds that have very different molecular structures. Those changes may be due to more general effects of the amphiphilic compounds on hydrophobic-hydrophilic interfaces in the cell such as at membrane surfaces. Of particular interest is the mechanism whereby local anesthetics can cause an increase in cell endocytosis rate.

In previous studies, an increase in endocytosis rate correlates with a decrease in the force on membrane tethers. Tethers can be formed by pulling on membrane-attached beads with laser tweezers and the displacement of the beads in the laser tweezers gives a readout of the tether force. The tether force has components of both cytoskeleton-membrane adhesion and a tension in the membrane but several different observations indicate that the cytoskeleton-membrane adhesion accounts for 60-90% of the tether force. Recent findings suggest that cytoskeleton-membrane adhesion can be regulated by plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) (Raucher et al., 2000). A pleckstrin homology (PH) domain of phospholipase C (PLC)δ fused with green fluorescent protein (GFP), which bound tightly to PIP_2, and a PIP_2 5′ phosphatase, which hydrolyzed PIP_2, both decreased membrane-cytoskeleton adhesion and tether force dramatically. It is possible that the mechanism of amphiphilic compound action could be through altering the activity of one or more of the enzymes involved in PIP_2 metabolism, particularly as those enzymes are active at the surface of the plasma membrane. One criterion for the compound acting in this way is that the compounds should act at the cytoplasmic surface of the plasma membrane, as that is where the PIP_2 metabolism is controlled. Local anesthetics, as other amphiphilic molecules, interact with the membrane by inserting into the bilayer-water interface

and causing alterations of membrane properties such as curvature and resistance to hypotonic lysis (Roth and Seeman, 1971; Seeman, 1972). Most local anesthetics at physiological pH are present in both the cationic (charged) and the base (uncharged) forms. Anionic drugs preferentially intercalate mainly into the lipid in the exterior half of the bilayer, expand that layer relative to the cytoplasmic half, and thereby induce the cell to crenate, whereas permeable cationic drugs do the opposite and cause erythrocytes to form cup-shapes (Sheetz and Singer, 1974). Impermeable amphiphilic drugs intercalate only into the exterior half of the bilayer, and therefore cause crenation. Pairs of permeable and impermeable analogs of anesthetics have been used to probe the inner versus outer surfaces of plasma membranes, respectively (Sheetz and Singer, 1974). Thus, if the site of action of the anesthetics on endocytosis rate and membrane-cytoskeleton adhesion is at the plasma membrane, an impermeable cationic anesthetic may not be effective.

The antipsychotic amine, chlorpromazine, which has local anesthetic effects, increases the turnover of phosphoinositides and elevates the steady-state level of phosphatidylinositol-4-phosphate (PIP) in human platelets (Frolich et al., 1992). However, biochemical mechanisms underlying this increase are poorly understood. Another effect of local anesthetics is to elevate cytoplasmic Ca^{2+} levels (Jaimovich and Rojas, 1994). Cytoplasmic Ca^{2+} elevation has been linked to the release of inositol 1,4,5 trisphosphate (IP_3) by the action of PLC on PIP_2. It is possible that the local anesthetics activate PLC and thereby increase both lipid turnover and cytoplasmic Ca^{2+}. To address this question we designed experiments to examine the effects of permeable and impermeable anesthetics on the PIP_2 hydrolyzing enzyme, PLC. Our results indicate that PLC stimulation may be an important factor in the modulation of PIP_2 concentration and cytoskeleton-membrane adhesion energy by anesthetics. These findings indicate that PLC in the plasma membrane is particularly sensitive to the effects of amphiphilic compounds.

MATERIALS AND METHODS

Materials

Myo-[^{3}H]inositol was purchased from Amersham Pharmacia Biotech. U71233 was purchased from Calbiochem. Chlorpromazine, lidocaine and bromo-lidocaine were purchased from Sigma, and met-chlorpromazine was a gift from F. Cohen (Department of Molecular Biophysics and Physiology, Rush Medical College, Chicago, IL). All other chemicals were of high-pressure liquid chromatography or analytical grade.

Endocytosis rate measurement

Endocytosis was determined by FACS® analysis. Cells were incubated for 10 minutes with 5 mg/ml fluorescein-dextran (average molecular weight 4000 kDa), gently rinsed and fixed for 5 minutes in 2% formaldehyde. The data are expressed as the relative fluorescence index (RFI), relative to control cells, and represent the mean of triplicate determinations±s.d. (for at least 10,000 cells in each measurement).

Transfection of cells for confocal microscopy

PH domain of PLCδ was fused to the NH_2 terminus of the GFP protein and this construct was transiently expressed in NIH-3T3 cells as previously described (Raucher et al., 2000).

Cells expressing PH-PLCδ–GFP were visualized by confocal microscopy before and 10-15 minutes after incubation with local anesthetics. The extent of PH-PLCδ–GFP membrane localization was calculated from I_{pm}/I_{cyt}, where I_{pm} is fluorescence intensity of the plasma membrane and I_{cyt} is fluorescence intensity of the cytosol.

Labeling of PI lipids with [^{3}H]inositol and analysis of inositol phosphates

NIH-3T3 cells were plated at 5-10% confluency in DMEM without inositol and 10% dialyzed FBS. [^{3}H]inositol was added at a final

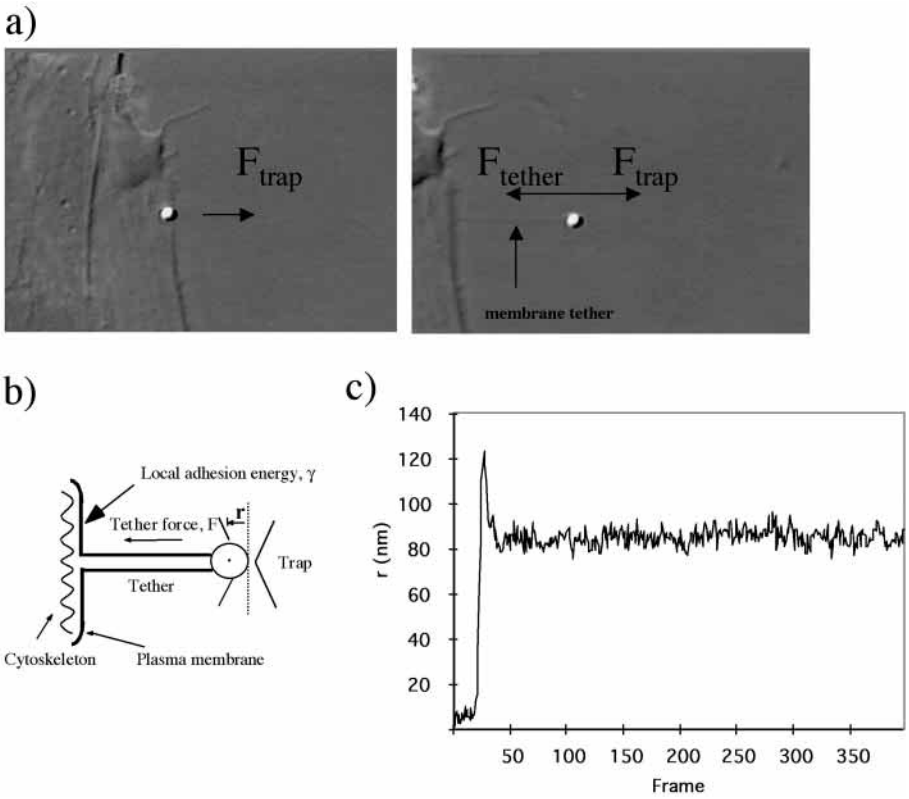

Fig. 1. Tether force measurements. (a) DIC image of a tether force measurement using optical tweezers. Polystyrene beads (1 μm diameter) coated with IgG were attached to the plasma membrane of NIH-3T3 fibroblasts using laser optical tweezers (left panel) and a membrane tether was formed by pulling out the bead (right panel). At a constant length, tether force (F_{tether}) is pulling the bead back towards the cell opposing the force of the trap (F_{trap}). (b) Schematic view of the optical tweezers force measurement that defines the local adhesion energy term. At a constant length, the force on the bead was measured by its displacement (r) from the center of the laser trap. (c) Typical displacement trace (r), showing how far the center of the bead has been moved away from the center of the optical tweezers. This tether force is a measure of the apparent membrane tension or the energy required to move membrane from the plasma membrane into the tether.

concentration of 10 μCi/ml. The cells were allowed to grow for 3 days at 37°C and 5% CO_2 before transfection. Cells were harvested 10-15 minutes after incubation with local anesthetics, and cellular lipids were extracted and deacylated (Guo et al., 1999). Briefly, cells were treated with 0.5 N HCl, harvested and their lipids isolated using a chloroform/methanol extraction. Lipids were treated with methamine as described and the resulting glycerophosphoinositols (gPIs) were stored at −80°C until use. The gPIs were separated by HPLC using conditions previously described (Guo et al., 1999) and the radioactivity of individual derivatives that co-eluted with gPI, gPI(3)P, gPI(4)P, gPI(3,4)P_2 and gPI(4,5)P_2 standards were quantified.

Quantification of PIP_2 distribution in presence of local anesthetics

Cellular expression of a PLCδ domain-GFP fusion construct and analysis of confocal images gives a measure of plasma membrane PIP_2 in living cells (Stauffer et al., 1998). The confocal images show that at low levels of expression the PLCδ-PH domain-GFP fusion protein is localized to the plasma membrane in transfected NIH-3T3 cells. The fluorescent intensities along the selected cross-sections of the cells were plotted as line intensity histograms. Dividing I_{cyt} by I_{pm} yields a ratio that can be used as an index of membrane localization. Therefore, the ratio I_{pm}/I_{cyt} was used to quantify plasma membrane binding before and after treatment with chlorpromazine.

RESULTS

Tether force measurements of the adhesion energy between the plasma membrane and the cortical cytoskeleton

To measure tether force, 1 μm diameter polystyrene beads coated with IgG were attached to plasma membranes of NIH-3T3 fibroblasts (Fig. 1a, left panel) (Dai and Sheetz, 1995; Raucher and Sheetz, 1999). In a typical experiment, the laser optical tweezers were used to pull the bead away from the cell surface, forming a thin membrane tether (Fig. 1a, right panel). The tether force was calculated from the displacement of the bead from the center of the laser trap during tether formation (schematically represented in Fig. 1b) using the same calibration protocol for the laser trap described by Dai and Sheetz (Dai and Sheetz, 1995). Fig. 1c represents a typical force record, showing the bead displacement from the center of the optical tweezers.

Permeable versus impermeable local anesthetic effects on tether force and endocytosis rate

We examined the effect of local anesthetic permeability on membrane-cytoskeleton adhesion and the endocytosis rate. To alter the outer membrane leaflet, we used membrane-impermeable forms of met-chlorpromazine or lidocaine bromide, which intercalate mainly into the lipid in the exterior half of the bilayer. To expand the cytoplasmic half we used chlorpromazine, which is membrane permeable. Fig. 2a shows the chemical structure of met-chlorpromazine and chlorpromazine. As measured by tether force there is a

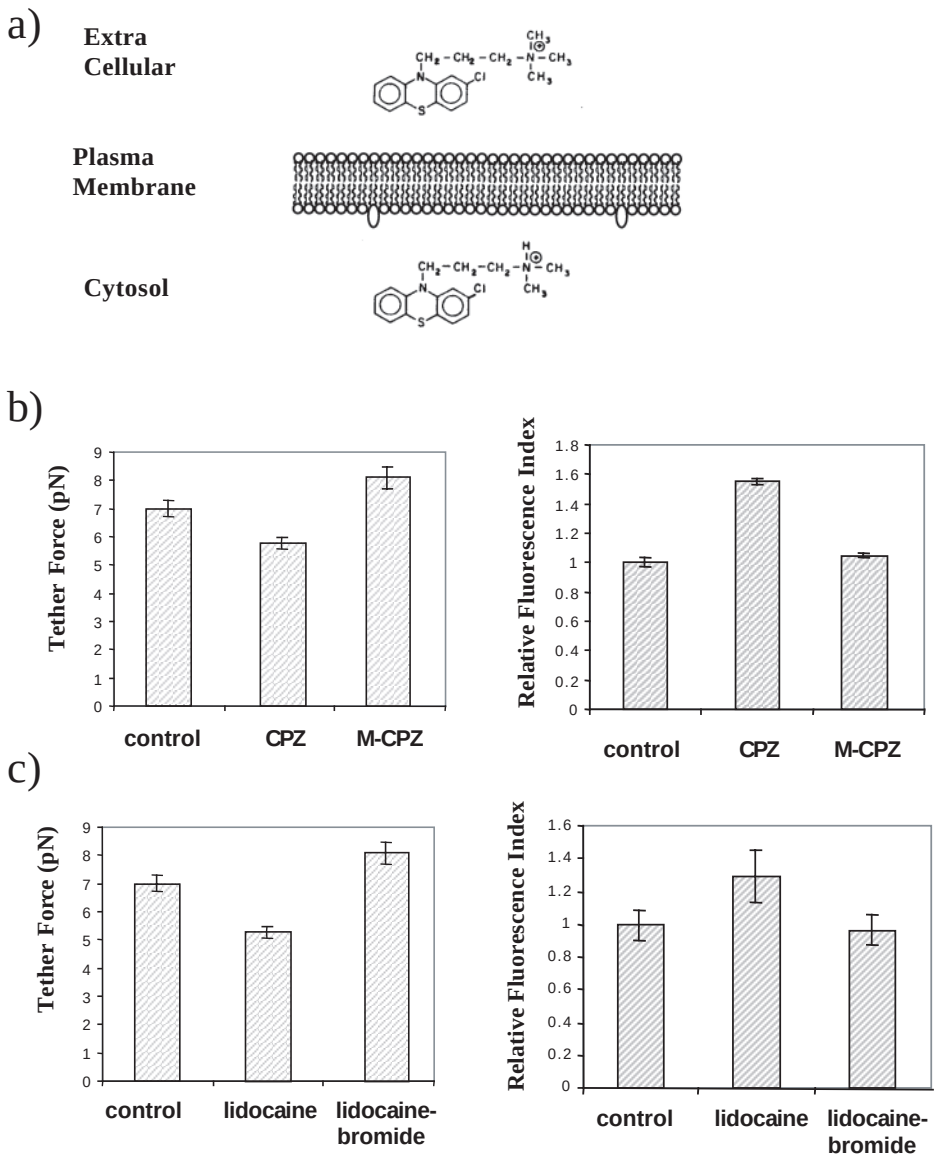

Fig. 2. Effects of different local anesthetics on membrane tension and the endocytosis rate. (a) Chemical structure of met-chlorpromazine, a membrane-impermeable local anesthetic, which intercalates mainly into the lipid in the exterior half of the bilayer and expands that layer relative to the cytoplasmic half, and chlorpromazine, its membrane-permeable analog, which expands the cytoplasmic half. (b) Tether force (left panel) and endocytosis rate, measured using fluorescence (right panel), in the presence of 30 μM chlorpromazine or met-chlorpromazine. (c) Tether force (left panel) and endocytosis rate (right panel) in the presence of 30 μM lidocaine or membrane-impermeable analog bromo-lidocaine. CPZ, chlorpromazine; M-CPZ, met-chlorpromazine.

decrease in membrane tension in cells treated with chlorpromazine or lidocaine, whereas there is a small increase in met-chlorpromazine or lidocaine bromide-treated cells (Fig. 2b,c; left panel). We believe that the effect is on membrane cytoskeleton adhesion and not on membrane tension because the cells have extensive blebbing at high concentrations of local anesthetics.

In our previous work we have shown that addition of amphiphilic compounds decreases membrane-cytoskeleton adhesion and increases endocytosis rate. To examine whether this relationship applies in the case of these anesthetics, we measured fluid phase endocytosis in NIH-3T3 fibroblasts before and after the drug treatment. As previously described (Raucher and Sheetz, 1999), endocytosis rate was determined from the total fluorescence intensity of cells after 10-15 minutes in the presence of fluorescent dextran. As shown in Fig. 2b,c (right panel), there is an increase in endocytosis in cells treated with chlorpromazine or lidocaine, but almost no change in those treated with met-chlorpromazine or lidocaine bromide.

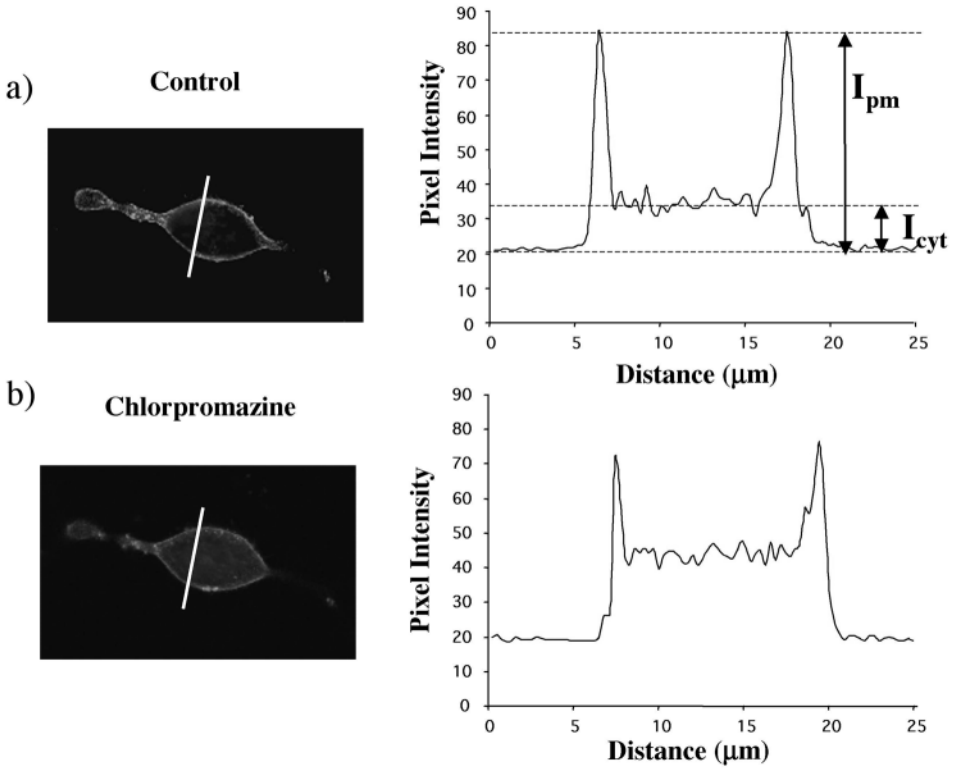

Fig. 3. PLCδ PH domain-GFP relocalization in chlorpromazine. (a) Confocal fluorescent image of membrane localization of PLCδ PH domain-GFP fusion protein in transfected NIH-3T3 cells. The fluorescent intensities along the white lines were plotted as line intensity histograms, shown in the right panels. Calculation of I_{pm}/I_{cyt} was used to quantify the extent of membrane localization. (b) Localization of PLCδ PH domain-GFP (left panel) and fluorescence intensity changes (right panel) after incubation with 30 μM chlorpromazine.

Visualization of PIP_2 distribution by PH domain in the presence of local anesthetics

One possible mechanism to decrease membrane-cytoskeleton adhesion is to decrease the membrane concentration of PIP_2 (Raucher et al., 2000). Plasma membrane PIP_2 pools can be measured by the binding of PH domains to the plasma membrane. Among the known PH domains reported to interact with PIP_2, the PH domain of PLCδ has the highest affinity (Ferguson et al., 1995). Cellular expression of a PLCδ domain-GFP fusion construct and analysis of confocal images gives a measure of plasma membrane PIP_2 in living cells (Stauffer et al., 1998). The confocal image in Fig. 3a (left panel) shows that at low levels of expression the PLCδ PH domain-GFP fusion protein is localized to the plasma membrane in transfected NIH-3T3 cells. Treatment of cells with chlorpromazine causes translocation of PLCδ PH domain-GFP fusion protein from the plasma membrane to the cytosol, as indicated by an increase in the amount of fluorescence in the cytosol (Fig. 3b). The redistribution of fluorescence was clearly demonstrated by comparing line intensity histograms calculated at selected cross sections of the cells. Dividing I_{cyt} by I_{pm} yielded a ratio that was used as an index of plasma membrane localization (Fig. 3a,b; right panel). There was about a twofold increase in the cytosol fluorescence relative to the membrane fluorescence after chlorpromazine addition (Fig. 3b).

Relationship between PIP_2 and membrane-cytoskeleton adhesion in the presence of local anesthetics

The local anesthetic could compete directly with the PH domain for PIP_2 binding but that effect should be rapid because of the rapid transport of the local anesthetics to the cytoplasm. Therefore, we measured the time course of changes in tether force and PLCδ PH domain-GFP fusion protein distribution during the treatment of cells with local anesthetics. Neither the I_{cyt}/I_{pm} ratio calculated from fluorescence histograms (Fig. 4a) or the tether force (Fig. 4b) was changed immediately. Both factors changed in parallel after 5-10 minutes. Thus, the decrease in membrane-cytoskeleton adhesion energy for membrane-permeable local anesthetics was temporally correlated with an apparent decrease in PIP_2 level at the plasma membrane. This suggests that local anesthetics that have access to the cytoplasmic side of the plasma membrane, such as chlorpromazine and lidocaine, may decrease membrane-cytoskeleton adhesion energy by decreasing PIP_2 levels in the plasma membrane.

Mechanism of membrane-cytoskeleton adhesion modulation by local anesthetics

Because the decrease in membrane-adhesion in the presence of local anesthetics was accompanied by an apparent decrease in PIP_2 in the plasma membrane, it was possible that local anesthetics modulated the PIP_2 level by directly or indirectly

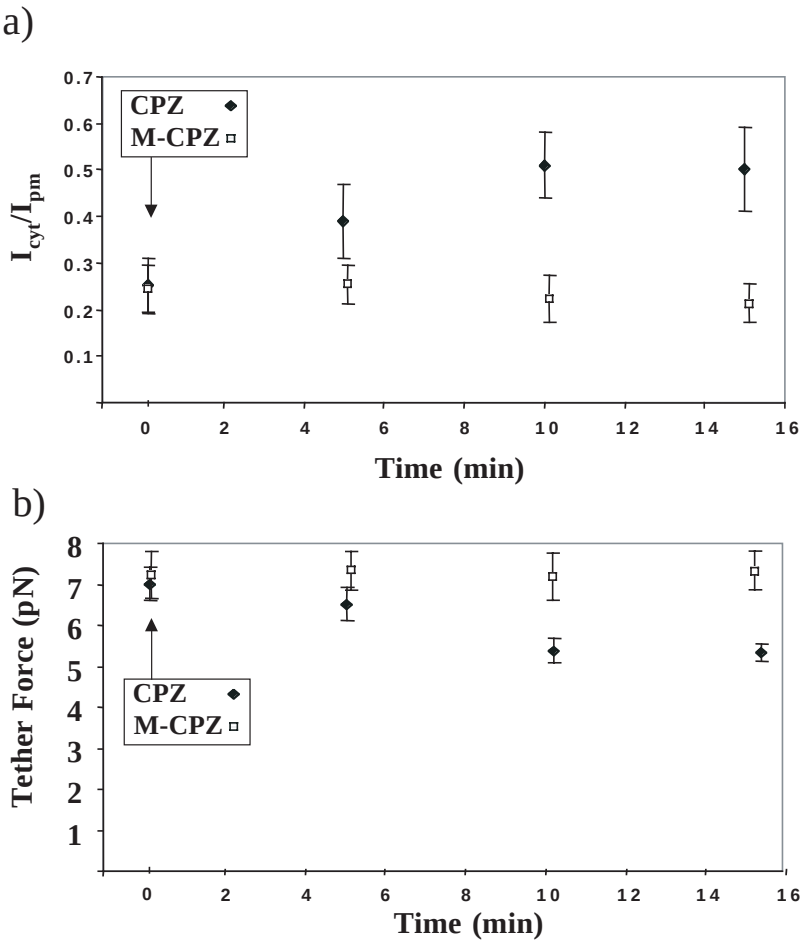

Fig. 4. Time course of PIP_2 relocalization and membrane tension in the presence of local anesthetics. (a) Fluorescence ratios calculated from line intensity plots (see legend to Fig. 3) were plotted against time. Chlorpromazine or met-chlorpromazine was added at 0 seconds, and images were taken at 5 minute intervals. (b) Tether force was measured as described in Fig. 1 and plotted against time. Chlorpromazine or met-chlorpromazine was added at 0 seconds, and measurements were made at 5 minute intervals. CPZ, chlorpromazine; M-CPZ, met-chlorpromazine.

acting on PIP_2 regulating enzymes. Therefore, to examine the mechanism of local anesthetic modulation of membrane-cytoskeleton adhesion, we explored the effect of chlorpromazine on the PIP_2 hydrolyzing enzyme PLC. The chlorpromazine-stimulated reduction of tether force was blocked by U73122 (Fig. 5), an inhibitor of PLC (Yule and Williams, 1992; Mogami et al., 1997; Jin et al., 1994), suggesting that PLC stimulation was an important factor in the modulation of PIP_2 concentration and adhesion energy by chlorpromazine.

It is well documented that the release of IP_3 by PLC increased the cytosolic concentration of Ca^{2+} (Petersen, 1992). If chlorpromazine stimulated IP_3 production, then intracellular free Ca^{2+} concentration would be increased in cells after incubation with chlorpromazine. We monitored Ca^{2+} using confocal time-lapse imaging of NIH-3T3 fibroblasts loaded with the high-affinity Ca^{2+} indicator fluo-3. We found that relative fluorescence of fluo-3 increased after incubation with chlorpromazine, indicating that there was Ca^{2+} release from internal stores that caused an increase in intracellular free Ca^{2+} concentration, initially (Fig. 6a). Furthermore, Ca^{2+} transients induced by chlorpromazine were shown to be coupled to the activity PLC, as the specific inhibitor U73122 completely blocked the response (Fig. 6b).

Chlorpromazine increased the turnover of PIP_2 and elevated PI(4)P

Because the experiments with the expressed PH-domain showed a decrease in plasma membrane PIP_2, we measured the effect of chlorpromazine on the level of total membrane-bound PIP_2. After steady-state labeling of NIH-3T3 cells with [^{3}H]inositol lipids, cells were incubated with 30 μM chlorpromazine for 15 minutes and we analyzed radiolabeled inositol lipids by HPLC (Fig. 7). A striking increase in extracted PI(4)P lipid (70% increase) was observed in cells treated with chlorpromazine, whereas the level of $PI(4,5)P_2$ was only slightly elevated (summarized in Fig. 7c). These results indicated that chlorpromazine perturbed polyphosphoinositide metabolism by increasing phosphatase activity in the plasma membrane and kinase activity in the cytosol such that the steady-state level of PIP_2 was constant and PIP was elevated.

DISCUSSION

The present experiments demonstrate that membrane-impermeable local anesthetics have little or no effect on cytoskeleton-membrane adhesion or on the endocytosis rate. However, their membrane-permeable analogs, which enter the cell and expand the cytoplasmic half of the bilayer, cause a dramatic decrease in cytoskeleton-membrane adhesion and an increase in the endocytosis rate. Although the anesthetics could act by modifying protein conformation, these studies suggest that they act at the inner plasma membrane bilayer surface, through modification of lipid metabolism and a decrease in plasma membrane-cytoskeleton adhesion (Sheetz, 2001).

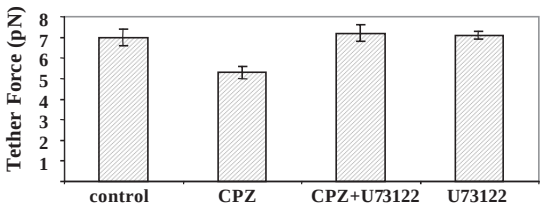

Fig. 5. Phospholipase C inhibitor, U73122, blocked the chlorpromazine (CPZ)-induced reduction in tether force. Incubation of cells with 30 μM chlorpromazine induces a decrease in tether force. Tether force was measured 10-15 minutes after chlorpromazine addition. Chlorpromazine-induced decrease in adhesion energy is likely mediated by activation of PLC, as 1 μM U73122, a PLC inhibitor, blocked the chlorpromazine-induced decrease in tether force.

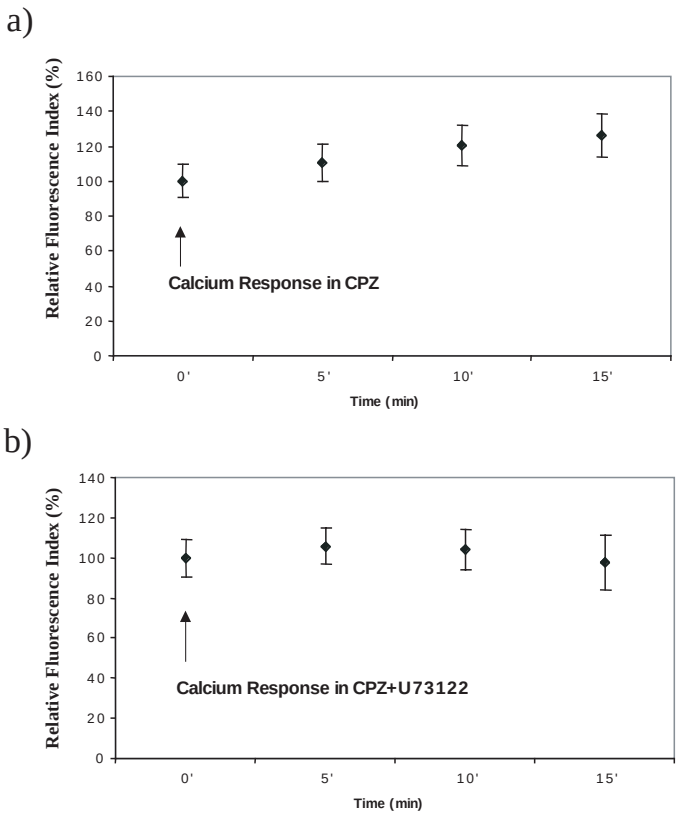

Fig. 6. Calcium response induced by (a) chlorpromazine (CPZ) alone and (b) chlorpromazine combined with the PLC inhibitor U73122. Fluorescence images were acquired using confocal time-lapse imaging of NIH-3T3 fibroblasts loaded with the $Ca(^{2+})$-sensitive dye fluo-3. Fluorescence intensity of each image was quantified as the sum of all pixel intensities.

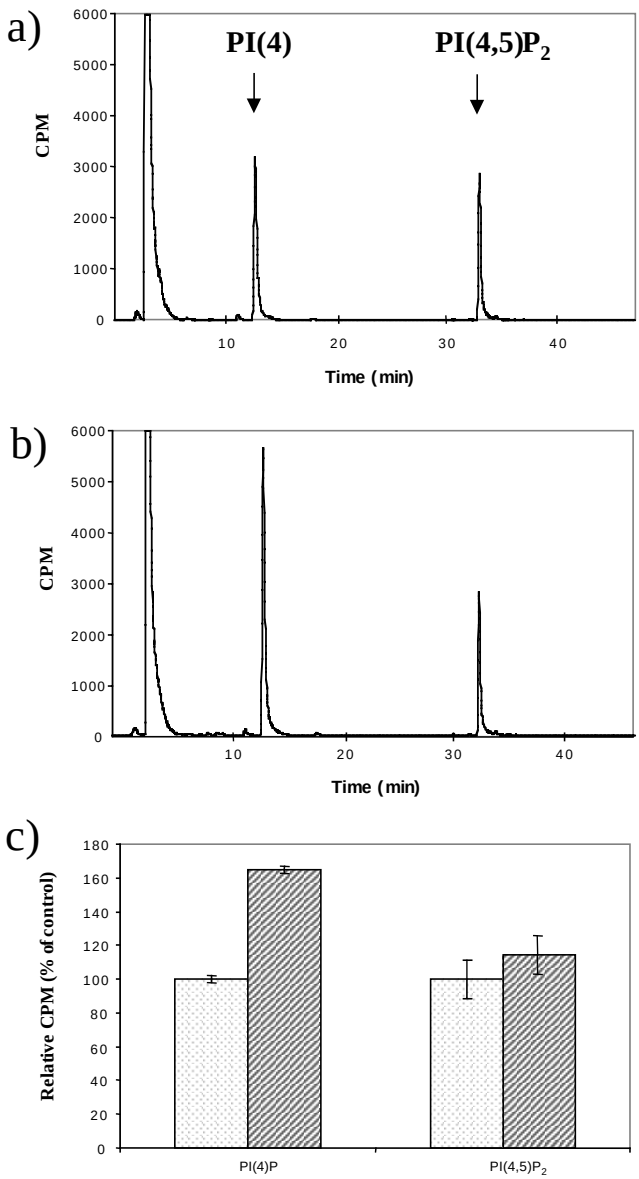

Fig. 7. The effect of 30 μM chlorpromazine on phospholipid metabolism, as measured by HPLC. Phosphatidylinositol-lipids in NIH-3T3 fibroblasts were radiolabeled with $[^3H]$inositol during a 24 hour period. Incubation and lipids were extracted from control cells and cells treated with chlorpromazine. (a) Typical HPLC radio-trace of separated phosphatidylinositols from control cells. (b) HPLC trace of phosphatidylinositol-lipids separated from cells after 30 minutes exposure to 30 μM chlorpromazine. (c) Bar chart representing summary of HPLC data. Integrated intensity of the PI_4 or PIP_2 peak after chlorpromazine treatment was calculated for each sample, normalized to control value and plotted as relative count per minute (CPM).

Previous studies have shown that local anesthetics have a dramatic effect on phosphoinositide metabolism in human platelets. Frolich et al. (Frolich et al., 1992) demonstrated that chlorpromazine increased the level of PIP and PIP_2 in platelets pre-labeled with $[^{32}P]P_i$, but the biochemical mechanism underlying this increase was poorly understood. In the present study, we have confirmed those findings in NIH-3T3 fibroblast cell line, i.e. chlorpromazine induced an almost twofold increase in PIP and a 20% increase in PIP_2 concentration. Although chlorpromazine induced an increase in the total pool of PIP_2, simultaneously it induced a reduction in the plasma membrane pool of PIP_2, as observed by relocalization of the PIP_2-specific PLCδ-PH domain. The time course of the chlorpromazine effects argues against the explanation that chlorpromazine competes with PH domains for PIP_2 binding sites in the plasma membrane or directly binds to PH domains, thereby inhibiting their binding to PIP_2. Direct binding events should occur within seconds, whereas it took 5-10 minutes for chlorpromazine's effect, which is indicative of an effect on lipid metabolism. Phospholipase C is an interesting candidate enzyme as it can directly hydrolyze PIP_2, thereby reducing the concentration in the plasma membrane, and it is activated in a number of signaling pathways. We find that the reduction in cytoskeleton-membrane adhesion, caused by chlorpromazine, is prevented by U73122, an inhibitor of PLC. Furthermore, chlorpromazine induces Ca^{2+} release from internal stores (Fig. 6), which is completely blocked by U73122. These results indicate that IP_3 is released by PLC cleavage of PIP_2 in the plasma membrane to cause Ca^{2+} release as well as a decrease in cytoskeleton-membrane adhesion energy. Previous studies (Goodman et al., 1996) have shown an increase in PLC activity by local anesthetics that modify the membrane bilayer surface. Similarly, it has also been shown that local anesthetics potentiate fMLP-stimulated PLC activity in human

promyelocytic leukemic HL-60 cells (Tan et al., 1999). We favor the hypothesis that plasma membrane PIP_2 is actually decreased by amphiphilic amine-dependent activation of PLC.

It is well documented that local anesthetics are capable of changing the activity of lipid-modifying membrane enzymes (Goodman et al., 1996; Tan et al., 1999). In addition, local anesthetics may affect the membrane-bound lipase activities by their direct interaction with the protein, through their effect on the physical state of the lipid micro-environment of the lipase or by a combination of those effects. For example, the rate of PIP_2 hydrolysis by PLC depends exponentially on the lipid monolayer surface pressure (Rebecchi et al., 1992). Thus, the physical chemical changes at the membrane surface that are expected from chlorpromazine binding could cause the observed changes in lipid metabolism.

The changes in lipid metabolism may be responsible for the observed changes in tether force. Several recent studies have shown that the tether force provides a rapid and reliable measure of membrane-cytoskeleton adhesion (Togo et al., 2000; Dai and Sheetz, 1999; Raucher et al., 2000), and a decrease in PIP_2 level in the plasma membrane is accompanied by a decrease in tether force and a marked decrease in cytoskeleton-membrane adhesion (Raucher et al., 2000). The overall interaction between the membrane and the cytoskeleton appears to be complex, given that many cytoskeletal proteins that bind to integral membrane proteins as well as to membrane phospholipids have been identified. Decreases in cytoskeleton-membrane adhesion correlate with decreases in membrane binding of GFP-PH domains, which bind PIP_2 with high affinity and specificity.

Actin filament assembly is dependent on the level of PIP_2 and some of the effects of anesthetics may be the result of decreased actin filament density. For example, Rabinovitch and DeStefano (Rabinovitch and DeStefano, 1975) have shown that lidocaine and chlorpromazine affect the cytoskeleton; they also induce cell rounding and inhibit motility in cultivated macrophages. Similar effects of local anesthetics are observed on BALB/3T3 cells (Nicolson et al., 1976). High concentrations of the anesthetics cause cell contraction, rounding and bleb formation, which is similar to overexpressed MARCKS (myristolylated alanine-rich C kinase substrate) mutant protein (which sequesters acidic phospholipids including PIP_2) (Myat et al., 1997). Furthermore, the selective reduction of PIP_2 level in plasma membrane causes NIH-3T3 fibroblasts to become round, lose their substrate attachments and form membrane blebs (Raucher et al., 2000). The tether force in blebs is very small, consistent with the absence of a membrane-cytoskeleton adhesion term (Keller and Eggli, 1998; Dai and Sheetz, 1999) and the appearance of the blebs after longer times or with higher concentrations of chlorpromazine indicates loss of cytoskeleton-membrane adhesion. Thus, the mechanism of local anesthetic action in disrupting cytoskeletal systems may be PIP_2 mediated by causing stimulation of phosphatidyl inositol turnover and a decrease in plasma membrane PIP_2.

An additional factor that may alter the endocytosis rate is the effect of bilayer couple asymmetry (Sheetz and Singer, 1974). In recent studies Farge et al. (Farge et al., 1999) tested the possibility that the phospholipid bilayer itself could generate the budding force for endocytosis in living cells. When asymmetry was generated by specific translocation to the inner layer by an endogenous flippase, bulk flow internalization was increased as the inner layer area was relatively increased. Similarly, Zha et al. (Zha et al., 1998) have found that hydrolysis of sphingomyelin in the outer half of the plasma membrane by sphingomyelinase treatment causes inward curvature of the plasma membrane and induces ATP-independent endocytosis. This is in qualitative agreement with the model, suggesting that an asymmetric expansion of the plasma membrane cytoplasmic surface by chlorpromazine or lidocaine may contribute a driving force for endocytosis; however, the drop in membrane-cytoskeleton adhesion is not explained. Thus, we suggest that the major factor causing the increase in endocytosis rate is the drop in membrane-cytoskeleton adhesion.

Several important cellular activities, including endocytosis, membrane extension and membrane resealing rates are controlled by membrane-cytoskeleton adhesion (Sheetz, 2001). Because of its importance, cells normally control the level of membrane-cytoskeleton adhesion quite tightly (the standard variance in the measurements of tether force is small) and the level varies in characteristic ways during mitosis, during stimulated secretion and in response to hormones or other treatments (Raucher and Sheetz, 1999; Dai et al., 1997; Raucher et al., 2000; Togo et al., 2000). Levels of phosphorylated inositol lipids play a major role in cytoskeleton assembly and dynamics as well as the adhesion between the membrane and the cytoskeleton. Many of the changes must occur at the cytoplasmic surface of the plasma membrane and it is expected that agents that alter that surface can have profound effects on cell functions through changes in the activities of lipid-modifying enzymes.

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