Acetylsalicylic acid enhances purinergic receptor-mediated outward currents in rat megakaryocytes

José P. Young,1 Jacob Beckerman,2 Stefano Vicini,1 and Adam Myers1

Departments of1 Physiology and Biophysics and 2 Biology, Georgetown University, Washington, District of Columbia

Submitted 17 September 2009; accepted in final form 23 December 2009

Young JP, Beckerman J, Vicini S, Myers A. Acetylsalicylic acid enhances purinergic receptor-mediated outward currents in rat megakaryocytes. Am J Physiol Cell Physiol 298: C602–C610, 2010. First published December 30, 2009; doi:10.1152/ajpcell.00422.2009.— Purinergic receptor activation increases cytosolic Ca2+ concentration in a fluctuating fashion, triggering oscillatory outward Ca2+-activated K+ currents in rat megakaryocytes (MKs). Whole cell and nystatin-perforated patch-clamp techniques were used to analyze changes in ionic conductance in MK with acetylsalicylic acid (ASA), a cyclooxygenase inhibitor, and ADP, a cyclooxygenase-1 inhibitor and antithrombotic agent. MKs are a model for platelet reactivity, particularly in ASA treatment failure (ASA resistance). Freshly isolated MKs were incubated 30 min in the absence or presence of 1 mM ASA. Using a K+-rich internal solution, we recorded outward currents in response to 10 μM ATP, 10 μM ADP, and 5 μM 2-methyl-thio-ADP (2MeSADP) in the voltage-clamp mode. Agonist-induced currents decreased in amplitude over time, but this decline was attenuated by ASA in both continuous and repeated agonist challenge, indicating increased MK reactivity with ASA treatment. In separate experiments, heterologous desensitization was observed when MKs were stimulated with ADP after exposure to a thromboxane receptor agonist (U46619), indicating cross talk between thromboxane and purinergic pathways. Different cells, treated with ASA or MRS2179 (P2Y1 receptor antagonist), were stimulated with 2MeSADP. The dose-response curve was shifted to the left in both cases, suggesting increased MK reactivity. ASA also caused an increased interval between currents (delay). ASA attenuated desensitization of purinergic receptors and increased delay, again suggesting cross talk between purinergic and thromboxane pathways. These findings may be relevant to ASA resistance, because individual variations in sensitivity to the multiple effects of ASA on signaling pathways could result in insensitivity to its antiplatelet effects in some patients.

platelets; patch clamp; ADP; ATP; thromboxane

CARDIOVASCULAR DISEASE is the leading cause of morbidity and mortality in the United States and developed countries (17, 54). Platelet increased reactivity is a key factor in the development of stroke and ischemic heart disease. Therefore, platelets have become a target for pharmacological treatment of cardiovascular complications after percutaneous coronary intervention (angioplasty and stent implant) (37) as well as in stroke prevention and management. Aspirin has been shown to be effective in the acute phase of recurrent stroke (49), whereas purinergic receptor blockade in the CAPRIE study showed more effectiveness than aspirin with lower side effects such as bleeding (1). The use of drug-eluting stents and dual therapy of more effectiveness than aspirin with lower side effects such as bleeding occurrence (47, 57). Common platelet evaluation tests demonstrate that patients on aspirin or clopidogrel treatment often have inadequate response to these drugs, which has been termed “resistance” (21, 42, 57, 58). However, few studies have been performed on the electrophysiological characteristics of these anucleated formed elements because of methodological limitations primarily related to platelet size. The megakaryocyte (MK), the large cellular precursor for platelets in the bone marrow, contains all the metabolic components present in platelets and has similar sensitivity to drugs such as aspirin (8, 11, 36, 52, 53). Platelets and MKs both exhibit ionic currents and changes in shape and adhesiveness after being activated by ADP, ATP, thromboxane, collagen, thrombin, and fibrinogen via increases in cytosolic Ca2+ concentration ([Ca2+]i) (19, 23–25, 32, 33, 52). Several sources and mediators have been identified in Ca2+ signaling in platelets (24, 31, 43, 44). Summarizing the results of multiple studies, the compartmentalized model of [Ca2+]i regulation pathways in platelets and MKs consists of a G protein-mediated, protein kinase C (PKC) regulation by phospholipase Cβ (PLCβ) followed by calcium release (stored in the dense tubular system) activating the phosphoinositide (PI) cascade (10, 43). The resulting increase in [Ca2+]i, activates phospholipase A2 (PLA2) and increases thromboxane A2 (TXA2) production (22). Because many different pathways converge upon Ca2+ release and storage in platelets, it has been difficult to distinguish the specific role of each pathway and how they interact with one another.

Two groups of purinergic (P2) receptors have been reported in MKs: the ATP receptor P2X1 has been characterized in mouse and rat (20, 34, 46, 52, 56), whereas two distinct types of P2Y receptors have been found, P2Y1 and P2Y12 (16, 35, 46, 52). P2 receptor agonists have been reported to induce distinct ionic currents in patch-clamp studies of mammalian MKs, depending on the agonist used and the species considered (23, 24, 33, 46, 56). For example, in the guinea pig and rat, ADP induces repetitive outward potassium currents characterized by a time-dependent rapid decay in amplitude explained by receptor desensitization (23, 24, 46, 53). In addition ADP-induced inward transient currents have also been reported in voltage-clamp studies of mouse MKs and human platelets using KCl internal solution at a holding potential (Vh) of −42 mV (24, 33). ATP-induced inward transient currents in rats and mouse MKs are sensitive to P2X blockers that reveal a Ca2+-dependent K+ outward current (20, 46, 53). Ionic currents in the MK show “considerable intercellular variation” (36), with the presence of inward current quite rare in MKs from rats (23), as was the case in our study. Studies using fluorescent dyes have shown the basal [Ca2+]i to be near 0.1 μM and the resting membrane potential in platelets to be around −60 mV (30–32). Cytosolic [Ca2+]i ([Ca2+]i) becomes elevated both in

Address for reprint requests and other correspondence: J. P. Young, 3900 Reservoir Rd., 228 Basic Science Bldg., Dept. of Physiology and Biophysics, Georgetown Univ., Washington, DC 20057 (e-mail: jpy9@georgetown.edu).
The TXA2 pathway in platelets is sensitive to COX-1 inhibition by aspirin (ASA) because it is sensitive to Gq and Gi, respectively, and consequent to ATP binding to the P2X1 receptor that directly gates a Ca²⁺/Na⁺ channel (2, 23, 24, 34, 46, 47, 52). However, ATP binds to the P2Y1 and P2Y₁₂ receptors and ADP binds to the P2X1 receptor with lower affinity, preventing the use of these agonists to definitively determine specific receptor activation (46). Stimulation by these and more selective agonists results in homologous desensitization, a decrease in receptor sensitivity as a result of the activation of a separate receptor pathway, has also been shown in studies with platelet aggregation using purinergic (P2) receptor agonists (3).

Acetylsalicylic acid (ASA) has been used to treat patients with a clinical history of platelet-related cardiovascular complications (60). In vitro studies have been performed with a broad range of ASA concentrations, from 5 µM to 4 mM (41). The TXA2 pathway in platelets is sensitive to COX-1 inhibition by ASA leading to less reactive platelets and reduced episodes of thrombosis (9). In some patients, however, ASA therapy fails to increase bleeding time or protect from thrombotic complications. Such “ASA resistance” is considered a treatment failure (21, 42). A recent study has shown cross-desensitization between ADP and thromboxane receptor (TP) signaling in human platelets indicating that changes in the activation state of one pathway may have effects upon the sensitivity of the other receptor system (3). Taking advantage of the MK model of platelet reactivity, we performed whole cell and the nystatin-perforated voltage-clamp experiments to investigate the effect of ASA on purinergic agonist-induced outward currents and their possible involvement in variability of platelet responses and the mechanism of ASA resistance. We describe changes in amplitude and time course of these currents in MKs with ASA treatment. Our results offer new insights into the role of the P2 and TXA2 pathways in [Ca²⁺], regulation and suggest a possible model of how these pathways interact with one another.

MATERIALS AND METHODS

Materials. ADP, ATP, ASA, 2-methyl-thio-ADP (2MeSADP) 9,11-dideoxy-11e,9e-epoxymethanoprostaglandin F₂₀ (U46619), indomethacin, and nystatin were all from Sigma-Aldrich (St. Louis, MO); 2’-deoxy-5’-methyladenosine 3’,5’-bisphosphate tetrasodium salt (MRS2179) was from Tocris Bioscience (Ballwin, MO). For most drugs stock solutions were prepared in distilled water and diluted as needed. Nystatin and MRS2179 were prepared fresh and diluted in DMSO.

MK isolation. Cells were collected from ~6-wk-old male Sprague-Dawley rats (Harlan Laboratories, IN), decapitated after complete anesthesia with 2,2,2-tribromoethanol, 240 mg/kg body wt ip, in a protocol approved by the Georgetown University Animal Care and Use Committee. Marrow containing MKs was isolated from the femoral bones (41) into extracellular recording solution (ES) containing (in mM) 145 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 n-glucose, adjusted to pH 7.2–7.3 with NaOH. We used apyrase (0.32 U/ml) in some experiments early; no difference was observed in terms of amplitude and duration of the outward currents as has been previously reported in some papers (23, 53). Experimental cells were incubated in 1 mM ASA or indomethacin 10 µM throughout (diluted in ES) for at least 30 min, and control cells were incubated in ES. An aliquot (100 µl) of cell suspension was plated directly onto the recording chamber, allowing 5–10 min for cell adhesion. MKs were identified by their size, the presence of several nuclei, and bright appearance with phase-contrast illumination (see Fig. 1 for examples). The experiments were carried out at room temperature (23–25°C). MKs were observed with Nikon upright or inverted phase-contrast microscopes (Tokyo, Japan), and photomicrographs were obtained with a Cohu 4912 CCD camera (Cohu, Poway, CA) using Scion software (Frederick, MD). Trypan blue exclusion test of cell viability was performed. Briefly, a sample of marrow tissue was suspended in 1 ml of extracellular solution and cells were dispersed by repetitive pipetting. One part of the cell suspension was mixed with one part of 0.4% Trypan blue, allowing 3 min for incubation. Living cells were unstained and appeared clear whereas dead cells were darkly stained (Fig. 1A) (28).

Electrophysiological studies. The nystatin-perforated patch-clamp technique was used in most experiments to record electrical activity in the MKs in the voltage-clamp mode, −40 mV holding potential, using an Axopatch 1-D amplifier (Molecular Devices, Sunnyvale, CA). To compare with previous studies (23, 46, 50) we also used the conventional whole cell configuration. Recording electrodes were pulled on a vertical pipette puller (Narishige, PP-83, Tokyo, Japan) from borosilicate glass capillaries (Witeg II, Drummond, Broomall, PA) and had resistance from 3 to 6 MΩ. The Clampex 9.2 software (Molecular Devices) was used for data acquisition with a previous junction potential correction. The internal pipette solution was modeled from Ref. 53 and had the following composition (mM): 150 KCl, 10 HEPES, pH 7.2–7.3 adjusted with NaOH. The extracellular recording solution had the same composition of that used for MK isolation listed above. ATP and ADP were applied by using a Y tube system into a chamber fed by gravity with ES (39). Applications were done soon after the whole cell configuration, or substantial decreases in access resistance were detected in the perforated recording mode and record-
Fig. 2. Representative traces illustrating variable responses of distinct MKs to ATP recorded with nystatin-perforated patch clamp. Traces shown are from 3 different experiments in which 10 μM ATP was applied during the time indicated by the horizontal bar at the top. The internal solution contained (in mM) 150 KCl, 10 HEPES, pH 7.2–7.3; holding potential (Vh) = –40 mV. A: ATP-induced oscillatory outward currents displaying gradual decrease in amplitude (desensitization). B: single current response in another MK. C: lack of response in a third example cell.

The desensitization of currents produced by the purinergic (P2) receptor activation in MKs has been reported to be fast followed by a time-dependent recovery (3, 4, 16, 20). Therefore, we examined current amplitude during the first 30 s of agonist applications. Amplitude of the initial ATP-induced current was significantly higher in the ASA-treated MKs (control 137 ± 57 pA, n = 11; ASA 289 ± 103 pA, n = 10; P < 0.05). In contrast, ADP-induced currents were not different between control and ASA-treated MKs (control 316 ± 262 pA, n = 13; ASA 328 ± 317 pA, n = 13). The normalized average amplitude of ADP- and ATP-induced outward currents were plotted over time (Fig. 3, B and C). The amplitude of the currents in the control group (not exposed to ASA) exhibited a natural decay over time in many cells, as illustrated (Fig. 3A, top left) and previously reported. In contrast, MKs previously incubated with ASA, displayed a trend to lower decay in the ATP-stimulated cells (Fig. 3C) but not in those stimulated with ADP (Fig. 3B). Current amplitude was significantly higher in the ASA-treated MKs after ATP stimulation at both the 10- and 20-s time points (P < 0.05). Nevertheless, no difference in current amplitude decline was observed between control and ASA-treated cells after ADP stimulation.

We also studied the changes in 10 μM ATP or 10 μM ADP evoked outward currents by repeating the 30-s exposures at 1-, 3-, and 5 min intervals after the initial application (Fig. 4) The amplitude of the first response of the oscillatory currents

<table>
<thead>
<tr>
<th>Control</th>
<th>OSC</th>
<th>SP</th>
<th>NR</th>
<th>Control</th>
<th>OSC</th>
<th>SP</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>60</td>
<td>27</td>
<td>13</td>
<td>62</td>
<td>23</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>83</td>
<td>11</td>
<td>6</td>
<td>53</td>
<td>41</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Percentage of MK displaying activity under control and experimental conditions in response to ADP and ATP 10 μM stimulation

Megakaryocytes (MKs) were studied by using the nystatin-perforated patch-clamp configuration. Holding potential (Vh) = –40 mV. Control and experimental cells were stimulated with 10 μM ADP or 10 μM ATP. Cells were grouped according to agonist and response: OSC, oscillatory currents; SP, single-peak currents; NR, no response. ASA, acetylsalicylic acid. Values represent percent of total N cells.
showed some decline with time in control conditions. In the presence of 1 mM ASA, however, the outward current was larger at each interval considered and increased progressively with time. As illustrated in Fig. 4, B and C, the amplitude of each subsequent response at the 1-, 3-, and 5-min intervals normalized to that of the initial current became significantly different between control and ASA treatments (P < 0.05) after 5 min of recovery time in the ATP experiment and at both the 3- and 5-min periods in the ADP experiment (P < 0.01 and P < 0.05, respectively).

To further investigate the individual roles of the P2Y and P2X pathways, the P2Y1 and P2Y12 agonist 2MeSADP was used in the same protocol as that used in Fig. 4. With this agonist, in conventional whole cell recordings, the decay in the outward oscillating current amplitude was remarkable in the agonist. In conventional whole cell recordings, the decay in the outward current amplitude was significant during continuous 10 μM ATP exposure in control cells (left) and cells exposed to 1 mM acetylsalicylic acid (ASA; right). B and C: effects of ASA on the progressive decay of outward current amplitude at distinct time points during the first 30 s of continuous application of ADP (B) and ATP (C). Each value represents the percent change of normalized current amplitude, setting the first peak amplitude as 100%. Values are means ± SE for 10 μM ADP: control n = 8, ASA n = 9; 10 μM ATP: control n = 9, ASA n = 8. *P ≤ 0.05.

The TXA2 pathway is sensitive to COX-1 inhibition by ASA, and we observed effects of ASA on P2-induced currents. Thus we investigated a possible heterologous desensitization between TXA receptor activation and subsequent P2 responses in an additional set of cells. In 58% of MKs (n = 19) exposed to the TXA2 agonist U46619 at a concentration of 2 μM for 30 s, we measured oscillatory outward currents upon subsequent exposure to ADP. The remaining cells exhibited single peak responses. These results were not different from those obtained in the control group in this cell set exposed to ES, of which 57% (n = 7) of cells responded to ADP with oscillatory currents. The initial response was selected to study changes in the current amplitude over time. The time-dependent decrease in the δ-induced outward current amplitude was significantly greater in the U46619-treated cells compared with control (Fig. 6A). Strikingly, after exposure to 2 μM U46619 and 10 μM ADP, significant differences were observed compared with control cells. Furthermore, even after a thorough washout for a period of 5 min, none of the 11 cells tested were able to respond to ADP whereas the control group responded (n = 7). In contrast, MKs stimulated with 10 μM ATP did not show significant differences between control and experimental cells (n = 4 and 3, respectively), with most MKs responding even 5 min after the last stimulation with ATP. These results after exposure to the selective agonist of the TXA2 pathway suggest that this pathway is heterologously related to that activated by ADP but not by ATP.

The increased amplitude of the 2MeSADP-induced outward currents indicates a possible change in sensitivity of MKs to P2Y agonist after exposure to ASA. We therefore investigated
reported for oscillatory outward potassium currents in rat MKs (23, 53). These outward currents have been shown to be produced by the opening of Ca$^{2+}$-activated K$^+$ channels, reflecting variations in [Ca$^{2+}$], (24, 35). Current oscillations displayed a time-dependent decrease in amplitude that could in part be described as desensitization (Figs. 3 and 4) (46, 58) and exhibited a wide variation in amplitude in both control and experimental MKs. The variability could be accounted for by the diversity in the degree of maturation of the cells studied; it might also be related to the recently reported variability in stromal interaction molecule 1 (STIM1) translocation next to the Orai1 channel in the plasma membrane and Ca$^{2+}$ leak currents through the store-operated Ca$^{2+}$ entry (SOCE) system reported to be present in MKs (5, 51, 55).

Our results show that pretreatment with ASA decreases desensitization during continuous exposure to the agonist and subsequent purinergic challenge following recovery (Figs. 3–5). The 30-s protocol of continuous agonist exposure demonstrated that ASA treatment results in higher ATP-induced normalized currents (Fig. 3C) with significance at the 0-, 5-, and 20-s time points for nonnormalized responses (data not shown) and indicates a similar trend for ADP-induced currents as raw data although not significant. A possible mechanism for this observation involves the proposed model of ASA action as

![Image](http://apjcell.physiology.org/)
antiaggregant (59). This model proposes that direct activation of P2 receptor causes a priming resulting in \([\text{Ca}^{2+}]_i\) increase. However, this is not sufficient to induce secondary aggregation and secretion. The critical factor for aggregation and secretion is the production of TXA2 that independently activates PLC and diacyl glycerol production. This in turn activates a PKC-dependent aggregation and secretion. Thus ASA treatment of platelets inhibits aggregation and secretion by blocking TXA2 production. We speculate that in MKs this secondary step, involving TXA2, may lead to a physiological feedback that decreases priming \(\text{Ca}^{2+}\) oscillations. Thus interfering with TXA2 production may allow \([\text{Ca}^{2+}]_i\) to remain in the priming oscillatory range.

An alternative possibility to explain desensitization of outward current and the effects of ASA is that the release of ATP and ADP from dense granules, resulting from activation, leads to excessive purine exposure and homologous desensitization. Thus ASA inhibiting ATP and ADP released from dense granules would prevent P2 receptor desensitization. Our results showed that ATP-induced currents were significantly higher under ASA treatment whereas ADP-induced currents were not (Fig. 3). This difference is not likely to be indicative of differences in ASA action between the P2X and P2Y receptors as the ATP and ADP solutions used were not pure and usually contained both compounds (12). More probable is that the agonistic profile of the ATP solution is such that it elicits a greater response from the P2Y pathway than the ADP solution, at least with the 30-s continuous application paradigm.

Desensitization was also observed with applications of agonist using the 0-, 1-, 3-, 5-min protocol. This was attenuated by ASA treatment. However, in this time course, the ADP-evoked current was more strongly affected by ASA treatment with two different points (3 and 5 min) whereas ATP-induced currents were significantly affected only at 5 min (Fig. 4). Because cross activation between the P2 agonists used and the receptors studied has been shown (52), and again because of the impurity in the ATP and ADP solutions, more specific agonists were necessary to study the individual roles of each of these receptors. Therefore, the repeated-exposure protocol was performed with the P2Y-nonspecific agonist 2MeSADP to study the P2Y pathway. As expected, the results were very similar to those with ADP exposure (Fig. 4 and 5), strongly supporting a role for P2Y receptors. The decline of the response with repeated application may be due to previously discussed
mechanisms. However, desensitization at longer time points may be also caused by intracellular changes such as temporary depletion of Ca\(^{2+}\) stores, uncoupling of the receptor from signaling molecules, PKC-mediated receptor internalization (40), or potassium current-related changes. Our results would rather support the hypothesis for a major role of TXA\(_2\) in decline of P2-induced current since similar results were observed with indomethacin (Fig. 5). We speculate that TXA\(_2\) has an inhibitory effect on the P2Y pathway because blocking the production of TXA\(_2\) with ASA increased P2Y receptor sensitivity in dose-response studies of outward current in MKs. To better understand how the TP pathway heterologously affects the P2 pathway, we used the TP receptor specific agonist U46619. We found a similar result in MKs: under the 0, 1, 3, and 5 min protocol, MKs exhibited significantly lower ADP-induced currents after 30 s of exposure to 2 \(\mu\)M U46619 than control cells (Fig. 6). Indeed, platelet aggregometry studies have recently reported that TP stimulation results in desensitization of the P2Y pathway (3, 7, 26).

In our studies with the TP agonist U46619 we observed that the desensitization to ADP was striking whereas ATP-induced currents were not affected. At both the 1- and 3-min time periods, ADP-induced currents were significantly lower in amplitude after U46619 exposure, and most notably no cells responded at the 5-min time point (Fig. 6). These results in combination with those shown in Fig. 5 indicate that the TP pathway heterologously desensitizes the P2Y pathway. Thus we conclude that by using ASA and indomethacin to block COX-1 and COX-2, highly expressed in MKs (6, 45), and therefore the production of TXA\(_2\), desensitization of the P2Y pathway is attenuated. TP activation leads to increased [Ca\(^{2+}\)]\(_i\) with PKC activation and decreases the response to ADP. When ATP was used as an agonist, less desensitization was observed, whereas the TP agonist also fails to change the response. This evidence further supports the hypothesis for a direct profile for ATP and ADP on P2Y pathway.

We also analyzed the dose-dependent activation of outward potassium current by measuring the response to a specific P2Y receptor agonist, 2MeSADP. To better understand the individual roles of the P2Y\(_1\) and P2Y\(_{12}\) receptors, we compared this dose response to that observed in the presence of MRS2179 to specifically block P2Y\(_1\) receptors. The maximal 2MeSADP-induced current recorded from MKs incubated in ASA and those exposed to MRS2179 were significantly higher than control (Fig. 7A). Interestingly, the current amplitude increased in both cases to about the same extent. Additionally, dose-response curves for 2MeSADP were obtained for control, for ASA treated cells, and in the presence of MRS2179 (Fig. 7B). These three curves together indicate that P2Y\(_1\) receptor activation in control conditions decreases sensitivity to the P2Y agonist and that a similar effect is seen with ASA treatment.

Comparing the control and MRS2179 dose response, we observed an increase in both efficacy and potency in the presence of drug (Fig. 7B). Thus blocking the P2Y\(_1\) receptor apparently resulted in greater and more sensitive activation of the P2Y pathway with 2MeSADP. However, we cannot definitively conclude that the P2Y\(_1\) receptor is inhibitory because of the confounding effect that desensitization has during measurement of the dose response in changing subsequent responses to increasing agonist doses in the same cell studied.

Nevertheless, when the P2Y\(_1\) receptor was blocked the maximal current amplitude was larger than control, suggesting that selective activation of P2Y\(_1\) receptor has an inhibitory role, perhaps via desensitization. Importantly, we observed that the 2MeSADP dose response was changed in a very similar way by ASA and the P2Y1 antagonist. This leads us to speculate that the mechanisms involved may be similar and may relate to desensitization occurring when P2Y\(_1\) and P2Y\(_{12}\) receptor are both active. This hypothesis is supported by previous reports on homologous desensitization of the P2Y1 receptor in platelets caused by PLC\(\beta\) inhibition by PKC (13, 40). The similarity between the MRS2179 and the ASA-treated maximal amplitude current values (Fig. 7A) suggests that ASA treatment might be preventing a PKC-regulated P2Y1 inhibition, increasing the amplitude of maximal currents similarly to MRS2179.

In addition to current amplitude, the delay and \(I_{\text{period}}\) of purine-induced outward currents were also studied. Although \(I_{\text{period}}\) did not change significantly after ASA and indomethacin treatment, 2MeSADP-induced oscillatory currents displayed a significantly higher average delay after ASA and indomethacin (Fig. 8). This increase in delay seems consistent with lower reactivity related to the “priming” mode of MK activation. At the same time, because ASA and indomethacin treatment did not affect the \(I_{\text{period}}\), it is unlikely that Ca\(^{2+}\) exit from the cytosol is affected. \(I_{\text{period}}\) is indicative of the time that [Ca\(^{2+}\)]\(_i\) is increased from baseline levels because outward K\(^{+}\) channels are open (46). Therefore, our data suggest that the way Ca\(^{2+}\) exits the cytosol, via sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase and Ca\(^{2+}\) ATPases, is not likely affected by COX inhibition. Rather, our results suggest an effect on the mobilization of intracellular Ca\(^{2+}\), as indicated by the increased delay under ASA and indomethacin treatment. Ca\(^{2+}\) increases in the cytosol from two sources: extracellular and from the dense tubular system. One or both of these systems may be altered by ASA in such a way that they act less frequently, but when they do act, they produce a higher concentration of Ca\(^{2+}\) in the cytosol as indicated by the increased current amplitude and delay (Figs. 3–5 and 8). Longer delay between currents during the oscillation implies more time to replenish calcium in intracellular stores. Thus, by increasing the delay, ASA treatment prevents the temporary depletion of intracellular Ca\(^{2+}\) stores that might represent an additional cause for the decrease of outward current observed in control conditions. The effect of ASA on delay may be linked to the reported acetylation of the DTS membrane with ASA (15). Entry of external Ca\(^{2+}\) to the cytosol contributes less to changes in cell reactivity, as evidenced by previous reports that the Ca\(^{2+}\) activated K\(^+\) currents occur even in Ca\(^{2+}\) free extracellular solution (24, 25, 35). The other possibility for ASA-reduced desensitization of MKs is that ASA might affect the STIM1 DTS-Ca\(^{2+}\) entry generating higher Ca\(^{2+}\)-induced K\(^+\) current amplitude. It has been reported that murine MKs express functional SOCE proteins and that the ADP-induced currents could be the result of Ca\(^{2+}\) release-activated Ca\(^{2+}\) (CRAC) and C-type transient receptor potential-6 (TRPC6) channels. ASA may generate high buffering with selective SOCE activation where ADP induces a typical CRAC channel. (51) In human platelets TRPC1-STIM1-IP3r interaction has been demonstrated suggesting a more complex system where ASA could play a role (29).
Despite the use of antiplatelet drugs, morbidity and mortality rates in cardiovascular disease continue to be high. The standard therapy to control thrombotic episodes leading to ischemic heart disease or recurrent stroke is not enough to ensure patient survival without complications such as bleeding and especially periprocedural myocardial infarction (18). A better understanding of platelet physiology will improve the development of new strategies to control platelet reactivity. Our results are a first step in elucidating ASA mechanisms with an electrophysiological approach. However, it is difficult at the present to make firm conclusion to ASA effects and resistance as further studies will be required.

In summary, using rat MKs as a model of platelet reactivity, our results provide significant insight into the actions of ASA on purinergic-induced platelet reactivity that may contribute to future understanding of aspirin control of clot formation and the ASA resistance phenomenon.

ACKNOWLEDGMENTS
We thank Drs. John Partridge and Zhanyan Fu and Megan Janssen for advice during these experiments.

GRANTS
Jose Young is supported by a National Secretariat for Science, Technology and Innovation of Panama (SENACYT) predoctoral scholarship, Panama. Jacob Beckerman is supported by the Howard Hughes Scholars Program.

REFERENCES


