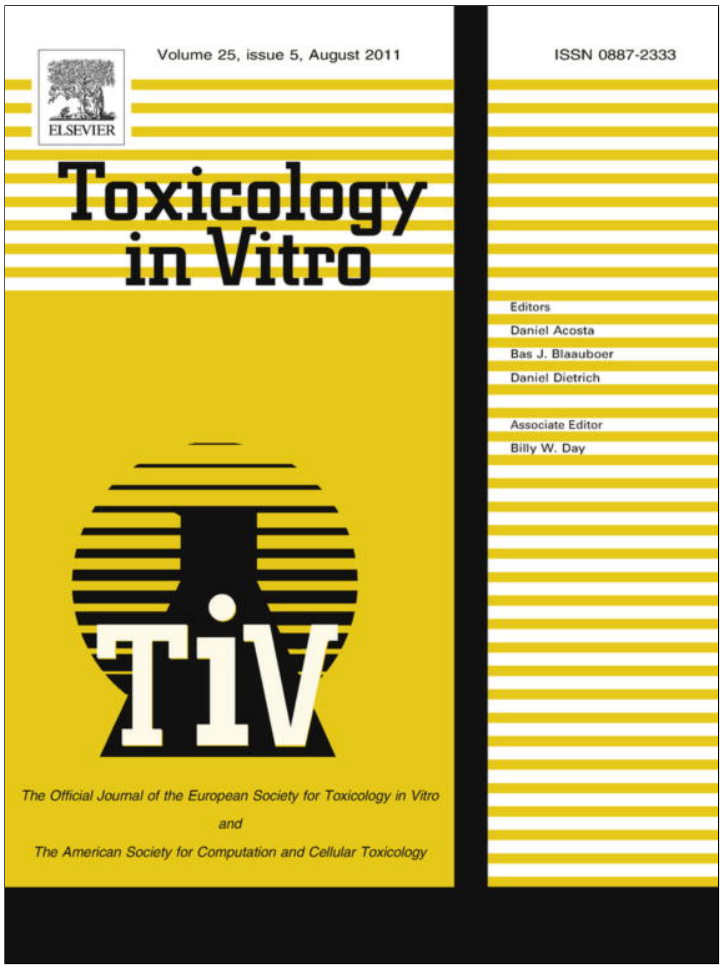

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# Plasma membrane damage sensing and repairing. Role of heterotrimeric G-proteins and the cytoskeleton

Rosa Calvello [a,*], Vincenzo Mitolo [b], Angela Acquafredda [b], Antonia Cianciulli [b], Maria Antonietta Panaro [b]

[a] Department of Pharmaco-Biology, University of Bari, Italy
[b] Department of Human Anatomy and Histology, University of Bari, Italy

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ABSTRACT

Different toxic agents, derived from bacteria, viruses or cells of the immune system, as well as mechanical forces generated during cell locomotion are able to open pores in the cell plasma membrane. Most of these biological agents operate through specific receptors. We studied the formation and resealing of the "non-specific" plasma membrane pores generated by the mild non-ionic detergent Triton X-100. In HL-60-derived granulocytic cells plasma membrane pore opening after a 1-h treatment with Triton X-100 is documented by entry into the cell of the membrane impermeant dye ethidium bromide. As a consequence of the opening of pores the intracellular $K^+$ concentration falls dramatically, the cytosolic pH diminishes and the cell membrane is depolarized. Furthermore the cells acquire a polarized morphology, demonstrating the involvement of the actin cytoskeleton. At the Triton concentration used the membrane lesions are progressively repaired and by 8 h the impermeability to ethidium bromide is restored and the intracellular $K^+$ concentration is virtually normal. Following treatments with Triton + Pertussis toxin, Triton + Cytochalasin, or Triton + Pertussis toxin + Cytochalasin the progress of membrane repair is dramatically slowed and is no longer completed by 8 h. It is concluded that the membrane damage activates pertussis-sensitive G-proteins which likely act as sensors of the damage, while both G-proteins and the actin cytoskeleton are involved in the membrane repair mechanism.



## 1. Introduction

The selective permeability of the intact cell membrane and the specific transport systems embedded in it guarantee the relative stability of different cytosolic parameters against a different composition of the milieu, ultimately ensuring cell viability. Agents which damage the cell membrane may eventually lead to cell lysis; however, due to the highly dynamic structure of the cell membrane systems, relatively minor lesions may be fully repaired by nucleated cells. In principle, the synthesis of new membrane patches or sliding on the horizontal plane of already existing membrane sections or else the recruitment of intracellular membranes may restore the membrane continuity; alternatively, injured membrane sections may be endocytosed and excluded from the cell surface (Idone et al., 2008a).

Several toxic agents, derived from bacteria, viruses or cells of the immune system, are able to open pores in the cell membrane. Depending on the nature of the toxin and its concentration as well as the cell type, the injured cell may eventually recover or else undergo lysis. In keratinocyte, lymphocyte and fibroblast cell membranes the alpha-toxin of Staphylococcus aureus may generate "channels" which permit selective transmembrane fluxes of monovalent ions. Following treatment with moderate concentrations of the toxin the cells may recover, whereas the larger pores created by *Escherichia coli* hemolysin or streptolysin *O* could not be repaired (Walev et al., 1994). However, a rapid-kinetics resealing of streptolysin *O*-opened transmembrane pores has been reported in more recent investigations (Idone et al., 2008a). Hemolytic paramyxoviruses create hydrophilic pores of approximately 1 nm in diameter, allowing ions and low molecular weight compounds, but not proteins, to leak into and out of cells. High concentrations of extracellular $Ca^{2+}$ inhibits leakage and aids recovery (Babiychuk et al., 2009). Similar results were obtained with the bee venom protein melittin, the alpha-toxin of Staphylococcus aureus and the activated complement (Pasternak et al., 1985).

The opening of pores in the cell membrane has been reported to activate pertussis toxin (PTX)-sensitive heterotrimeric guanine-nucleotide binding proteins (G-proteins). Typically, these are indirectly activated by different agents through the ligation of specific seven-transmembrane receptors, e.g., the ligation of the human granulocyte formyl peptide receptor 1 (FPR1) by the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Panaro and Mitolo, 1999; Panaro et al., 2006).

The first aim of the present investigation was to evaluate the non receptor-mediated membrane damage of granulocytes brought about by treatment with detergents which are devoid of

* Corresponding author. Address: Department of Pharmaco-Biology, University of Bari, Via Orabona 4, Bari I-70125, Italy.
*E-mail address:* r.calvello@anatomia.uniba.it (R. Calvello).

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any specific biological activity but are expected to solubilize some membrane components and open artificial (non-specific) pores through which some ions could leak out of the cell or enter it in an unspecific manner (Senkovich and Chernitsky, 1998; Walev et al., 1994).

A second aim of this investigation was to monitor the processes of possible membrane recovery after withdrawal of the detergent. Finally, we focused on the role of the PTX-sensitive heterotrimeric G-proteins and the actin cytoskeleton in sensing and repairing the membrane damage, by studying alterations of the processes of membrane repair following Triton damage in cells which had been additionally treated with appropriate tools to block the PTX-sensitive heterotrimeric G-proteins or interfere with the organization of the actin cytoskeleton.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Chemicals

Cell culture media [RPMI 1640; Hanks balanced salt solution with $Ca^{2+}$ and $Mg^{2+}$ (HBSSw) or without $Ca^{2+}$ and $Mg^{2+}$ (HBSSw/o)] were obtained from Invitrogen (Milan, Italy). The fluorescent probes EtBr (ethidium bromide), EthD-1 (ethidium bromide homodimer-1), PBFI-AM ($K^+$-binding benzofuran isophthalate, acetoxymethyl ester), DiSC3(5) (3,3′-dipropylthiadicarbocyanin iodide), and Fluo-3-AM were all purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS), EGTA [ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], Triton X-100 (octyl-phenoxyl-polyethoxyethanol), DMSO (Me2SO; dimethyl sulfoxide), MOPS (Morpholinopropane sulfonic acid), pluronic F-127, HEPES buffer, Ficoll–Hypaque, Trypan blue, PTX, Cytochalasin B, Glutaraldehyde, Valinomycin, Nigericin were all purchased from Sigma–Aldrich (Milan, Italy). The 96-well culture plates (Black/White Isoplate) were from PerkinElmer (Milan, Italy); all other tissue culture plasticware was from Falcon (Milan, Italy).

### 2.2. Methods

#### 2.2.1. Culture of HL-60 cells

HL-60 promyelocytes (obtained from Interlab Cell Line Collection, ICLC, Genoa, Italy) were cultured at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS (referred to as complete RPMI medium), in a humidified atmosphere of 5% CO2 and 95% air. The HL-60 cells were induced to differentiate into granulocytic cells by treatment with DMSO (1.3% for 3 days and 0.65% for a further 2 days).

Cell viability (>90%) was tested by Trypan blue exclusion and experiments were carried out immediately.

#### 2.2.2. Cell treatment

A first series of experiments was devised to create small lesions in the cell membrane and then follow the spontaneous recovery. HL-60 granulocytes ($2 \times 10^6$ cells/ml) were resuspended in HBSSw containing 20 mM HEPES (pH 7.4) and Triton X-100 at the final concentration of 0.001% v/v ($1.25 \times 10–5$ M) was added to the medium. Control cells were kept in the same medium without Triton. After incubation for 60 min at 37 °C, cells were washed twice in fresh HBSSw medium. A first aliquot of cells was immediately tested with probes to measure the ethidium bromide (monomer and dimer) incorporation and the $K^+$ cytosolic concentration (probes used: EtBr, EthD-1, PBFI-AM, respectively; see below) which had been added to the medium. After incubation with each probe, the cells were washed twice and resuspended in fresh HBSSw medium for fluoresce readings. Further aliquots of Triton-treated cells were kept in complete RPMI medium and were similarly tested after 2, 4, 6 or 8 h. In other experiments, the immediate effects (during the first 10 min) of the Triton treatment on the cell membrane potential and the cytosolic free $Ca^{2+}$ concentration were monitored. For potentiometric tests HL-60 granulocytes ($2 \times 10^6$ in 1 ml) were suspended in HBSSw containing 20 mM HEPES; the probe used was the DiSC3(5) (see below). For cytosolic free $Ca^{2+}$ concentration tests cells ($2 \times 10^6$ in 1 ml) were suspended in the $Ca^{2+}$- and $Mg^{2+}$-free medium HBSSw/o; the probe used was the Fluo-3 (see below).

Additional experiments were set up to evaluate the roles of the PTX-sensitive heterotrimeric G-proteins and the actin cytoskeleton in the membrane damage sensing and/or repair. PTX is an agent that selectively blocks the activity of several heterotrimeric G-proteins belonging to the Gi/o family and the cell permeant Cytochalasin B inhibits actin assembly. Tests for EtBr incorporation and the $K^+$ cytoplasmic concentration were made as indicated below, except that PTX (100 ng/ml) or Cytochalasin B (8 μM) or both were added to the medium of both control and Triton-treated cells.

Fluorescence readings were performed with a Victor2 Multilabel counter fluorometer equipped with a thermostated plate holder and an injector (PerkinElmer, Milan, Italy). The filters used (PerkinElmer) exhibited a range of fluorescence readings which covered the maximal emission and excitation wavelengths of the probes used in the present experiments.

#### 2.2.3. Measurements of ethidium bromide and ethidium homodimer-1 cell entry

EtBr is a fluorescent dye (max excitation = 518 nm; max emission = 605 nm) impermeant to cells with intact membranes. Thus, entry of the dye into cells indicates the presence of lesions in the cell membrane (Krause et al., 1998). One hundred micromolars EtBr (dissolved in water) were added to the aliquots of Triton-treated and control cells. After 15 min incubation at 37 °C, in the dark, the cells were washed twice in fresh HBSSw and the fluorescence intensity at 605 nm (upon excitation at 520 nm) was measured. The same treatment (EtBr for 15 min, followed by washing) was applied to aliquots of experimental and control cells stored in complete medium for 2, 4, 6, or 8 h after 1 h Triton treatment. Results are here expressed as: (Fluorescence intensity in the experimental specimen/Fluorescence intensity in the control specimen) × 100.

In addition, an aliquot of cells was tested for incorporation of the EtD-1 probe. EthD-1 is a fluorescent EtBr homodimer (max excitation = 528 nm; max emission = 617 nm). EthD-1 molecules are larger than the EtBr molecules and their entry into cells indicates the presence of comparatively much larger lesions in the cell membrane (Krause et al., 1998). Six nanomolars EthD-1 (dissolved in DMSO/water 1:4) were added to the aliquots of Triton-treated and control cells. After 15 min incubation at 37 °C, in the dark, the cells were washed twice in fresh HBSSw and the fluorescence intensity at 595 nm (upon excitation at 530 nm) was measured. The same treatment (EtD-1 addition and washing) was applied to aliquots of experimental and control cells stored for 2, 4, 6, or 8 h.

#### 2.2.4. Cytosolic $K^+$ concentration ($[K^+]c$)

PBFI-AM is a very weakly fluorescent, cell-permeant form of PBFI. Once within the cell, non-specific cytosolic esterases hydrolyze the acetoxymethyl ester (AM), yielding the non-permeant, intensely fluorescent, PBFI, a probe for cytosolic $K^+$. The excitation maximum is at 340 nm when the probe is bound to K ions and at 380 nm for the unbound probe; maximum emission is at 510 nm. Thus, the ratio Emission at 510 nm after excitation at 340 nm/Emission at 510 nm after excitation at 380 nm is a measure of cytosolic $K^+$ concentration (Kasner and Ganz, 1992). PBFI-AM (5 μM in DMSO containing 0.02% pluronic F-127) was added to the aliquots

of Triton-treated and control cells. After a 60-min incubation period at 37 °C, in the dark, the cells were washed twice in fresh HBSSw and the fluorescence intensity at 510 nm (following excitation at 340 and 380 nm) was measured. The same treatment (PBFI-AM addition and washing) was applied to aliquots of experimental and control cells stored for 2, 4, 6, or 8 h. The F340/F380 ratio was calibrated for $[K^+]c$ in situ at the end of the experiments. In situ calibration was performed using valinomycin and nigericin in extracellular solutions of variable $[K^+]$ as described by Kasner and Ganz, Results are here expressed as the ratio indicated above, the value for untreated cells being normalized to 100.

*2.2.5. Membrane potential measurements*

DiSC3(5) is a permeant fluorescent cationic dye. Membrane depolarization decreases the level of the intracellular nonfluorescent aggregates and induces the release of the positively charged dye into the extracellular space, increasing the fluorescence emission. Thus, fluorescence emission intensity at 670 nm (following excitation at 651 nm) parallels the membrane depolarization. (Krause et al., 1998; Patrat et al., 2002). In our experiments 200 nM DiSC3(5) were added to the HBSSw medium and, after 15 min equilibration (37 °C in the dark), the cells were washed twice in fresh medium and the experiment was started by the injection of 0.001% Triton X-100. Control cells were similarly treated, except for the addition of Triton. Fluorescence intensity was read at 1–8 mins at 670 nm (upon excitation at 650 nm). Maximal fluorescence was determined at the end of the experiment by adding nigericin (5 μM) and valinomycin (2 μM). Potential readings are here expressed as Actual fluorescence intensity/Maximal fluorescence intensity.

*2.2.6. Intracellular calcium concentration ($[Ca^{2+}]i$)*

Fluo-3-AM is a non-fluorescent cell permeant derivative of fluo-3. Within the cell, non-specific cytosolic esterases hydrolyze the acetoxymethyl ester (AM), yielding non-permeant Fluo-3, which is also non-fluorescent unless bound to $Ca^{2+}$ (max excitation at 488 nm; max emission at 526 nm) (Boston et al., 2004; He et al., 2009). In our experiments 4 μM Fluo-3-AM were added to the Ca/Mg-free cellular medium HBSSw/o and after 30 min equilibration (at 37 °C in the dark), the cells were washed twice in HBSSw/o to remove nonincorporated extracellular fluo3-AM. Then the experiment was started by injecting 0.001% Triton X-100. Control cells were similarly treated, except for the addition of Triton. Fluorescence was read at 1–8 mins. Immediately after the readings at 8 mins, 1 mM calcium chloride was added to the medium in order to determine the influence of the external calcium on the cytosolic free $Ca^{2+}$ concentration under the above-mentioned experimental conditions. At the end of each experiment, the fluo-3 fluorescence signal was calibrated for each sample by addition 1 μM ionomycin to acquire maximal fluorescence (Fmax), followed by 20 mM EGTA, 75 mM Tris, pH 9, to obtain minimal fluorescence (Fmin).Intracellular calcium was calculated using the following equation: $[Ca^{2+}]i = Kd\ (F-Fmin)/(Fmax-F)$, where Fmin is the fluorescence intensity of the indicator in the absence of calcium, Fmax is the fluorescence of the calcium-saturated indicator, $F$ is the fluorescence at intermediate calcium levels, and Kd was 400 nM for the dissociation constant of the intracellular dye. Fluorescence intensity was read at 535 nm (upon excitation at 485 nm) and values of cytosolic free $Ca^{2+}$ ions are here expressed in nM.

*2.2.7. Microscopical analysis of HL-60 granulocytes*

The general morphology of HL-60 human granulocytes treated with Triton alone or in presence of PTX and Cytochalasin. $2 \times 10^6$ granulocytes treated as above indicated were incubated in HBSSw or HBSSw/o with addition 10 mM MOPS for 60 min at 37 °C. At the end of the assay, glutaraldehyde was added to the cell suspension at the final concentration of 2.5%. After 10-min fixation, the cells were washed three times in fresh medium, stained with Türk method and the cell morphology was examined at phase contrast microscopy using a 40× objective.

*2.3. Statistical analysis*

Data are presented as the mean ± SD. Statistical determinations of the difference between means of experimental groups were performed using one-way ANOVA (software MINITAB release 15.1). $P$ values < 0.05 were considered statistically significant.

## 3. Results

*3.1. EtBr and EthD-1 incorporation into cells following treatment with Triton*

In this set of experiments, 1 h Triton treated cells, after washing, were stored for different times (2, 4, 6, and 8 h) in complete medium (Fig. 1A) These cells were stained with EtBr for 15 min and after soon washed in order to evaluate the resealing of membrane pores induced by Triton treatment. This was carried out measuring the amount of dye incorporation which indirectly refers about membrane damage. In control cells some EtBr fluorescence could be detected, possibly due to dye trapped in cells damaged during the experimental procedures or some residual dye in the medium even after cell washing. If this base fluorescence is made equal to 100, after 1-h Triton treatment the measured fluorescence is 120. This significantly augmented ($P < 0.05$) EtBr entry is attributable to the opening of small artificial pores in the cell membrane by Triton treatment. Triton-treated cell sampling after 2, 4, 6, and 8 h recovery in complete RPMI demonstrates a progressive decrease of EtBr incorporation, which is attributable to resealing of a quota of Triton-opened pores. By 8 h the measured fluorescence is only 102.5, not statistically different from control values. Thus, after an 8-h recovery period the great majority of the Triton pores are likely to have resealed, with practically complete cell recovery.

Alternatively, EthD-1 molecules, which are significantly larger than the EtBr molecules, did not penetrate the cell membrane to any significant extent, even after the Triton treatment (data not shown). This indicates that the Triton-opened pores, at the concentrations used in the present experiments, are relatively small. However, the present experiments do not allow the actual dimensions of such pores to be determined.

*3.2. Intracellular $K^+$ concentration following treatment with Triton*

Triton induced change in intracellular $K^+$ was evaluated by monitoring the changes of PBFI fluorescence after loading the cells with this dye as described in Materials and Methods.

After 1-h treatment with Triton, a very significant ($P < 0.01$) drop of intracellular $K^+$ concentration was observed (Fig. 1B), attributable to $K^+$ loss through the Triton-opened pores since in control cells any changes of intracellular $K^+$ have been observed. This efflux of $K^+$ could, at least in part, cause a depolarization of the cell membrane (see below). Recovery, presumably in parallel with closure of Triton-opened pores, is progressive and by 8 h the intracellular $K^+$ concentration of treated cells does not differ significantly from that of untreated control cells.

*3.3. Cell membrane potential modifications following treatment with Triton*

To investigate the ability of Triton to cause depolarization consequent to opening artificial pores on cell membrane, we have used

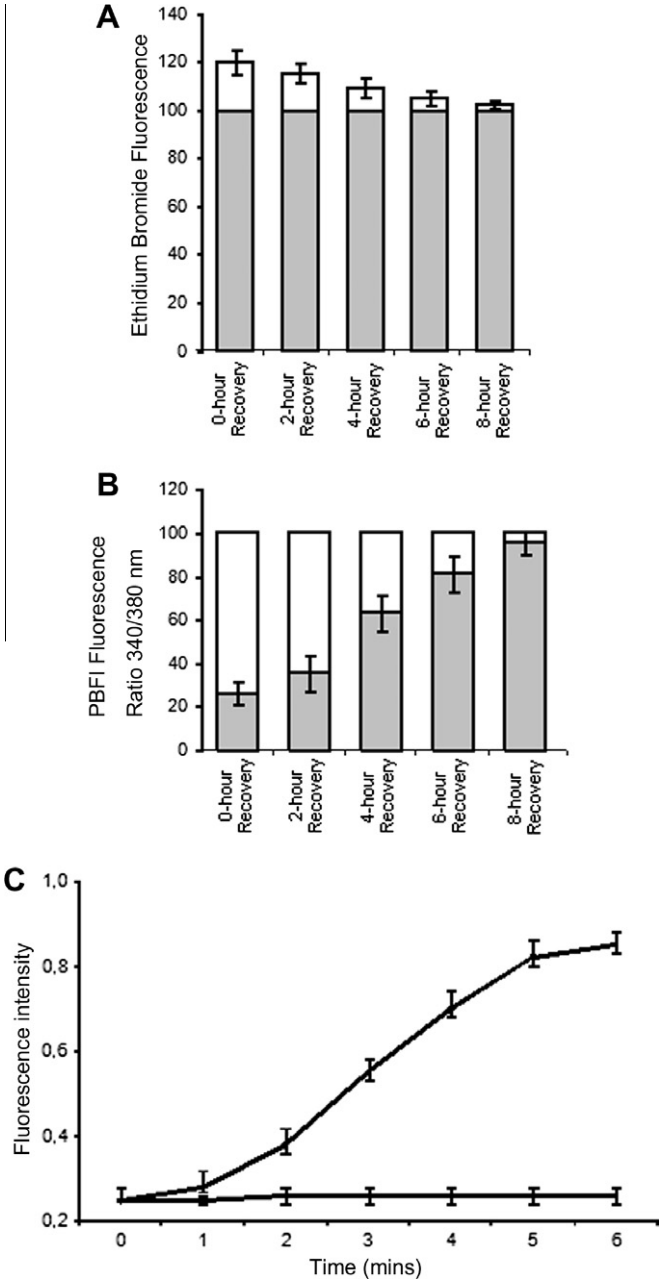


**Fig. 1.** Permeabilization the plasma membrane of HL-60 cells. (A) Membrane permeability of cells to EtBr at different time intervals after 1 h treatment with Triton. Fluorescent intensity of untreated control cells = 100. (B) Cytosolic concentration of $K^+$ in treated HL-60 cells for 1 h with Triton and recovery intracellular potassium after 2, 4, 6, and 8 h detergent withdrawal. Probe: PBFI-AM. Values of experimental cells (gray section of the columns) are expressed as the ratios: emission at 510 nm after excitation at 340 nm/Emission at 510 nm after excitation at 380 nm, the value for untreated cells being normalized to 100. (C) Changes induced by triton (upper tracing) of the fluorescence of the membrane potential sensitive probe DiSC3(5) after uptake by HL-60 cells; Lower tracing: fluorescence intensity of untreated control cells. Ratios: Actual fluorescence intensity/Maximal fluorescence intensity. Maximal fluorescence (=1.0), corresponding to the maximal depolarization, was measured after the addition of nigericin and valinomycin. Values represent the means ± SDs of six independent experiments.

the fluorescent probe DISC3(5) which has been extensively employed for this purpose (Patrat et al., 2002). Cell membrane depolarization was monitored during the first 6 min following Triton contact (Fig. 1C, upper tracing). Depolarization starts immediately after the addition of Triton to the culture medium and by 6 min has reached about 85% of the maximal depolarization achievable with the addition of nigericin and valinomycin. In untreated cells, no significant depolarization was observed, as reported in Fig. 1C, lower tracing.

### 3.4. Cytosolic free $Ca^{2+}$ modifications following treatment with Triton

Cytosolic free $Ca^{2+}$ modifications in a Calcium-free medium were monitored during the first 8 min following Triton contact. Cytosolic free $Ca^{2+}$ rises steeply within the first min and then at a lower pace during the following 7 min. After 8 min Calcium was added to the medium and the cytosolic free $Ca^{2+}$ concentration rose very steeply within 1 min (Fig. 2, upper tracing). The rise of cytosolic free $Ca^{2+}$ concentration has the characters of a "transient", since after 1 h of Triton exposure the $Ca^{2+}$ concentration is not significantly different in treated cells, as compared to controls (data not shown).

Cytosolic free $Ca^{2+}$ modifications following treatment with Triton + PTX (Fig. 2, lower tracing). These experiments were devised to determine whether the "Calcium transient" observed following treatment with Triton depended upon the activation of the PTX-sensitive heterotrimeric G-proteins. The experimental setting was the same as above described, except that PTX (100 ng/ml) was added to the medium of experimental (tritonated) and control cells. The rise of cytosolic free $Ca^{2+}$ concentration due to Triton treatment proved to be completely abrogated by the addition of PTX.

### 3.5. EtBr incorporation into cells following treatment with Triton, Triton + PTX, Triton + Cytochalasin, or Triton + PTX + Cytochalasin)

In Fig. 3 all controls were normalized at 100 which represents the average EtBr fluorescence of HL-60 cells not treated with Triton in medium alone (dark columns), or in the presence of PTX, or Cytochalasin, or both (controls). Columns represent the EtBr incorporation following the treatments with Triton alone (white dotted

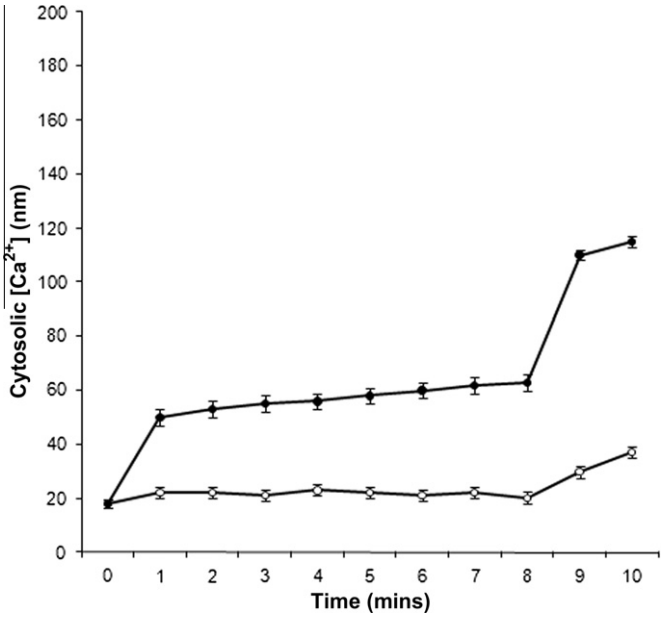


**Fig. 2.** Cytosolic free $Ca^{2+}$ of HL-60 cells after different (as described in the text). Upper tracing (closed circle): treatment with Triton; Lower tracing (open circles): treatment with Triton + PTX. Probe: Fluo-3. Mins 0–8: in the absence of external Calcium; Mins 9–10: after the addition of external Calcium. Fluorescence expressed in arbitrary units. Values represent the means ± SDs of six independent experiments.

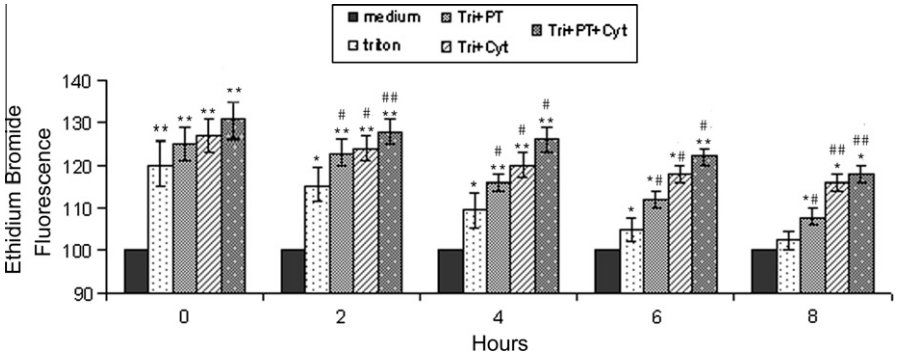


**Fig. 3.** Membrane permeability of HL-60 cells to EtBr at different time intervals (0, 2, 4, 6, and 8 h): medium alone (dark columns); after treatment with Triton (white dotted columns); Triton + PTX (grey columns); Triton + Cytochalasin (white lined columns); or Triton + PTX + Cytochalasin (grey dotted columns). Fluorescent intensity of control cells = 100. Values represent the means ± SDs of six independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0001$ significantly different vs. triton; $^{*}p < 0.05$ and $^{**}p < 0001$ significantly different vs. medium (control).

columns) or with Triton in presence of PTX (grey columns), or Cytochalasin (white lined columns), or both PTX and Cytochalasin (grey dotted columns) at time 0 (i.e., immediately after the end of the treatment) and after 2, 4, 6, and 8 h. Cell recovery is significantly slowed down in the presence of PTX, and even more so in the presence of Cytochalasin or PTX + Cytochalasin, and after 8 h the cells have not completely recovered, whereas after treatment with Triton alone complete recovery is virtually achieved. Experiments included negative controls represented by cells treated with PTX, Cytochalasin or both in absence of Triton. In this case EtBr incorporation was comparable to that observed in untreated cells (medium alone) (data not represented).

### 3.6. Intracellular $K^+$ concentration following treatment with Triton, Triton + PTX, Triton + Cytochalasin, or Triton + PTX + Cytochalasin

In Fig. 4 the horizontal reference line at 100 represents the average intracellular $K^+$ concentration of HL-60 cells not treated with Triton, but in the presence of PTX, or Cytochalasin, or both (controls). Tracings 1–4 represent the average intracellular $K^+$ concentration following the treatments with Triton only or with Triton together with PTX, Cytochalasin, or both PTX and Cytochalasin, at time 0 immediately after the end of the treatment, and after 2, 4, 6, and 8 h. As in the preceding experiment, cell recovery is significantly slowed down in the presence of PTX, and even more so in the presence of Cytochalasin or PTX + Cytochalasin, and after 8 h the cells have not completely recovered, whereas after treatment with Triton alone complete recovery is virtually achieved.

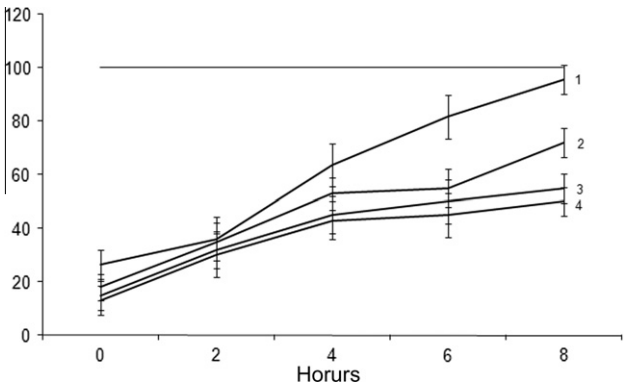


**Fig. 4.** Cytosolic concentration of $K^+$ in HL-60 cells at different time intervals after treatment with Triton (tracing 1), Triton + PTX (tracing 2), Triton + Cytochalasin (tracing 3), or Triton + PTX + Cytochalasin (tracing 4). Probe: PBFI-AM. Experimental data are ratios: Emission at 510 nm after excitation at 340 nm / Emission at 510 nm after excitation at 380 nm. Values are normalized to control cells = 100. Values represent the means ± SDs of six independent experiments.

### 3.7. Morphological changes

Granulocytes submitted to Triton treatment acquire an irregular morphology: they extend dynamic lamellipods and the contour of the cell becomes irregular, furthermore the cells become markedly elongated (anisodiametric), while untreated cells (control) appear mostly round in shape. These morphological changes are largely abrogated by PTX (100 ng/ml) and Cytochalasin B (8 μM) treatments, suggesting that these responses depend on the activation of PTX-sensitive G-proteins and are affected by the cell actin cytoskeleton (Fig. 5).

## 4. Discussion

In the present report we studied the formation and resealing of the "non-specific" plasma membrane pores generated by Triton, a mild non-ionic detergent which does not possess ligands selective for divalent cations and protons and for which no cell membrane receptor has been described, while most of the toxins act through specific receptors. HL-60 myelocytes were treated with Triton X-100 for 1 h and opening of artificial membrane pores was demonstrated by the penetration of the cell-impermeant fluorescent dye EtBr. The pores were likely to be relatively small since the larger dimer (EthD-1) did not penetrate treated cells, as described in Results. Similar results have been reported for granulocytes and other cell types treated with the lymphocyte perforin or the aerolysin and for fibroblasts contracting collagen matrices (Jones et al., 1990; Krause et al., 1998; Lin et al., 1997). As a direct or indirect consequence of the opening of pores, some cytosolic parameters were modified: namely, a loss of intracellular $K^+$ and plasma membrane depolarization. Leakage of intracellular $K^+$ following application of different pore-forming agents has also been reported (Walev et al., 1994; Abrami et al., 2003; Knapp et al., 2010). The plasma membrane depolarization is in part due to the efflux of $K^+$ and it has been observed in granulocytes and other cell types following treatments with different pore-forming agents as described by a number of reports (Pasternak et al., 1985; Bashford et al., 1988; Krause et al., 1998).

Following Triton treatment in the absence of external Calcium there is a steep rise of the cytosolic free $Ca^{2+}$ within the first minute of contact with the chemical; then the concentration of the cytosolic free $Ca^{2+}$ rises more slowly, but significantly, at least until min 8, but after 1 h it has reverted to baseline. Under these exper-

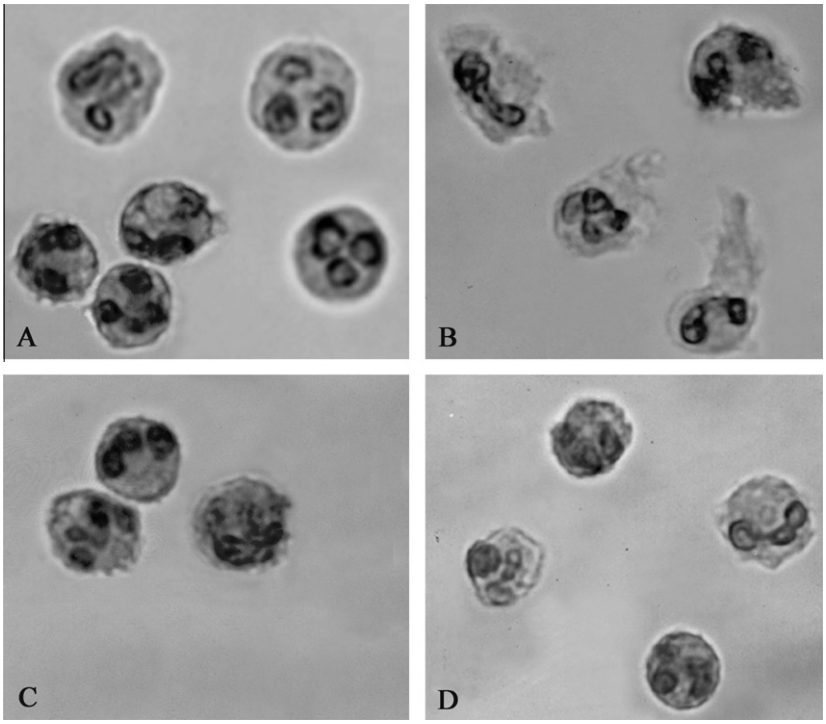


**Fig. 5.** Representative microphotographs of morphological changes of HL-60 granulocytes in untreated cells (A), after treatment with Triton (B), and after treatment with Triton + PTX (C) or Triton + PTX + Cytochalasin (D). Türk staining, phase contrast microscopy with 40× objective.

imental conditions the additional $Ca^{2+}$ ions must obviously be released from the intracellular stores. Treatment with PTX blocks the cytosolic free $Ca^{2+}$ rise, suggesting that it depended upon the activation of PTX-sensitive heterotrimeric G-proteins. A G-protein dependent cytosolic free $Ca^{2+}$ rise was observed in different cell types following treatment with a variety of natural pore-forming toxins and the pulmonary surfactant (Jones et al., 1990; Krause et al., 1998; Boston et al., 2004). In particular, based on several evidences, Krause et al. argued that activation of PTX-sensitive heterotrimeric G-proteins appears to be a consequence of pore formation, rather than receptor activation through aerolysin-binding (Krause et al., 1998).

In another set of experiments we studied the roles of the PTX-sensitive heterotrimeric G-proteins and the actin cytoskeleton in the processes of pore resealing. The activity of the PTX-sensitive heterotrimeric G-proteins was blocked by PTX administration, which ADP-ribosylates the latter; the cytoskeleton was partly disassembled through the use of Cytochalasin. The course of pore resealing was monitored using the tests of EtBr incorporation and intracellular $K^+$ concentration, the two tests which had proven to yield the most clear-cut results.

Although the mechanisms effecting plasma membrane repair are not completely understood, previously published and present results allow some of the players in this process to be defined.

An immediate result of plasma membrane injury in eukaryotic cells is the influx of $Ca^{2+}$, down an approximately 10,000-fold concentration gradient (Bhakdi et al., 1986; Reddy et al., 2001). Even in the absence of external calcium, the concentration of the cytosolic free $Ca^{2+}$ increases by release from the intracellular Ca stores. According to some reports, the cytosolic free $Ca^{2+}$ rise is of paramount importance for pore resealing, since it is achieved through endocytosis or exocytosis processes or through an orderly combination of the two processes, both of which require relatively high levels of cytosolic free $Ca^{2+}$. Lysosome and endosome exocytosis, regulated by the $Ca^{2+}$ sensor molecule synaptotagmin VII, might move these organelles (including the azurophil granules of neutrophils) to the surface so that their membrane can fuse with the plasma membrane and repair the damaged section. (Terasaki et al., 1997; Reddy et al., 2001; Keefe et al., 2005; Togo, 2006; Idone et al., 2008b). Endocytosis might internalize damaged patches of the plasma membrane (Idone et al., 2008a). Alternatively, the damaged endocytosed patches of plasma membrane (with the bound toxin) could be secondarily expelled through exocytosis (Husmann et al., 2009).

Loss of intracellular Potassium ions has also been advocated as an event triggering the membrane repair processes, by means of the activation of the mitogen activated protein kinase (MAPK) p38, a "survival" protein (Kloft et al., 2009).

PTX-sensitive heterotrimeric G-proteins are certainly involved in cases of plasma membrane damage. In cases of damage by natural toxins binding to specific receptors PTX-sensitive heterotrimeric G-proteins are activated directly and not via the receptor molecule (Krause et al., 1998). Non-receptor chemicals, such as detergents, also activate PTX-sensitive heterotrimeric G-proteins, as evidenced by the response of the actin cytoskeleton. These proteins are located immediately beneath the membrane to which they are anchored; it may be hypothesized that mere exposure to the extracellular medium through the newly formed pores may bring about their activation. Thus, PTX-sensitive heterotrimeric G-proteins appear to be "sensors" of membrane damage. Heterotrimeric G-proteins are known to have a crucial role as molecular switches in signal transduction pathways (Panaro and Mitolo, 1999), triggering actin assembly through complex transduction cascades, involving as downstream effectors phosphatidylinositol 3-kinase, some small GTPases of the Rho family and their regulators (Glogauer et al., 2000; Cicchetti et al., 2002). Modulation of the actin cytoskeleton by PTX-sensitive heterotrimeric G-proteins is well described (Panaro and Mitolo, 1999; Calvello et al., 2002; Panaro et al., 2006). Tubulin assembly has also been reported to be regulated by some heterotrimeric G-proteins

families (Dave et al., 2009). Even some transcription factors, such as STAT3 and NF-κB, are modulated by individual heterotrimeric G protein subfamilies (Ho et al., 2009). Furthermore, there is now accumulating evidence for a number of noncanonical functions of some heterotrimeric G-proteins, including the Gi proteins, independently of activation by seven transmembrane domain receptors (Marty and Ye, 2010). As a result of these different actions, the heterotrimeric G-proteins have been implicated in several intracellular processes, including membrane trafficking and budding of membrane vesicles from cell organelles, vesicular transport, and endocytosis (Siegel et al., 1999). Since some of these cellular activities have been suggested to play a direct or indirect role in membrane repairing (see above), we investigated the progress of membrane repair following Triton treatment, in the absence or presence of PTX, using the tests of EtBr incorporation and Intracellular $K^+$ concentration. Cell recovery was significantly slowed down in the presence of PTX, showing that the PTX-sensitive heterotrimeric G-proteins act not only as sensors of membrane damage but are also positive players in membrane repair.

We also investigated the role of the actin cytoskeleton in the progress of membrane repair, by monitoring the course of the latter in the absence or presence of Cytochalasin B, using the tests of EtBr incorporation and intracellular $K^+$ concentration. In this case, too, cell recovery was significantly slowed by the disruption of the cytoskeleton, showing that the latter is also a positive player in membrane repair. Accordingly, the presence of both PTX and Cytochalasin further slowed the progress of membrane repair. These effects could not be attributed to a general toxicity of these drugs, since in control cells PTX and Cytochalasin did not alter the membrane permeability. Although our results clearly indicate that the integrity of the actin cytoskeleton favors membrane recovery, earlier reports are conflicting in this respect. Idone et al. maintained that disassembly of the actin cytoskeleton markedly facilitates membrane resealing through the enhancement of endocytosis in cells permeabilized by streptolysin *O* pores or mechanical wounds (Idone et al., 2008a). On the contrary, other investigators concluded for a positive role of the cytoskeleton in the processes of endocytosis and exocytosis and ultimately in membrane repair (Sokac et al., 2003; Zhou et al., 2008). Also microtubules seem to participate locally in the resealing of wounded membranes (Togo, 2006).

PTX-sensitive heterotrimeric G-proteins appear to play a positive role of paramount importance in the plasma membrane repair. First of all, they might be the first "sensors" of the membrane damage, as discussed above. Secondarily, upon their activation, they trigger a series of cellular responses, involving phospholipase C cascade, that ultimately trigger the endocytosis/exocytosis processes for membrane repair (Sokac et al., 2003; Cicchetti et al., 2002). Furthermore, they regulate tubulin assembly (Dave et al., 2009) and activate some transcription factors (Ho et al., 2009), with possible positive effects on membrane repair. In this context, the actin cytoskeleton seems to be an important positive player in the membrane repair, since its disruption markedly slows down this process. It may also be hypothesized that it could play a more direct role by driving patches of the plasma membrane in a horizontal direction to effect the closure of small artificial pores.

To conclude, PTX-sensitive heterotrimeric G-proteins are likely sensors of plasma membrane damage and both the PTX-sensitive heterotrimeric G-proteins and the actin cytoskeleton are likely major positive players in the processes of membrane repair.

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