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Original Paper

Salicylate Inhibits Thrombopoiesis in Rat Megakaryocytes by Changing the Membrane Micro-Architecture

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Key Words
Salicylate • Megakaryocytes • Membrane capacitance • Thrombopoiesis • Membrane micro-architecture

Abstract
Background/Aims: Salicylate causes drug-induced immune thrombocytopenia. However, some clinical studies indicate the presence of additional mechanisms in the drug-induced thrombocytopenia, by which the platelet production from megakaryocytes may directly be affected. Since salicylate is amphiphilic and preferentially partitioned into the lipid bilayers of the plasma membrane, it can induce some structural changes in the megakaryocyte membrane surface and thus affect the process of thrombopoiesis. ***Methods:*** Employing the standard patch-clamp whole-cell recording technique, we examined the effects of salicylate on the membrane capacitance in rat megakaryocytes. Taking electron microscopic imaging of the cellular surface, we also examined the effects of salicylate on the membrane micro-architecture of megakaryocytes. ***Results:*** Salicylate significantly decreased the membrane capacitance of megakaryocytes, indicating the decreased number of invaginated plasma membranes, which was not detected by the fluorescent imaging technique. As shown by electron microscopy, salicylate actually halted the process of pro-platelet formation in megakaryocytes. ***Conclusion:*** This study demonstrated for the first time that salicylate inhibits the process of thrombopoiesis in megakaryocytes, as detected by the decrease in the membrane capacitance. Salicylate-induced changes in the membrane micro-architecture are thought to be responsible for its effects.

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Introduction

Salicylate is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAIDs) to relieve fever, pain and inflammatory symptoms [1]. Besides its anti-inflammatory properties, recent studies also indicated its pharmacological efficacies as an immunosuppressive, anti-neoplastic or anti-atherosclerotic reagent [2-4]. However, differing from its acetylated derivative, aspirin, which exerts antiplatelet effects and is used to prevent cardiovascular diseases [5-7], salicylate does not inhibit cyclooxygenase-1 (COX-1)-dependent aggregation of platelets [1]. Nevertheless, this drug frequently causes hemorrhagic complications [8, 9], and is one of the leading causes of drug-induced immune thrombocytopenia [10, 11]. As for the mechanisms, salicylate stimulates the production of auto-antibodies to proteins within the platelet membranes, and the antibodies then bind to the drug-protein complexes to destroy the platelets [10, 11] . In some clinical studies, the use of NSAIDs was associated with the development of aplastic anemia, which is characterized by a decrease in the hematopoietic function [12-14]. These studies indicated the presence of additional mechanisms in the drug-induced thrombocytopenia, by which the platelet production from megakaryocytes may directly be affected. In our recent study, salicylate, which partitions preferentially into the lipid bilayers of the plasma membranes [15], induced membrane surface deformation in mast cells [16], and thus modulated the process of exocytosis. Since degranulating mast cells share common membranous features with megakaryocytes in that they have many blebs on their cell surface [17], salicylate would also likely induce some structural changes on the membrane surface of megakaryocytes and thus affect their thrombopoietic activity. To test this, employing the standard patch-clamp whole-cell recording technique in rat megakaryocytes, we examined the effects of salicylate on the membrane capacitance. By electron microscopic imaging of the cellular surface, we also examined the effects of this drug on the membrane micro-architecture of the cells. Here, we show for the first time that salicylate inhibits the process of thrombopoiesis in megakaryocytes, as detected by the decrease in the membrane capacitance. We also show that the salicylate-induced changes in the membrane micro-architecture are responsible for its effects.

Materials and Methods

Cell Sources and Preparation

Male Wistar rats more than 25 weeks old, supplied by Japan SLC Inc. (Shizuoka, Japan), were deeply anaesthetized with isoflurane and then killed by cervical dislocation. The protocol for animal use was approved by the Animal Care and Use Committee of Tohoku University Graduate School of Medicine. Single megakaryocytes were isolated from rat bone marrow as described in our previous studies [18, 19]. Briefly, bone marrow obtained from femoral bones was flushed with standard external (bathing) solution containing (in mM): NaCl, 145; KCl, 4.0; $CaCl_2$, 1.0; $MgCl_2$, 2.0; HEPES, 5.0; bovine serum albumin, 0.01 % (pH 7.2 adjusted with NaOH), and dispersed by repetitive pipetting. After removing large pieces of tissue, cells were washed twice by gentle centrifugation for a minute and resuspended in the standard external solution. They were maintained at room temperature (22-24°C) for use within 8 hours.

Di-8-ANEPPS Staining

After incubating single megakaryocytes in the external solution at room temperature, they were further incubated for 30 min in the external solution containing styryl membrane indicator, di-8-ANEPPS (Biotium, Hayward, Calif., USA; final concentration 20 μM), as previously described [19]. Salicylate was then added to reach the final concentration of 3 mM. Fluorescent images were taken using a TE 2000-E Nikon Eclipse confocal microscope (Nikon, Tokyo, Japan).

Electrical Setup and Patch-Clamp Recordings

We conducted standard whole-cell patch-clamp recordings using an EPC-9 patch-clamp amplifier system (HEKA Electronics, Lambrecht, Germany) as described previously [2, 18-20]. The patch pipette

resistance was 1-3 MΩ when filled with internal (patch pipette) solution containing (in mM): KCl, 145; $MgCl_2$, 1.0; EGTA, 10; Hepes, 5.0 (pH 7.2 adjusted with KOH). After the giga-seal formation, we applied suction briefly to the pipette to rupture the patch membrane. The series resistance of the whole-cell recordings was maintained below 10 MΩ during the experiments. All experiments were carried out at room temperature.

Drug Delivery

Salicylate, purchased from Wako Pure Chem Ind. (Osaka, Japan), and margatoxin, from Peptide Institute (Osaka, Japan), were separately dissolved in the external solution at a final concentrations of 3 mM and 100 nM. We delivered the reagents to the cells by the standing hydrostatic pressure of 3 cmH_2O from a nearby pipette as described previously [2, 19, 20]. Then, whole-cell membrane currents were recorded before and after 1 min exposure to the drug and after a 2 min washout. To rule out the possibility that the observed effect just resulted from the procedure of the reagent application, we simply applied the external solution to the cells and confirmed the absence of any significant changes in the channel currents (Fig. 4A).

Membrane Capacitance Measurements

To measure the membrane capacitance of the megakaryocytes, we employed a sine plus DC protocol using the Lock-in amplifier of the EPC-9 Pulse program, as described previously [16, 19, 21]. An 800-Hz sinusoidal command voltage was superimposed on the holding potential of -80 mV. The membrane capacitance (Cm), as well as membrane conductance (Gm) and series conductance (Gs), was continuously recorded before and after 30 s exposure to salicylate during the whole-cell recording configuration. Specific Cm was expressed per unit spherical cell surface area ($\mu F/cm^2$).

Electron Microscopy

The megakaryocytes, incubated in the external solutions containing no drug, 3 or 0.3 mM salicylate and 100 nM margatoxin for 10 min, were fixed with 4% paraformaldeyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 2 h at room temperature. After being trimmed into small pieces, the above specimen containing the cells was postfixed in 1% osmium tetroxide for 1 h at 4°C, rinsed in PBS, dehydrated in a graded series of alcohol and propylene oxide, and finally embedded in epoxy resin. Ultrathin (80 nm) sections were prepared on an ultramicrotome (Ultracut R, Leica, Heerbrugg, Switzerland) with a diamond knife and then were stained with uranyl acetate and lead citrate, and viewed using an electron microscope (JEM-1200, JOEL, Tokyo, Japan).

Statistic Analyses

Data were analyzed using PulseFit software (HEKA Electronics, Lambrecht, Germany), IGOR Pro (WaveMetrics, Lake Oswego, Oreg., USA) and Microsoft Excel (Microsoft Corporation, Redmond, Wash., USA) and reported as means ± SEM. Statistical significance was assessed by two-way ANOVA followed by Dunnett's or Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Effects of salicylate on membrane architecture of rat megakaryocytes

In previous *in vitro* studies, the physiological and therapeutic levels of salicylate ranged from 0.1 to 1 mM [22, 23]. However, according to our recent study using mast cells, salicylate at a concentration as high as 3 mM was required to effectively elicit its pharmacological property on the membrane curvature of the cells [16]. Therefore, in the present study, we used salicylate at 3 mM. As previously demonstrated in outer hair cells [15], salicylate induced deformation of the membrane surface in red blood cells (Fig. 1Ab vs a). Such an effect of salicylate may be ascribable to its pharmacological property as a negatively charged membrane amphipath [15, 16, 24], which is partitioned preferentially into the outer leaflet of the plasma membrane and generates outward membrane bending (Fig. 1Ac).

In megakaryocytes, however, salicylate did not affect the general appearance of the cells, which was characterized by a number of wrinkles on their cell surface (Fig. 1Bb vs a). During the process of thrombopoiesis, invagination of the plasma membrane occurs

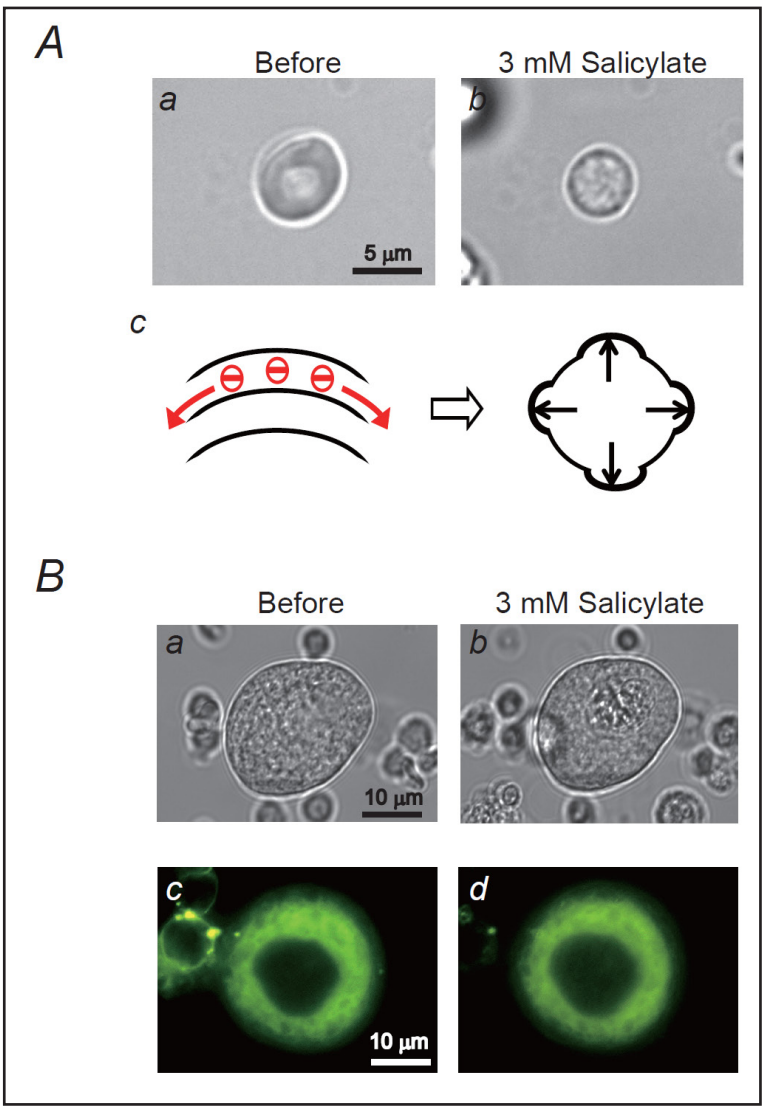

Fig. 1. Effects of salicylate on membrane architecture of megakaryocytes. A: (a, b) Differential-interference contrast (DIC) microscopic images of red blood cells before (a) and after incubation in the external solution containing 3 mM salicylate (b). (c) Illustrations of the salicylate-induced plasma membrane curvature in red blood cells. Negatively charged salicylate is partitioned preferentially into the positively charged outer leaflets of the lipid bilayers and generates outward membrane bending (left). It induces outer leaflet expansion in red blood cells (right). B: (a, b) DIC microscopic images of megakaryocytes before (a) and after incubation in the external solution containing 3 mM salicylate (b). (c, d) Confocal fluorescence images of di-8-ANEPPS-labeled megakaryocytes before (c) and after incubation in the external solution containing 3 mM salicylate (d).

in megakaryocytes, providing the extracellular space required for the formation of proplatelets [25-27]. Therefore, these wrinkles were thought to represent the membrane surface deformation induced by thrombopoiesis. To examine the effects of salicylate on this membrane surface deformation in megakaryocytes, we stained the cells with di-8-ANEPPS, a membrane-impermeant fluorescent dye better retained in the outer leaflet of the plasma membrane [28]. In megakaryocytes incubated in the external solution, the dye penetrated deep into the cells and stained throughout the extranuclear cellular volume (Fig. 1Bc), indicating its retention into the folds created in the membranes during thrombopoiesis [25-27]. However, the external addition of salicylate did not affect these findings (Fig. 1Bd), suggesting that salicylate did not morphologically alter the membrane architecture of megakaryocytes.

Effect of salicylate on whole-cell membrane capacitance in rat megakaryocytes

From our results, we could not obtain morphological evidence that salicylate may affect the membrane architecture of megakaryocytes (Fig. 1B). However, since salicylate is amphiphilic [15, 16, 24] and is known to interact with the lipid bilayers of the plasma membrane [29], this drug would generate some microscopic changes in the megakaryocyte membrane surface. In spherical cells, such as in thymocytes and mast cells, microscopic changes in the cell surface area were best monitored by measurement of the whole-cell

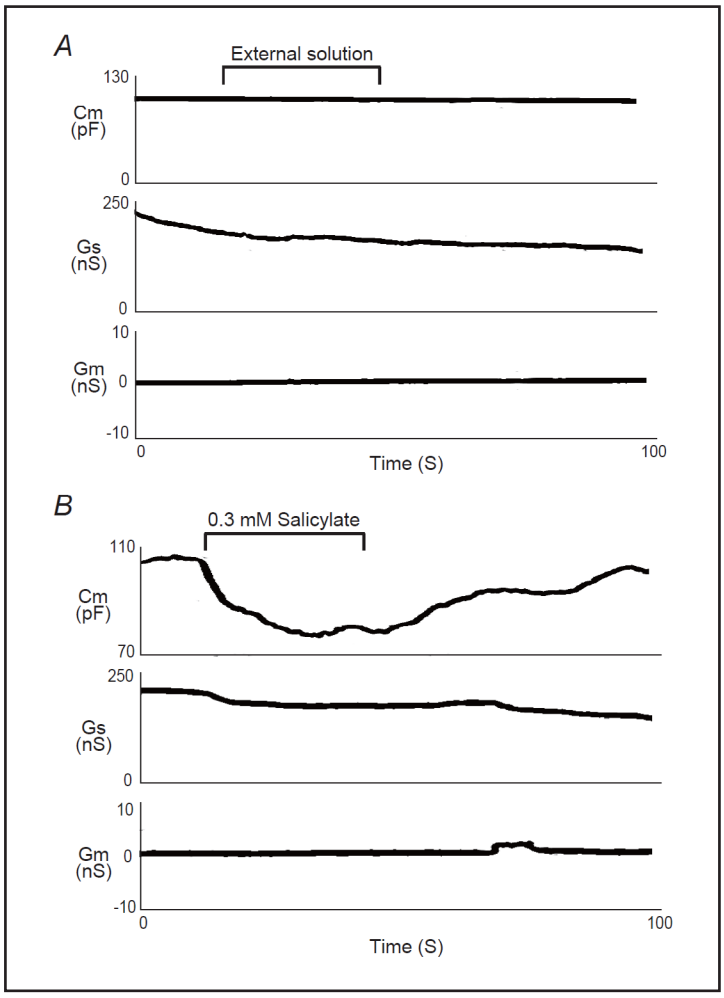

Fig. 2. Salicylate-induced changes in megakaryocyte membrane capacitance, series and membrane conductance. After establishing the whole-cell configuration, external solutions containing no drug (A) and 0.3 mM salicylate (B) were delivered for 30 sec to single megakaryocytes. Membrane capacitance, series and membrane conductance were monitored for at least 100 sec. N=5 for each trace. Cm, membrane capacitance; Gs, series conductance; Gm, membrane conductance.

Table 1. Summary of changes in membrane capacitance after application of salicylate

Agents	*N*	*Cm* before drug application ($\mu F/cm^2$)	*Cm* after drug application ($\mu F/cm^2$)	ΔCm ($\mu F/cm^2$)
External solution (control)	5	9.28 ± 0.95	9.10 ± 0.85	0.17 ± 0.13
0.3 mM Salicylate	5	9.71 ± 0.84	5.75 ± 0.65 #	3.96 ± 0.56

Values are means ± SEM. *Cm* = specific membrane capacitance. # $p < 0.05$ vs. *Cm* before drug application.

membrane capacitance (Cm) [2, 16, 21, 30-32]. Therefore, in the present study, we employed this electrophysiological approach to detect the microscopic changes in the megakaryocyte membrane surface (Fig. 2). Numerical changes in the specific Cm expressed per unit spherical cell surface area ($\mu F/cm^2$) are summarized in Table 1. By simply applying the external solution alone to megakaryocytes (Fig. 2A), we confirmed that our procedure of reagent application, a puff application by constant hydrostatic pressure with a nearby pipette, was without significant effects on the Cm and the other parameters, such as membrane conductance (Gm) and series conductance (Gs) (Fig. 2A, Table 1). Since the pipette inclusion of 3 mM salicylate caused erratic changes in the Gm and Gs, which made the measurement of the Cm less reliable [33, 34], we applied this drug at a lower concentration, 0.3 mM. The pipette inclusion of 0.3 mM salicylate induced a significant decrease in the Cm immediately after

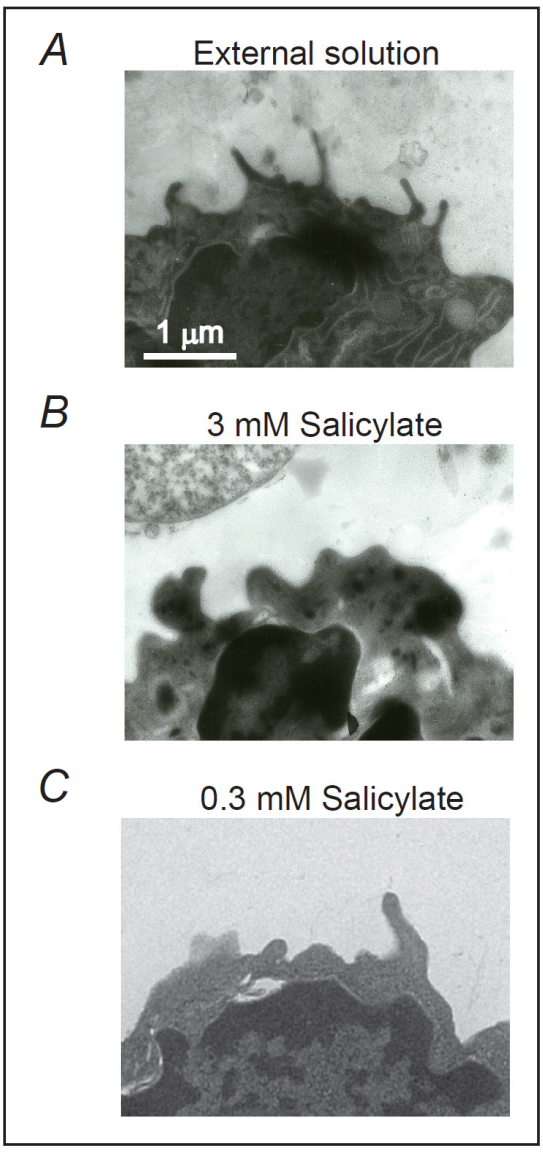

Fig. 3. Electron microscopic images of salicylate-induced changes in membrane micro-architecture in megakaryocytes. Thin-section electron micrographs of megakaryocyte plasma membranes after incubating the cells in the external solutions containing no drug (A), 3 mM salicylate (B) or 0.3 mM salicylate (C).

the application (Fig. 2B, Table 1), but with minimal changes in the Gm and Gs. These results indicated that salicylate actually induced microscopic structural changes in the megakaryocyte membrane surface, which was not morphologically detected by the differential-interference contrast (DIC) microscopy or the fluorescent imaging technique (Fig. 1B). In megakaryocytes, since the value of specific Cm reflects the number of invaginated plasma membranes during thrombopoiesis [18, 19, 28], the decrease in the Cm suggested a decrease in thrombopoietic activity caused by salicylate.

Electron microscopic images of salicylate-induced changes in membrane micro-architecture of megakaryocytes

Since salicylate was likely to induce microscopic structural changes in the megakaryocyte membrane surface (Fig. 2), it should affect the micro-architecture of the membrane, which could not be detected by DIC microscopy or the fluorescent imaging technique (Fig. 1B). Therefore, we took the electron microscopic images of megakaryocytes after incubating the cells in the external solutions containing no drug, 3 mM or 0.3 mM salicylate (Fig. 3). On the membrane surface of megakaryocytes incubated in the external solution, there were a number of fine protrusions of pseudopodia (Fig. 3A), which were in the middle of the elongation or the branching process [17, 25, 35]. Some of them actually released pro-platelets (Fig. 3A), indicating the ongoing process of thrombopoiesis [17, 25, 35]. However, in megakaryocytes incubated in the salicylate-containing solutions, instead of the formation these podia, there were bolder bulging structures continuously formed on the membrane surface (Fig. 3B, C). These results suggested that salicylate changed the micro-architecture of the megakaryocyte membrane surface, which was likely to be associated with the decreased pro-platelet production.

Effects of salicylate on Kv1.3-channel currents in rat megakaryocytes

Megakaryocytes predominantly express delayed rectifier K^+-channels (Kv1.3) in their plasma membranes [19, 36], and these channels play crucial roles in facilitating the calcium influx necessary to stimulate the megakaryocyte activity [36, 37]. Therefore, to determine the involvement of the channels in the effects of salicylate (Fig. 2 and 3), we applied the external solution containing 3 mM salicylate to megakaryocytes and examined the changes in the whole-cell currents (Fig. 4). Stepwise changes in the membrane potential, from the holding

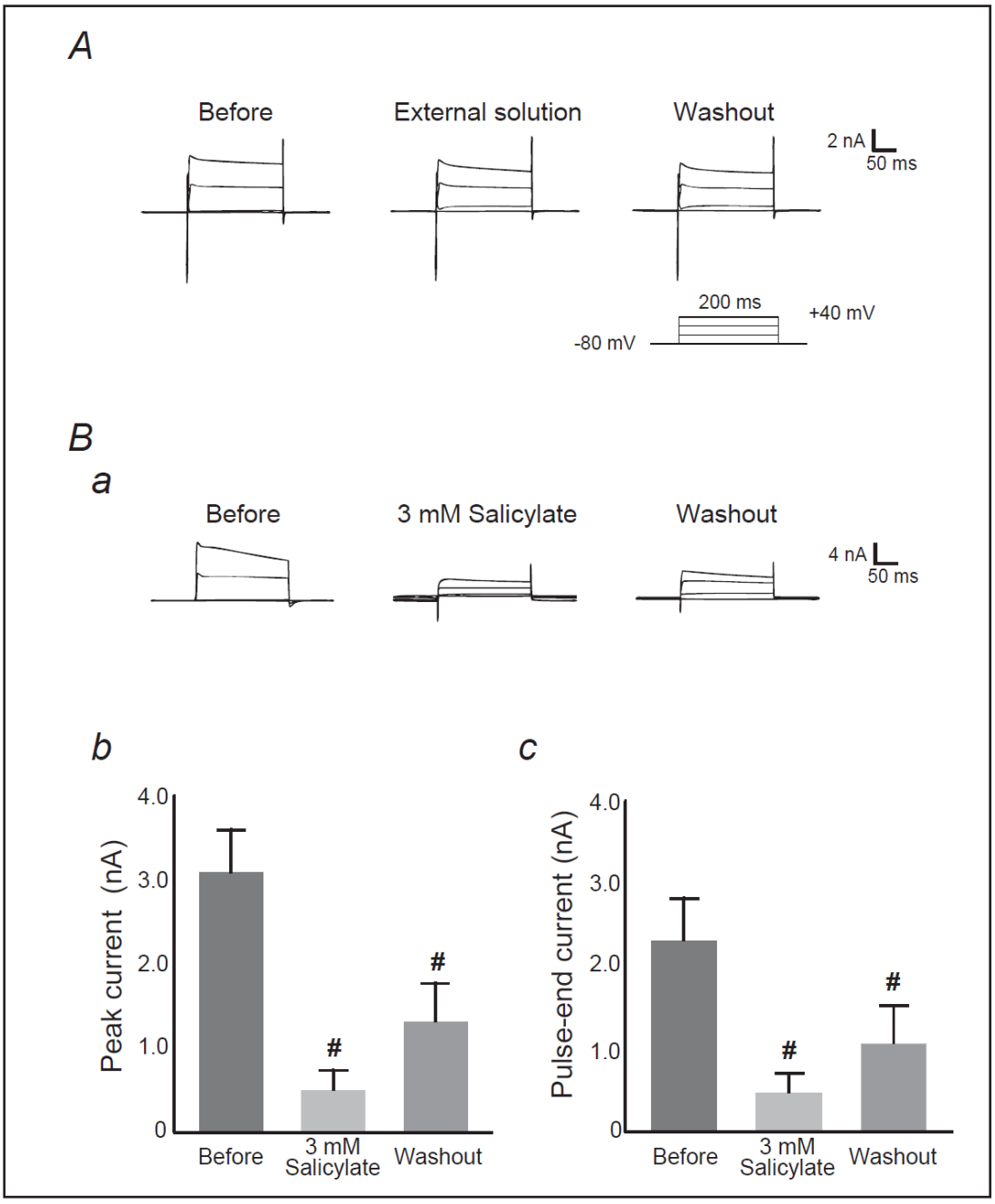

Fig. 4. Effects of salicylate on Kv1.3 channel currents in rat megakaryocytes. Effects of the external solutions containing no drug (A) and 3 mM salicylate (B). A, Ba: typical whole-cell current traces at different voltage-steps recorded before and after the reagent application. The currents were elicited by voltage-steps from the holding potential of -80 mV to -40, 0 and 40 mV, as depicted in the voltage protocol in A. Each pulse was applied for a 200-ms duration between 10-second intervals. Bb: peak current amplifications obtained from the records in a at the voltage-step of 0 mV. Bc: pulse-end current amplifications obtained from the records in a at the voltage-step of 0 mV. $^{\#}P<0.05$ vs. before the reagent application. Values are means ± SEM (n=6). Differences were analyzed by ANOVA followed by Dunnett's or Student's t test.

potential of -80 mV to the various depolarizing potential levels, evoked membrane currents in the megakaryocytes, showing a voltage-dependent activation and inactivation pattern characteristic to Kv1.3 (Fig. 4A) [19, 36]. As we previously demonstrated in thymocytes

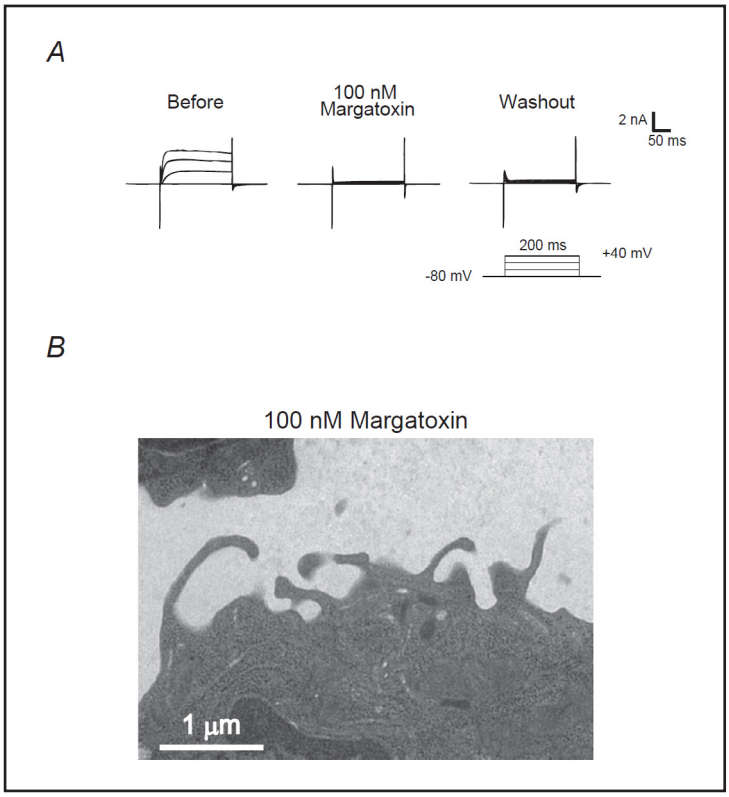

Fig. 5. Effects of margatoxin on Kv1.3 channel currents and membrane micro-architecture in rat megakaryocytes. Effects of the external solutions containing 100 nM margatoxin. A: typical whole-cell current traces at different voltage-steps recorded before and after toxin application. The currents were elicited by voltage-steps from the holding potential of -80 mV to -40, 0 and 40 mV, as depicted in the voltage protocol. Each pulse was applied for a 200-ms duration between 10-second intervals. B: a thin-section electron micrograph of megakaryocyte plasma membranes after incubating the cells in the external solution containing 100 nM margatoxin.

[2], salicylate suppressed the Kv1.3-channel currents in megakaryocytes (Fig. 4Ba). It both lowered the peak (from 3.10 ± 0.51 to 0.50 ± 0.23 nA , n=6, P<0.05, Fig. 4Bb) and the pulse-end currents significantly (from 2.31 ± 0.52 to 0.47 ± 0.23 nA , n=6, P<0.05, Fig. 4Bc). After the withdrawal of the drug, the suppressed currents remained significantly lower than those before the drug application during the observation period (Fig. 4Bb, c). However, consistent with the results obtained from the capacitance experiments (Fig. 2B), the currents tended to recover (Fig. 4Ba), which finally approached the baseline levels with longer washout periods (data not shown). These results indicated that salicylate reversibly suppressed the Kv1.3-channel currents in megakaryocytes when it modulated the thrombopoietic activity of the cells.

Effects of margatoxin on membrane micro-architecture of megakaryocytes

To reveal the actual contribution of the Kv1.3-channels to the decreased thrombopoietic activity in megakaryocytes, we examined the effects of margatoxin, a selective Kv1.3-channel inhibitor, on the membrane micro-architecture of megakaryocytes (Fig. 5). As previously demonstrated in murine megakaryocytes or thymocytes [20, 36], 100 nM margatoxin almost totally abolished the Kv1.3-channel currents in rat megakaryocytes (Fig. 5A). However, as shown by electron microscopy, margatoxin did not affect the elongation or the branching process of megakaryocytes, well preserving the fine protrusions of pseudopodia on the membrane surface (Fig. 5B). The results indicated that the channel inhibition alone did not contribute to the decreased thrombopoietic activity by salicylate.

Discussion

In megakaryocytes, invagination of the plasma membrane takes place during one of the initial steps of thrombopoiesis, as it provides the extracellular space necessary for the proplatelet formation [25-27]. In the present study, however, observation by DIC microscopy or the fluorescent imaging technique could not provide morphological evidence that salicylate

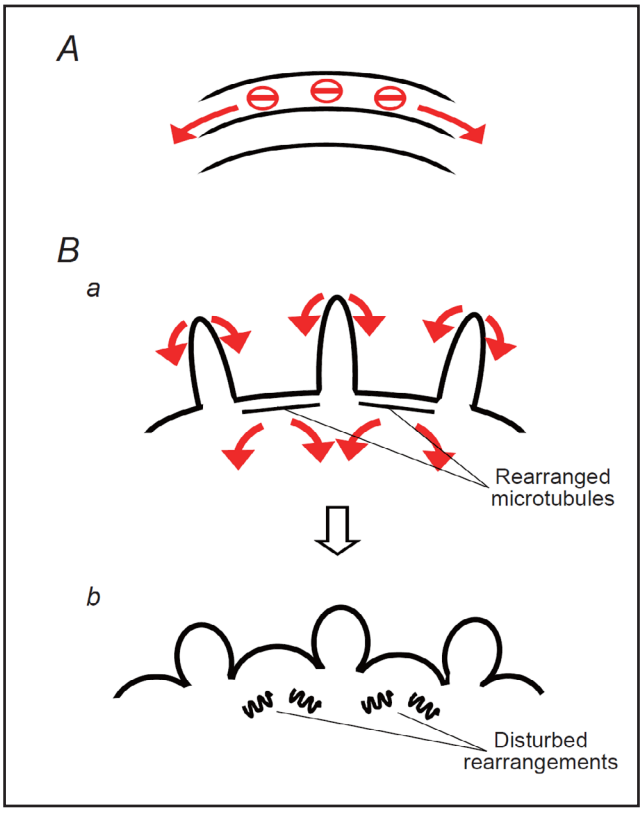

Fig. 6. Illustrations of the salicylate-induced plasma membrane curvature and the proposed effects on thrombopoiesis in megakaryocytes. A: Negatively charged salicylate is partitioned preferentially into the outer leaflet of the lipid bilayers. It induces outer leaflet expansion and generates outward membrane bending. B: On the surface of the pseudopodia, which protrude from the megakaryocyte membrane during thrombopoiesis (a), such induced outward membrane stretch (red arrows) would mechanically widen the heads of the podia (b), thereby halting their further elongation or the branching process required for the pro-platelet release. On the flat parts between the pseudopodia (a), the outward stretch of the membranes (red arrows) would generate the bold bulging structures on the membrane surface (b) and mechanically disturb the cytoskeletal rearrangements required for the pro-platelet formation (b).

affected the membrane invaginations in megakaryocytes (Fig. 1B). On the other hand, a whole-cell Cm, which can be measured by the electrophysiological method, is mathematically calculated from a parallel-plate capacitor formula: $Cm = \varepsilon A/d$, where ε is the dielectric modulus of the plasma membrane; A, the membrane surface area and d, the membrane thickness [38]. Assuming that ε and d are relatively constant under a physiological condition, the changes in Cm are primarily considered to be ascribable to the changes in A [39]. Therefore, as previously demonstrated in our study [19], the value of specific Cm in megakaryocytes reflects the number of invaginated plasma membranes as a result of thrombopoiesis [18, 28]. In the present study, using this electrophysiological approach, we demonstrated for the first time that salicylate decreased the number of invaginated folds in the megakaryocyte membranes (Fig. 2). The findings suggested that salicylate may directly inhibit the process of thrombopoiesis, showing an additional mechanism in the drug-induced thrombocytopenia.

As shown by the electron microscopy (Fig. 3), salicylate actually induced changes in the membrane micro-architecture of megakaryocytes. In many types of secretory cells, the exocytotic process can be modulated by mechanical stimuli, such as changes in the membrane tension, shear stress, hydrostatic pressure and compression [40]. Therefore, the salicylate-induced changes in the membrane micro-architecture of megakaryocytes may also contribute to its inhibitory effect on the thrombopoiesis. As we schematized in Figure 6A, salicylate, a negatively charged membrane amphipath, is partitioned preferentially into the outer leaflet of the lipid bilayers. It induces outer leaflet expansion and generates outward membrane bending [15] (Fig. 6A). On the surface of the pseudopodia, which protrude from the megakaryocyte membrane during thrombopoiesis (Fig. 6Ba), such induced outward membrane stretch (red arrows) would mechanically widen the heads of the podia (Fig. 6Bb), thereby halting their further elongation or the branching process required for the pro-platelet release. On the flat parts between the pseudopodia (Fig. 6Ba), the outward stretch of the membranes (red arrows) would generate the bold bulging structures on the membrane surface (Fig. 6Bb) and mechanically disturb the cytoskeletal rearrangements required for the pro-platelet formation (Fig. 6Bb), which include the disassembly of centrosomes and the

translocation of microtubules to the cellular cortex [26, 35, 41].

In the present study, salicylate induced the inactivation of Kv1.3-channel currents on a slower time scale than that before the drug application (Fig. 4Ba). This represented a "C-type inactivation" pattern in kinetic studies [42], suggesting that salicylate induced a conformational collapse of the selectivity filter (inactivation gates) within a pore-forming domain of the K^+-channel [20]. Since salicylate is amphiphilic [15], it tends to accumulate between the lipid bilayers of the plasma membrane after its penetration [43]. Then, in addition to disturbing lipid-lipid interactions, the accumulated salicylate may directly perturb the composite domains of the channels from inside the membrane. Previously, we actually measured the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse megakaryocytes and revealed the physiological mechanisms of its regulation [37]. Recently, McCloskey et al. further demonstrated in mouse megakaryocytes and platelets that Kv1.3-channels expressed in these cells regulate agonist-evoked Ca^{2+} increases and influence the circulating platelet counts [36]. In the present study, we did not directly examine Ca^{2+} influx in response to K^+ efflux through the channels. However, the previous findings strongly indicate that the $[Ca^{2+}]_i$ in megakaryocytes is determined by the changes in the outward K^+ currents. In the present study, since salicylate markedly suppressed the Kv1.3-channel currents in megakaryocytes (Fig. 4B), the $[Ca^{2+}]_i$ was thought to be decreased in the cells. In cultured megakaryocytes, adenosine diphosphate (ADP)-induced spreading or shape changes, which are deeply associated with the pro-platelet formation [26, 35, 41], were primarily dependent on the Ca^{2+} signaling [44]. Therefore, the decrease in the $[Ca^{2+}]_i$ in megakaryocytes may also have additional effects on the salicylate-induced inhibition of thrombopoiesis. In the present study, however, since a selective inhibition of the Kv1.3-channels alone did not affect the membrane micro-architecture of megakaryocytes (Fig. 5), the contribution of the channels to the decreased thrombopoietic activity was thought to be small.

In summary, this study demonstrated for the first time that salicylate may inhibit the process of thrombopoiesis in megakaryocytes, as detected by the decrease in the membrane capacitance. The salicylate-induced changes in the membrane micro-architecture are thought to be responsible for its effects.

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Disclosure Statement

The authors declare no conflicts of interest.

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