Baseline and Activated Platelet Cytoplasmic Ionized Calcium in Acute Ischemic Stroke

Effect of Aspirin

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We measured cytoplasmic ionized calcium concentrations ([Ca$^{2+}$]) in aequorin-loaded gel-filtered platelets from 38 patients with acute occlusive stroke (12 treated with aspirin, 26 untreated) and 25 healthy controls. Compared with controls, baseline [Ca$^{2+}$] was higher in untreated patients (p<0.002), maximal 36–72 hours after the onset of neurologic dysfunction (p<0.0001), in those patients with as well as those without major stroke risk factors. The increase in [Ca$^{2+}$] after stimulation with 0.5 and 1.0 unit/ml thrombin (p<0.05), 2 and 4 μg/ml collagen (p<0.02), and 0.5 and 1.0 mM platelet activating factor (p<0.05) were also greater in untreated patients, but the profiles of these changes were parallel to those in controls. Even though the platelets of stroke patients are more sensitive to activation, they are functionally similar to those of controls. Aspirin treatment reduced baseline [Ca$^{2+}$] as well as thrombin- and collagen-induced [Ca$^{2+}$] changes. Platelet activating factor-induced increase in [Ca$^{2+}$] was not altered by aspirin treatment. Our results suggest that the usefulness of aspirin in stroke is limited because aspirin does not suppress platelet responsiveness to all in vivo thrombogenic stimuli. Specific platelet activating factor antagonists may prove to be useful therapeutic agents in stroke. (Stroke 1988;19:1234–1238)

One reason for the limited usefulness of aspirin in the treatment of ischemic stroke may be that aspirin does not suppress platelet responsiveness to all in vivo thrombogenic stimuli. Among the known in vivo mediators of platelet activation, thrombin, collagen, and platelet activating factor (PAF) are believed to be important. Cytoplasmic ionized calcium is the pivotal second messenger coupling platelet stimulation and response. Therefore, we studied the effect of aspirin treatment on cytoplasmic ionized calcium concentration ([Ca$^{2+}$]) following stimulation with thrombin, collagen, and PAF. Baseline and stimulated [Ca$^{2+}$] is reliably measured by loading washed platelets with aequorin, a Ca$^{2+}$-sensitive photoprotein. Using this technique, we show that the increase in [Ca$^{2+}$] following stimulation with PAF is not suppressed by aspirin treatment.

Subjects and Methods

We studied 25 healthy people (nine men, 16 women) ranging in age from 20 to 74 (mean ± SD 45 ± 14) years. These controls were free of all antiplatelet medication (aspirin, nonsteroidal anti-inflammatory drugs) for at least 10 days before the study.

We also studied 26 patients with acute ischemic stroke (13 men, 13 women) ranging in age from 24 to 88 (mean ± SD 55 ± 17) years. The patients were also free of all antiplatelet medications for at least 10 days before the study (untreated). Twelve patients had large, cortical infarcts attributed to major-vessel occlusion and 14 had small, subcortical infarcts attributed to small-vessel occlusion; the diagnosis was based on clinical examination and was supported by computed tomography, magnetic resonance imaging, or four-vessel cerebral angiography. Nine patients were studied <24 hours, nine 36–72 hours, and eight 4–35 days after the onset of neurologic dysfunction. Sixteen patients suffered from essential hypertension, one had insulin-dependent diabetes mellitus and hypertension, and nine had neither; treatment of hypertension was limited to thiazide diuretics and/or clonidine.

In addition, we studied 12 patients with acute ischemic stroke (nine men, three women) treated with 325 mg/day aspirin for 1–4 days. These aspirin-treated patients ranged in age from 39 to 82 (mean ± SD 64 ± 15) years and were studied within 96 hours after the onset of neurologic dysfunction.

Sixty milliliters of blood from the antecubital vein was collected from all subjects in the resting state...
via a 19-gauge butterfly needle into syringes containing 3.8% citrate in a 1:10 (vol:vol) dilution; no subject had had any invasive vascular procedure such as angiography before blood collection. Samples were prepared as described. Whole blood was centrifuged at 185g for 10 minutes, and platelet-rich plasma was separated. Prostaglandin E2 (PGE2) was added to the platelet-rich plasma (final concentration 1 µM), centrifuged at 1660g for 15 minutes, and the supernatant was removed. PGE2 stabilizes platelets and prevents activation during the loading process; there is no evidence that PGE2 affects [Ca2+]i.

The platelet pellet was washed in 1 ml of modified N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-Tyrode’s buffer (pH 7.3) containing 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 1 µM PGE2, and was then centrifuged at 17,400g for 8 seconds. The platelet pellet was suspended in 300 µl Ca2+-free solution containing 0.2 mg/ml aequorin, 10 mM EGTA, 2 mM MgCl2, 5 mM adenosine 5′-triphosphate (ATP), 150 mM NaCl, 5 mM HEPES, and 1 µM PGE2 (pH 7.3). EGTA/ATP make the platelet membrane permeable to aequorin. The aequorin-loaded platelet suspension was then incubated at 0° C for 1 hour and centrifuged at 17,400g for 8 seconds; the supernatant was removed and the aequorin-loaded platelet pellet was resuspended in a solution containing a high concentration of Mg2+ (0.1 mM EGTA, 10 mM MgCl2, 5 mM ATP, 150 mM NaCl, 5 mM HEPES, and 1 µM PGE2, pH 7.3), which helped repair the platelet membrane. The second suspension was incubated at 0° C for 1 hour, and then CaCl2 was added to give a final concentration of 300 µM.

The aequorin-loaded platelets were then warmed to room temperature and gel-filtered in HEPES-Tyrode’s buffer containing 1 mM Ca2+ (calcium buffer) through a 10-ml bed volume of acetone-washed Sepharose 2B. Extracellular Ca2+ is necessary for full platelet activation. The eluent (the gel-filtered aequorin-loaded platelet suspension) was collected, and the platelet count was adjusted to 7.5 x 107/ml using the calcium buffer.

Upon binding Ca2+, aequorin emits a blue luminescence that increases as the 2.5th power of the Ca2+ concentration within the physiologic range (10−7 to 10−4 M).8 This blue luminescence can be detected using a highly sensitive photomultiplier tube. A Chrono-Log PICA instrument (Haverton, Pennsylvania) that contains such a photomultiplier tube and a BBC (Model SE-120) strip chart recorder (Broomfield, Colorado) were used to measure the luminescence and hence [Ca2+]i. To estimate the rate of decay of luminescence in aequorin-loaded platelets, 7.5 x 107 platelets (1 ml) in calcium buffer were warmed 2–3 minutes at 37° C, stirred at 1200 rpm with a Teflon-coated magnetic stirrer bar, and lysed with 0.1% Triton X-100. This signal output represented the maximum luminescence (Lmax) obtainable from aequorin-loaded platelets. Lmax was measured at the beginning and at the end of the experiment (usually within 15 minutes, chart speed 3 cm/min).

The aequorin signal was calibrated as described.2,3 The binding of aequorin to Ca2+ is competitively inhibited by Mg2+; therefore, a stable intracellular Mg2+ concentration ([Mg2+]i) must be present.8 Briefly, the luminescence obtained after Triton lysis in the presence of a saturating concentration of Ca2+ (Lmax) was divided by the baseline luminescence or the luminescence obtained after stimulation (L). The logarithm of L/Lmax was compared with a calibration curve provided with each lot of aequorin, and the [Ca2+]i corresponding to 1.25 mM Mg2+ (by analogy to lymphocytes since the exact [Mg2+]i in platelets is not known) was read. More recently, using nuclear magnetic resonance spectroscopy and atomic absorption, the platelet [Mg2+]i has been measured to be 0.05–0.35 mM, which is substantially lower than that in lymphocytes.9 If these results are substantiated, then reconstruction of the [Ca2+]i calibration curve for aequorin at lower [Mg2+]i will indicate lower baseline and stimulated [Ca2+]i values than that obtained with current calibration curves.

One-milliliter aliquots of the gel-filtered aequorin-loaded platelet suspension in calcium buffer were used to measure baseline and stimulated [Ca2+]i. Each aliquot was warmed and stirred as described above. The baseline [Ca2+]i was the signal output from 1 ml (7.5 x 107 platelets) of unstimulated platelet suspension. Thereafter, 1-ml aliquots of the suspension were stimulated with thrombin of bovine origin (0.5 and 1.0 unit/ml applied in volumes of 5 and 10 µl, respectively); native collagen from equine tendon (2 and 4 µg/ml applied in volumes of 2 and 4 µl, respectively), and PAF (0.5 and 1.0 mM applied in volumes of 3 and 6 µl, respectively). Thrombin, collagen, and PAF are potent inducers of platelet activation and may play an important role in the pathogenesis of thrombosis.10 Thrombin interacts with the glycoprotein receptors GPIb and GPV on the platelet membrane and activates platelets by adenosine 5′-diphosphate (ADP)-dependent (at low concentrations) and ADP-independent (at high concentrations) mechanisms. The quaternary structure of collagen is considered critical for its interaction with platelets; the effects of collagen are believed to be mediated by both the release of ADP and the oxygenation of arachidonic acid.10 PAF (1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine), a naturally occurring phospholipid, is a potent in vivo inducer of thrombosis and is present in vascular endothelial cells and leukocytes, so it may be expected to be present in high concentrations in regions of vascular injury.11,12 PAF was stored at −70° C in aliquots of 10 mg/ml in chloroform; for each experiment an aliquot was blown dry under air and resuspended in Tyrode’s-albumin buffer. It was our intention to study maximal responses to PAF; therefore, final concentrations were chosen arbitrarily.13

Aequorin was purchased from Dr. John Blinks (Mayo Clinic, Rochester, Minnesota), thrombin from...
Parke Davis (Morris Plains, New Jersey), PAF from Avanti Polar Lipids (Pelham, Alabama), and collagen from Chrono-Log Corp. (Haverton, Pennsylvania). Sepharose 2B was obtained from Pharmacia Fine Chemicals (Piscataway, New Jersey), and Triton X-100 from Kodak (Rochester, New York). The remainder of the reagents were obtained from either Sigma Chemical Co. (St. Louis, Missouri) or Fisher Scientific (Pittsburgh, Pennsylvania).

We used Student's $t$ test to assess differences between groups for baseline $[\text{Ca}^{2+}]$. To answer the following questions we used profile analysis. First, do the groups follow the same trend over the three levels (baseline and two activated states) measured for each inducing agent (i.e., are the profiles parallel), and second, if the groups do follow the same trend, are they equal? We used analysis of variance (ANOVA) to compare baseline $[\text{Ca}^{2+}]$ between groups at different times after the onset of neurologic dysfunction.

**Results**

**Demographics**

We compared the sex distribution in the 25 controls and 26 untreated patients. There were nine (36%) male controls compared with 13 (50%) male patients ($p<0.4$, Fisher's exact test). Among the controls, the mean $\pm$ SD age of the men was $46.7 \pm 15.3$ years compared with $44.0 \pm 14.0$ years for the women ($p>0.66$, two-sample $t$ test). No significant sex difference was observed for any measure in the controls. Therefore, in further analyses sex was not considered.

We compared the age distribution in the two groups. The mean $\pm$ SD age of the controls was $45.0 \pm 14.2$ years compared with $55.4 \pm 17.2$ years for the untreated patients ($p<0.03$, two-sample $t$ test).

Repeateed-measures ANOVA using age and group as the independent variables was done to determine if age had a significant effect on the variables of interest (the inducing agents). The effect of age was not significant for any inducing agent including baseline $[\text{Ca}^{2+}]$ ($p>0.37$, $p>0.15$, and $p>0.16$, for thrombin, collagen, and PAF, respectively). Thus, in further analyses age was not considered.

**Baseline Cytoplasmic Ionized Calcium Concentration**

Mean $\pm$ SD baseline $[\text{Ca}^{2+}]$ was higher in the 26 untreated patients ($4.95 \pm 1.60 \times 10^{-6}$ M) than in the 25 controls ($3.78 \pm 0.71 \times 10^{-6}$ M, $p<0.002$) (Table 1). Compared with the 25 controls, mean $\pm$ SD baseline $[\text{Ca}^{2+}]$ in nine patients 0–24 hours following the onset of dysfunction was $4.62 \pm 1.17 \times 10^{-6}$ M ($p=0.07$), in nine patients 36–72 hours after onset was $5.75 \pm 0.54 \times 10^{-6}$ M ($p=0.0001$), and in eight patients 4–35 days after onset was $4.44 \pm 0.63 \times 10^{-6}$ M ($p=0.18$). Patients with and without major stroke risk factors had equally elevated baseline $[\text{Ca}^{2+}]$. No differences in $[\text{Ca}^{2+}]$ were observed relative to the size of the infarction. Aspirin treatment in 12 patients reduced baseline $[\text{Ca}^{2+}]$ to control levels (Table 1).

**Activated Cytoplasmic Ionized Calcium Concentration**

Using profile analysis, thrombin-induced $[\text{Ca}^{2+}]$ increases from baseline were greater in untreated patients at both concentrations ($p<0.05$) (Table 1). However, the profiles of these responses in controls and these patients were parallel ($p>0.23$). The higher $[\text{Ca}^{2+}]$ in response to thrombin in untreated patients could be accounted for by their higher

| TABLE 1. Platelet Cytosolic Ionized Calcium Concentrations in Ischemic Stroke |
|----------------|-------------------------------|----------------|----------------|----------------|
|                | Controls (n = 25) | Untreated patients (n = 26) | p*             | Aspirin-treated patients (n = 12) | p†              |
| Age (yr)       | 45 ± 14            | 55 ± 17                     |                | 64 ± 15                  | <0.008          |
| Sex (males: females) | 9:16 | 13:13                      |                | 9:3                      |                 |
| Ionized calcium concentration |                |                             |                |                          |                 |
| Baseline       | 3.78 ± 0.71        | 4.95 ± 1.60                 | <0.002         | 3.83 ± 0.85              | <0.008          |
| Thrombin-induced |                  |                             |                |                          |                 |
| 0.5 unit/ml    | 6.49 ± 2.89        | 7.60 ± 2.44                 | <0.05          | 4.87 ± 0.84              | <0.0002         |
| 1.0 unit/ml    | 7.28 ± 3.51        | 9.33 ± 2.80                 |                | 5.62 ± 0.72              |                 |
| Collagen-induced |                  |                             |                |                          |                 |
| 2.0 μg/ml      | 4.69 ± 2.40        | 5.85 ± 1.62                 | <0.02          | 3.23 ± 0.86              | <0.0002         |
| 4.0 μg/ml      | 5.15 ± 2.62        | 6.76 ± 1.89                 |                | 3.55 ± 0.76              |                 |
| Platelet activating factor-induced |          |                             |                |                          |                 |
| 0.5 mM         | 47.0 ± 35.6        | 125.3 ± 183.3               | <0.05          | 35.0 ± 22.7              | >0.11           |
| 1.0 mM         | 76.7 ± 81.1        | 446.8 ± 905.3               |                | 50.4 ± 29.4              |                 |

Values are $\times 10^{-6}$ M/7.5 $\times 10^7$ platelets, mean ± SD. Student's $t$ test was used only to test for differences in baseline concentration. Using profile analysis to test for differences in induced concentrations, thrombin-, collagen-, and platelet activating factor-induced ionized calcium concentration increases were greater in untreated patients; however, profiles of their responses were parallel to those of controls ($p>0.23$, $p>0.56$, $p>0.11$, respectively).

*Untreated patients vs. controls.
†Untreated patients vs. aspirin-treated patients.
baseline \([\text{Ca}^{2+}]\). Aspirin treatment reduced the thrombin-induced increase in \([\text{Ca}^{2+}]\) (Table 1).

The effect of collagen was similar to that of thrombin. Using profile analysis, collagen-induced \([\text{Ca}^{2+}]\) increases from baseline were greater in untreated patients at both concentrations \((p<0.02)\) (Table 1). However, the profiles of these responses in controls and these patients were parallel \((p>0.56)\). The higher \([\text{Ca}^{2+}]\) in response to collagen induction in untreated patients could be accounted for by their higher baseline \([\text{Ca}^{2+}]\). Aspirin treatment reduced the collagen-induced increase in \([\text{Ca}^{2+}]\) (Table 1).

The effect of PAF was similar to those of thrombin and collagen. Using profile analysis, PAF-induced \([\text{Ca}^{2+}]\) increases from baseline were greater in untreated patients at both concentrations \((p<0.05)\) (Table 1). However, the profiles of these responses in controls and these patients were parallel \((p>0.11)\). The higher \([\text{Ca}^{2+}]\) in response to PAF induction in untreated patients could be accounted for by their higher baseline \([\text{Ca}^{2+}]\). Unlike with thrombin and collagen, aspirin treatment did not decrease PAF-induced increase in \([\text{Ca}^{2+}]\) (Table 1).

**Discussion**

Platelet \([\text{Ca}^{2+}]\) is the common pivotal second messenger governing the extent of platelet activation following agonist stimulation.\(^1\) Accordingly, changes in \([\text{Ca}^{2+}]\) may precede changes in platelet shape, adhesion, aggregation, and secretion.\(^1\) Until recently, the direct measurement of platelet \([\text{Ca}^{2+}]\) was impossible because the small size of the platelet did not permit microinjection of \([\text{Ca}^{2+}]\)-sensitive indicators.\(^2\) The synthesis in 1980 of quin-2, a \([\text{Ca}^{2+}]\)-sensitive fluorophore that diffuses across the plasma membrane, has led to better understanding of platelet \([\text{Ca}^{2+}]\) homeostasis.\(^14\) However, there are significant limitations to the use of quin-2, including the high intracellular concentrations required, low sensitivity to changes in \([\text{Ca}^{2+}]\), and alteration in platelet function caused by the loading process. Newer \([\text{Ca}^{2+}]\)-sensitive fluorophores such as fura-2 and indo-1 are brighter and produce better signal-to-noise ratios than quin-2 but are still not free of the problems seen with quin-2.\(^15,16\) Aequorin \(\left(M_r = 20,000\right)\), a photoprotein isolated from the photocytes of the jellyfish *Aequorea aequorea*, binds three moles \(\text{Ca}^{2+}\) per molecule and is simultaneously converted to apoaequorin, which emits a quantifiable blue luminescence.\(^17-19\) In 1985, Johnson et al\(^12\) nondestructively loaded platelets with aequorin by modifying a technique introduced by McClellan and Winegrad\(^8\) and others\(^6,7\): EGTA and ATP were used to induce selective permeability of the platelet membrane to aequorin, with the subsequent addition of \(\text{Mg}^{2+}\) to repair the membrane; the platelet is not "skinned" or made grossly permeable to other molecules during this procedure.\(^2,20\) Platelet cytoplasmic \(\left(^{3}\text{H}\right)\text{adenine and lactate dehydrogenase}\), dense-granule \(\left(^{14}\text{C}\right)\text{serotonin}\) and \(\alpha\)-granule \(\left(\beta\text{-thromboglobulin}\right)\) markers were retained, and serial electron micrographs confirmed the structural integrity of the platelets at all times during the loading procedure.\(^2\) Furthermore, platelet function measured by ATP secretion and aggregation was unaltered by the presence of aequorin in the platelet.\(^2,21\) Though the exact mechanism for the uptake of aequorin into platelets is unknown, aequorin is believed to be located in the cytoplasm outside the granules.\(^2\) In the measurement of local \([\text{Ca}^{2+}]\) in the cytoplasm aequorin may have an advantage over quin-2, which indicates an average \([\text{Ca}^{2+}]\) from both high- and low-\([\text{Ca}^{2+}]\) regions of the platelet.\(^2,16\) We chose the aequorin technique because platelet function was not altered following loading,\(^2,3\) and no substantial additional information was likely to be derived by simultaneously employing other fluorophores.

We used aequorin-loaded gel-filtered washed platelets in calcium buffer to study \([\text{Ca}^{2+}]\) homeostasis following the onset of cerebral ischemia. Compared with controls, baseline and stimulated platelet \([\text{Ca}^{2+}]\) were both increased in untreated patients with acute ischemic stroke. Our results are consistent with other observations of increased platelet activation during cerebral ischemia.\(^22-24\) Even though thrombin-, collagen-, and PAF-stimulated \([\text{Ca}^{2+}]\) were greater in patients, the profiles of these changes were similar to those of controls, suggesting that the changes were functionally similar. Accordingly, the higher baseline \([\text{Ca}^{2+}]\) in the unstimulated (in vitro) platelets of acute stroke patients may represent an increased sensitivity to activation following exposure to agonists. The reason for higher baseline \([\text{Ca}^{2+}]\) in stroke patients is not known. It may be caused by a plasmatic factor released secondarily during cerebral infarction. The secondary nature of platelet involvement in cerebral infarction is also supported by the fact that platelet baseline \([\text{Ca}^{2+}]\) was maximally increased only 36–72 hours after the onset of neurologic dysfunction. We found no relation between \([\text{Ca}^{2+}]\) and the size of the infarction; the converse would be expected if platelet activation were of primary importance in causing cerebral infarction. There is some evidence, based on measurements of plasma concentrations of \(\beta\)-thromboglobulin and platelet factor 4, that platelet activation does not occur in patients suffering small infarcts.\(^23\) This observation is not supported by direct measurements of platelet \([\text{Ca}^{2+}]\); patients with both large and small infarcts had increased \([\text{Ca}^{2+}]\).

Aspirin treatment decreased the effects of thrombin and collagen but did not significantly alter the effect of PAF on platelets. For unknown reasons, the effect of PAF was highly variable in both aspirin-treated and untreated patients. The mechanism by which PAF activates platelets is also not known, but it appears to involve a cyclooxygenase-independent pathway. A damaging effect on the platelet membrane is unlikely since the response to PAF was dose dependent. PAF is a potent inducer of platelet activation.\(^25-27\)
thrombosis, and ischemia and has been postulated to play an important role in the pathogenesis of microangiopathy. Therefore, suppression of PAF-induced platelet responses, in addition to those of thrombin and collagen induction, may be necessary to more effectively limit pathologic thrombosis. Accordingly, an approach to improving stroke therapy might be the use of agents that also inhibit PAF-induced platelet activation. Analogues of PAF and structurally unrelated compounds, such as kadsurenone, gingko extracts, certain triazolobenzodiazepines, and calcium channel blockers, may prove to be useful agents in the prevention of cerebral thrombosis.

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References


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