Short-term potentiation of membrane resealing in neighboring cells is mediated by purinergic signaling

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Abstract Resealing of a disrupted plasma membrane in the micron-size range requires Ca^{2+} -regulated exocytosis. When cells are wounded twice, the second membrane disruption reseals more quickly than the initial wound. This response is protein kinase C (PKC)-dependent and protein kinase A-dependent in the early stages. In the long term (24 h), potentiation of membrane resealing in a wounded cell depends on gene expression mediated by a transcription factor, cyclic adenosine monophosphate response element binding protein (CREB), which is activated by a PKC-dependent and p38 mitogen-activated protein kinase-dependent pathway. In addition, a recent study demonstrated that wounding of Madin–Darby canine kidney (MDCK) cells potentiates membrane resealing in neighboring cells by activating CREB-dependent gene expression through nitric oxide (NO) signaling. The present study demonstrated that wounding of MDCK cells induces short-term potentiation of membrane resealing in neighboring cells in addition to a long-term response. Inhibition of purinergic signaling suppressed short-term potentiation of membrane resealing in neighboring cells, but not long-term potentiation. By contrast, inhibition of NO signaling did not suppress the short-term response in neighboring cells. These results suggest that cell membrane disruption stimulates at least two intercellular signaling pathways, NO and purinergic signaling, to potentiate cell membrane resealing in neighboring cells.

Keywords Membrane resealing · ATP · Ca^{2+} · Nitric oxide

Introduction

Mechanical stress under physiological conditions induces disruptions of plasma membranes in many animal tissues [1]. The disruption of the cell membrane must be resealed rapidly to ensure the viability of the cell. Mechanisms for membrane resealing may differ depending on the extent of the membrane lesion [1]. If a cell experiences a membrane disruption in the micrometer diameter range, Ca^{2+} influx at the wound site triggers exocytosis that is essential for successful cell membrane repair [2–12]. Exocytosis is accompanied by a decrease in membrane tension, which has been shown to be necessary for successful membrane resealing [6, 7].

Previously, it was demonstrated that exocytosis induced by small micron-sized disruption is potentiated following an initial wound, and repeated membrane disruptions reseal more quickly than the initial wound [6, 9–12]. This response is dependent on protein kinase C (PKC) and protein kinase A (PKA) in the early stages [6, 9, 12]. In the long term (24 h), potentiation of membrane resealing in a wounded cell depends on the activation of a transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), via the PKC- and p38 mitogen-activated protein kinase (MAPK)-dependent pathway [9, 11].

In addition to wounded cells, it has been demonstrated that wounding of Madin–Darby canine kidney (MDCK) cells potentiates membrane resealing in neighboring cells for long term by stimulating CREB-dependent gene expression [13]. Intercellular signaling is mediated by several forms including paracrine signaling with extracellular molecules as well as signaling via gap junctions between two adjacent cells. Among them, the long-term response observed in neighboring cells upon cell membrane disruption requires nitric oxide (NO)/protein kinase G (PKG) signaling to stimulate CREB [13]. Although neighboring cells show long-term responses to membrane disruption, it is still unclear if these cells also have

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short-term responses upon cell membrane disruption, as observed in a wounded cell.

The aim of the present study was to investigate whether cell membrane disruption potentiates membrane resealing in neighboring cells in the short and long terms. Results revealed that paracrine signaling mediated by adenosine 5'-triphosphate (ATP) was involved in short-term potentiation of membrane resealing in neighboring MDCK cells.

Materials and methods

Cell culture

MDCK cells were cultured as described previously [13]. Cells for experiments were plated on glass-based 35 mm dishes (Iwaki) and were grown for 2–3 days before use. During the wounding experiments, cells were maintained in 1.8 mM Ca^{2+} Ringer's solution containing 1 % fetal bovine serum (FBS). The Ca^{2+} -free Ringer's solution contained 138 mM NaCl, 2.7 mM KCl, 1.06 mM MgCl_2 , 5.6 mM D-glucose, and 12.4 mM HEPES (pH 7.25). A stock solution of 100 mM CaCl_2 was used to adjust the concentration of Ca^{2+} .

Membrane resealing assay

Cells were loaded with CellTrace calcein red–orange AM (1 μM ; Life Technologies) in culture medium for 1 h at 37 °C. Changes in the fluorescence intensity of calcein red–orange were monitored with an LSM510 laser scanning confocal microscope (ver. 3.2; Carl Zeiss) equipped with Axiovert (C-Apochromat 40X/1.2 W Corr objective). To disrupt the plasma membrane, glass needles were made from Narishige G-1000 glass rods by pulling with a Narishige PC-10. Wounding of cells was performed using an InjectMan 5179 and a FemtoJet 5247 (Eppendorf) equipped with a microscope. The time setting for wounding was 0.3 s. A slowly declining fluorescence possibly caused by photobleaching was subtracted from raw recordings before plotting the traces. A transient decrease of fluorescence intensity indicated successful resealing (see Fig. 1a).

Scratch wound

The marker dye, Alexa 488-dextran (10,000 MW; Life Technologies) was dissolved at a concentration of 0.5 mg/ml in 1.8 mM Ca^{2+} Ringer's solution containing 1 % FBS. MDCK cells were rinsed once with 1.8 mM Ca^{2+} Ringer's solution containing 1 % FBS at 37 °C before adding the above Alexa solution at 37 °C. Cells were then wounded by multiple slow scratches with a sterile 27G needle in the presence of the marker dye. The cells were allowed to stand for 5 min and then were returned to normal culture conditions.

Fig. 1 Cell membrane disruption potentiates membrane resealing in neighboring cells. **a** Typical recordings of the resealing assay. Cells loaded with calcein red–orange AM were wounded with a glass needle, and changes in fluorescence intensity of calcein red–orange were monitored. After 5 min, an adjacent cell was also wounded, and changes in fluorescence intensity were recorded. Units of fluorescence intensity were normalized to 100 % before wounding. Arrows indicate the time of membrane disruption. Bars indicate the completion time of membrane resealing. **b** Comparison of membrane resealing rates of initial wound and second wound created in neighboring MDCK cell. The resealing rate was defined as the reciprocal of the resealing time in seconds. For cells that failed to reseal, the rate was defined as zero. Numbers of cells observed are indicated in parentheses. * $P < 0.01$

Ca^{2+} imaging

A stock solution of calcium green-1 (CG-1) acetoxymethyl (AM) ester (Life Technologies) in dimethyl sulfoxide (DMSO) was mixed with equal volume of 20 % Pluronic F-127 in DMSO (Life Technologies) before dilution to a final loading concentration of 1 μM in 1.8 mM Ca^{2+} Ringer's solution containing 10 % FBS. CG-1 AM was loaded at room temperature (23–25 °C) for 1 h and then washed with 1.8 mM Ca^{2+} Ringer's solution containing 1 % FBS. Image data acquisition was performed using the same system as for the membrane resealing assay.

Statistical analysis

All values are shown as mean±SEM. Statistical comparisons were performed by Prism 6 (GraphPad Software). The Mann–Whitney test was used for comparison of two groups. One-way ANOVA followed by Bonferroni's multiple comparison test was used for comparison of more than two groups. A value of $P<0.05$ was considered statistically significant.

Results

Cell membrane disruption induces short-term potentiation of membrane resealing in neighboring cells

To assess cell membrane resealing, MDCK cells loaded with calcein red–orange AM (1 μ M) were wounded using a glass needle in 1.8 mM Ca^{2+} Ringer's solution, and the changes in fluorescence intensity of calcein red–orange were monitored. As described previously [13, 14], cell membrane disruption was indicated by a decrease in fluorescence intensity (arrows in Fig. 1a). In most cases, the decrease in fluorescence intensity stopped (bars in Fig. 1a). When cells were wounded under conditions that inhibited membrane resealing, the intensity persistently decreased [13, 14], indicating that successful membrane resealing resulted in a transient decrease in fluorescence intensity. To compare the timing of membrane resealing of initial wound and second wound created in neighboring cells (adjacent to the initially wounded cells), the resealing time was measured. Since it was impossible to determine resealing time in the cells that failed to reseal, the reciprocal of resealing time was calculated. These values were termed “resealing rates” as reported previously, and the value was defined as zero for cells that failed to reseal [2, 6, 7, 9–14]. When non-wounded cells were initially wounded in 1.8 mM Ca^{2+} Ringer's solution, the resealing rate was 0.026 ± 0.002 ($n=39$; Fig. 1b). The resealing rate for neighboring cells increased significantly to 0.042 ± 0.003 ($n=22$) when neighboring cells were wounded 5 min after the initial wound (Fig. 1b). These results indicate that membrane resealing of neighboring MDCK cells is potentiated within 5 min.

Short-term potentiation of membrane resealing in neighboring cells is dependent on the substrate of apyrase

Cell membrane disruption can stimulate at least two intercellular signaling systems: the purinergic and NO signaling systems [13, 15]. Although NO signaling has been shown to be involved in long-term potentiation of membrane resealing in neighboring cells [13], the role of purinergic signaling in neighboring cells is not yet clear. To investigate whether short-term potentiation of membrane resealing is mediated by purinergic signaling, cells were wounded in the

presence of apyrase, an enzyme that degrades ATP and adenosine diphosphate to AMP [16]. When non-wounded cells were initially wounded in the presence of 20 U/ml apyrase, the resealing rate was 0.019 ± 0.001 ($n=36$; Fig. 2a). When neighboring cells were wounded 5 min later, the resealing rate of the neighboring cell was 0.017 ± 0.002 ($n=19$; Fig. 2a). These results indicate that degradation of extracellular ATP by

Fig. 2 Degradation of extracellular ATP suppresses short-term potentiation of membrane resealing in neighboring cells, but not the long-term response. **a** Effect of extracellular apyrase on the potentiation of membrane resealing in neighboring MDCK cells. For the short-term assay, cells loaded with calcein red–orange AM were initially wounded by a glass needle in the presence of 20 U/ml apyrase, and the changes in fluorescence intensity of calcein red–orange were monitored. Neighboring cells were then wounded 5 min later, and the changes in fluorescence intensity were analyzed. For the long-term assay, cells were scratched in the presence of Alexa 488-dextran along with 20 U/ml apyrase. These cells were loaded with calcein red–orange AM 24 h after scratching, and the resealing rates of neighboring cells were analyzed. **b** Effect of NO synthase inhibitor on the short-term potentiation of membrane resealing in neighboring MDCK cells. Cells loaded with calcein red–orange AM were wounded by a glass needle in the presence of L-NAME (300 μ M), and the resealing rates of initially wounded and neighboring cells were compared. Numbers of cells observed are indicated in parentheses. * $P<0.01$; # $P<0.05$. The effect of L-NAME on the long-term potentiation of membrane resealing has already been reported previously [13]

apyrase suppressed short-term potentiation of membrane resealing in neighboring cells.

To determine if purinergic signaling also affects long-term potentiation of membrane resealing in neighboring cells, MDCK cells were initially wounded by scratching in 1.8 mM Ca^{2+} Ringer's solution containing 20 U/ml apyrase and the marker dye Alexa 488-dextran. Alexa 488-dextran enters cells that incur cell membrane disruption and is retained in wounded cells that successfully reseal [13]. Then cells adjacent to Alexa 488-positive cells were wounded by a glass needle 24 h later and resealing rates were analyzed. As shown in Fig. 2a, resealing rate was significantly increased to 0.035 ± 0.002 ($n=11$), suggesting that long-term potentiation of membrane resealing in neighboring cells was not mediated by purinergic signaling.

To investigate the involvement of NO signaling in short-term potentiation of membrane resealing in neighboring cells, cells were initially wounded in the presence of 300 μM L- N^{G} -nitroarginine methyl ester (L-NAME), a NO synthase inhibitor. Resealing rate for initial wounding was 0.023 ± 0.003 ($n=11$), whereas the rate was increased to 0.047 ± 0.011 ($n=7$) when neighboring cells were wounded 5 min later (Fig. 2b). These results suggest that short-term potentiation of membrane resealing in neighboring cells was mediated by ATP but not NO. Contrary to the short-term response, previous [13] and present studies indicate that long-term potentiation of membrane resealing in neighboring cells was mediated by NO but not ATP.

Cell membrane disruption induces intercellular Ca^{2+} signaling in MDCK cells

It has been reported that mechanically scratching cell monolayers induces intercellular Ca^{2+} waves in bovine pulmonary endothelial cells and mouse mammary epithelial cells [17] and that ATP is involved in intercellular Ca^{2+} signaling in response to membrane disruption in sea urchin embryo [15]. Thus, the short-term potentiation of membrane resealing mediated by ATP may require the increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{i}}$) in neighboring cells. To investigate if cell membrane disruption stimulates intercellular Ca^{2+} signaling in MDCK cells, cells were loaded with CG-1 AM and changes in $[\text{Ca}^{2+}]_{\text{i}}$ upon cell membrane disruption were observed in wounded and neighboring cells (Fig. 3). A cell indicated by an arrow (Fig. 3a) was wounded by a glass needle which immediately evoked a rise in $[\text{Ca}^{2+}]_{\text{i}}$ in the cell (Fig. 3b, W). In addition to a wounded cell, increase in $[\text{Ca}^{2+}]_{\text{i}}$ was also observed in cells neighboring the wounded cell following a short delay (Fig. 2b, 1–4). The same results were obtained from all experiments (three repetitions). To observe the involvement of ATP in intercellular Ca^{2+} signaling, cells loaded with CG-1 AM were wounded by a glass needle in the presence of

Fig. 3 Cell membrane disruption induces intercellular Ca^{2+} signaling in MDCK cells. **a** Cells loaded with CG-1 AM were wounded with a glass needle, and changes in fluorescence intensity of CG-1 were monitored. The arrow in the differential interference contrast (DIC) image indicates a wounded cell (W in CG-1 image). Cells adjacent to the wounded cell were labeled 1, 2, etc., in order of their relationship with the wounded cell. **b** The time course of changes in fluorescence intensity of CG-1 ($\Delta F/F$) are plotted for wounded (W) and neighboring cells (1–4). The fluorescence change in W does not reflect the precise changes in $[\text{Ca}^{2+}]_{\text{i}}$ since cell membrane disruption induces the efflux of CG-1

20 U/ml apyrase. Although cell membrane disruption resulted in an increase in $[\text{Ca}^{2+}]_{\text{i}}$ (Fig. 4, W), apyrase attenuated the Ca^{2+} signaling in neighboring cells (Fig. 4, 1–4). The same conclusions were reached from 8 out of 10 measurements. Finally, addition of ATP (100 μM) to the medium resulted in a transient increase in $[\text{Ca}^{2+}]_{\text{i}}$ (Fig. 5). Similar responses were observed in all experiments (four repetitions). These results suggest that purinergic signaling evoked by a cell membrane disruption leads to an increase in $[\text{Ca}^{2+}]_{\text{i}}$ in neighboring MDCK cells.

Fig. 4 Extracellular apyrase inhibits wound-induced intercellular Ca^{2+} signaling in MDCK cells. Cells loaded with CG-1 AM were wounded with a glass needle in the presence of 20 U/ml apyrase, and the changes in fluorescence intensity of CG-1 were monitored. The arrow in the DIC image indicates the wounded cell. Cells were numbered as per Fig. 3. The fluorescence change in *W* does not reflect the precise changes in $[\text{Ca}^{2+}]_i$ since cell membrane disruption induces the diffusion of CG-1

An increase in $[\text{Ca}^{2+}]_i$ is required for short-term potentiation of membrane resealing in neighboring cells

To investigate the involvement of intercellular Ca^{2+} signaling in short-term potentiation of membrane resealing in neighboring cells, cells were treated with BAPTA-AM (50 μM), a membrane permeable Ca^{2+} chelator, for 30 min. As shown in Fig. 6, treatment with BAPTA-AM completely suppressed changes in $[\text{Ca}^{2+}]_i$ in neighboring cells (Fig. 6, 1–4). By contrast, a rise in $[\text{Ca}^{2+}]_i$ was observed in a wounded cell (Fig. 6, W), probably because influx of extracellular Ca^{2+} overcomes the chelating ability of BAPTA. The treatment with BAPTA-AM abolished the potentiation of membrane resealing in neighboring cells. When non-wounded cells were initially wounded, the resealing rate was 0.026 ± 0.002 ($n=14$;

Fig. 5 Extracellular ATP induces an increase in $[\text{Ca}^{2+}]_i$ in MDCK cells. Cells loaded with CG-1 AM were treated with ATP (100 μM). The time course of CG-1 fluorescence ($\Delta F/F$) is plotted for nine regions of interest. ATP was applied at time zero

Fig. 7a). When neighboring cells were wounded 5 min later, the resealing rate of the neighboring cell was 0.025 ± 0.003 ($n=13$).

To confirm the involvement of ATP and Ca^{2+} in short-term potentiation of membrane resealing, ATP or AMP (100 μM) was applied to non-wounded cells, and membrane resealing was assessed 5–20 min after nucleotide application (Fig. 7b). The results clearly indicated that ATP potentiates membrane resealing. Resealing rates for ATP- and AMP-treated cells were 0.048 ± 0.003 ($n=28$) and 0.028 ± 0.003 ($n=10$), respectively. When cells were treated with BAPTA-AM (50 μM) for 30 min before addition of ATP, ATP did not potentiate cell membrane resealing, and the resealing rate was 0.029 ± 0.003 ($n=27$; Fig. 7b). These results indicate that an increase in $[\text{Ca}^{2+}]_i$ induced by ATP is required for short-term potentiation of membrane resealing in neighboring cells.

Discussion

Ca^{2+} -regulated exocytosis, which requires vesicle docking/fusion SNARE proteins, has been shown to be essential for resealing of micrometer-sized membrane disruptions in mammalian cells and invertebrate embryos [2–12]. It was demonstrated that exocytosis of wounded cells is potentiated following an initial wound, and repeated membrane disruptions reseal more quickly than the initial wound [6, 9–12]. This

Fig. 6 BAPTA inhibits wound-induced intercellular Ca^{2+} signaling in MDCK cells. Cells loaded with CG-1 AM were incubated with 50 μM BAPTA-AM for 30 min and wounded with a glass needle. The arrow in the DIC image indicates the wounded cell. Cells were numbered as per Fig. 3. The fluorescence change in *W* does not reflect the precise changes in $[\text{Ca}^{2+}]_i$ since cells contained BAPTA and cell membrane disruption induces the diffusion of CG-1

potentiation in membrane resealing is achieved by various signaling cascades in a wounded cell. For example, it has been demonstrated that PKC and PKA are involved in short-term potentiation of membrane resealing and wound-induced exocytosis [6, 9, 12]. PKC is also involved in the activation of CREB-dependent gene expression through p38 MAPK in a wounded cell [11].

In addition to intracellular signaling, a previous study has revealed that cell–cell signaling by NO, which is stimulated by cell membrane disruption, potentiates membrane resealing in neighboring cells over the long term in a CREB-dependent manner in MDCK cells [13]. The present study further demonstrates that cell membrane disruption stimulates an increase in $[\text{Ca}^{2+}]_i$ in neighboring cells through purinergic signaling. Purinergic signaling induced

Fig. 7 An increase in $[\text{Ca}^{2+}]_i$ induced by ATP is required for short-term potentiation of membrane resealing in MDCK cells. **a** Cells loaded with calcein red-orange AM were incubated with BAPTA-AM (50 μM), and resealing rates of the initial and secondary wounds created in neighboring cells were compared. **b** BAPTA-AM-treated and -untreated cells were wounded by a glass needle after addition of ATP (100 μM), and the resealing rates were analyzed. As a control, cells treated with AMP (100 μM) were wounded by a glass needle. Resealing rates were analyzed 5–20 min after addition of nucleotides. Numbers of cells observed are indicated in parentheses. * $P < 0.01$

by cell membrane disruption has been described in detail in sea urchin embryo [15], but the role of the increase in $[\text{Ca}^{2+}]_i$ in neighboring cells is not yet clear. The present study demonstrates that this signaling pathway potentiates membrane resealing in neighboring cells, at least in MDCK cells. Signals mediated by NO and ATP are independently involved in long-term and short-term potentiation of membrane resealing of neighboring MDCK cells, respectively. NO stimulates CREB phosphorylation through PKG in neighboring cells [13], but do not affect short-term potentiation of membrane resealing in neighboring cells (Fig. 2). Contrary to NO, ATP induces short-term potentiation of membrane resealing, but does not affect long-term potentiation in neighboring cells (Fig. 2).

It is well established that cell–cell communication can also be mediated by molecules moving via gap junctions [18]. However, the signaling mediated by gap junctions may not

be involved in the short-term potentiation of membrane resealing of neighboring MDCK cells as this potentiation is completely blocked by apyrase that degrades extracellular ATP (Fig. 2). Elevated $[Ca^{2+}]_i$ have been shown to reduce the permeability of gap junctions in many cells [19]. Thus, in MDCK cells, it is possible that massive Ca^{2+} influx upon cell membrane disruption protects intact neighboring cells from leakage of metabolites through gap junctions by disconnecting them from wounded cell. It is still not determined if gap junction is affected by Ca^{2+} transients induced by cell membrane disruption in MDCK cells.

Previous studies demonstrate that the amount of exocytosis evoked at a wound is closely correlated with the rate of membrane resealing in all cell types tested [2, 4–6, 9]. Thus, it is possible that exocytosis is also potentiated in neighboring cells upon cell membrane disruption in MDCK cells. Exocytosis can be enhanced by protein kinases in neurons and endocrine cells. For example, in insulin-secreting β -cells and in adrenal chromaffin cells, PKC and PKA amplify the size of the readily releasable pool of vesicles [20–23]. In wounded cells, both PKC and PKA are involved in short-term potentiation of membrane resealing and wound-induced exocytosis of mouse 3T3 fibroblasts and rabbit corneal epithelial cells [6, 9, 12]. Similarly, although this remains to be demonstrated, protein phosphorylation by PKC and/or PKA might be involved in short-term potentiation of membrane resealing in neighboring MDCK cells. The nucleotides mediate their effects through specific receptors that are either metabotropic (P2Y) or ionotropic (P2X) [24, 25]. MDCK cells have been reported to express at least three P2Y receptors; P2Y₁, P2Y₂, and P2Y₁₁ [26], and these receptors are involved in an increase in $[Ca^{2+}]_i$ and PKC activity [27]. In addition, P2Y receptors are involved in an increase in cytosolic cAMP concentration [26, 28]. Further studies are required to elucidate the involvement of PKC and PKA in short-term potentiation of membrane resealing of neighboring MDCK cells.

In summary, wounding of MDCK cells stimulates at least two intercellular signaling pathways, NO and purinergic signaling, and potentiates membrane resealing in neighboring cells. Many organs in animals normally generate and/or receive considerable levels of repetitive mechanical stress, and these stresses often result in cell membrane disruptions [1]. Thus, the multicellular adaptive response leading to faster cell membrane resealing at subsequent wounds may minimize the damage of excessive Ca^{2+} influx into cells [29–33] and may also lessen the loss of crucial cellular constituents from cells. These mechanisms may, therefore, efficiently protect tissues from mechanical stresses.

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