To study the pathogenesis of platelet activation in ischemic stroke, ionized calcium ([Ca\(^{2+}\)]) was measured in aequorin-loaded gel-filtered platelets in the basal and stimulated state. Basal [Ca\(^{2+}\)] was increased in stroke patients maximally 36–72 hours after onset. The increase in [Ca\(^{2+}\)] after stimulation with thrombin, collagen, and platelet-activating factor were also greater in stroke patients, but the profiles of these [Ca\(^{2+}\)] changes were parallel to control. Cross incubation of control platelets with plasma from stroke patients resulted in raised basal [Ca\(^{2+}\)] and caused the release of serotonin from platelets. These results indicate that the higher platelet basal [Ca\(^{2+}\)] in stroke patients represents a lowered threshold for activation and that this may be due to a plasmatic factor rather than a primary platelet defect. (Circulation Research 1989;65:1679–1687)
utes, and the supernatant was removed. The platelet pellet was washed in 1 ml modified HEPES-Tyrode's buffer (pH 7.3) containing 10 mM EGTA and 1 mM MgCl₂, and then centrifuged at 17,400g for 8 seconds. The pellet was washed in 300 μl Ca²⁺-free solution containing 0.2 mg/ml aequorin, 10 mM EGTA, 2 mM MgCl₂, 5 mM ATP, 150 mM NaCl, 5 mM HEPES, and 1 μM PGE₁ (pH 7.3). EGTA/ATP helped to make the platelet membrane permeable to aequorin. The preparation was then incubated at 0°C for 1 hour and centrifuged at 17,400g for 8 seconds. The supernatant was removed, and the platelet pellet was resuspended in a solution containing a high concentration of Mg²⁺ that helped to repair the platelet membrane: 0.1 mM EGTA, 10 mM MgCl₂, 5 mM ATP, 150 mM NaCl, 5 mM HEPES, and 1 μM PGE₁ (pH 7.3). The preparation was then incubated again at 0°C for 1 hour. After the second incubation, CaCl₂ was added (final concentration, 300 μM). The platelets were then warmed to room temperature and gel-filtered in HEPES-Tyrode's buffer containing 1 mM Ca²⁺ through a 10-ml bed volume of acetone-washed Sepharose 2B. The eluent (gel-filtered platelets) was collected, and the platelet count was adjusted to 7.5×10⁸/ml by use of the same buffer.

[Ca²⁺] Measurements

When aequorin binds Ca²⁺ ions, it emits a blue luminescence that increases with an increase in the Ca²⁺ concentration within the physiological range (10⁻²⁻¹⁰⁻⁴ M) by a power of 2.₃,₄ This blue luminescence can be detected by a highly sensitive photomultiplier tube. A platelet-ionized calcium aggregometer (Chrono-Log, Havertown, Pennsylvania), which contains such a photomultiplier tube, and a strip chart recorder (model SE-120, Brown Boveri, Vienna, Austria) were used to measure [Ca²⁺]. To allow estimation of the rate of decay of the aequorin luminescence in platelets, the equivalent of 1 ml platelet suspension (7.5×10⁹ platelets) in HEPES-Tyrode's buffer containing 1 mM Ca²⁺ was warmed (2-3 minutes at 37°C), stirred (1,200 rpm with a Teflon-coated magnetic stirrer bar), and lysed with Triton X-100 (0.1%). This signal output represented the maximum luminescence (Lₘₐₓ) obtainable from the aequorin-loaded platelets. Lₘₐₓ was measured at the beginning and end of the experiment (usually within 15 minutes, chart speed 3 cm/min). One millilitre (i.e., 7.5×10⁹ platelets) aliquots of gel-filtered aequorin-loaded platelet suspension in a 1 mM Ca²⁺ media (HEPES-Tyrode's calcium buffer) were used to measure basal and stimulated cytosolic [Ca²⁺]. Each aliquot was warmed and stirred as stated above. The platelet basal cytosolic [Ca²⁺] was the aequorin signal output indicated by 1 ml unstimulated platelet suspension. Thereafter, 1-ml aliquots of the platelet suspension were stimulated with two concentrations each of thrombin (0.5 and 1.0 unit/ml) applied in volumes of 5 and 10 μl, respectively; collagen (2 and 4 μg/ml) was applied in volumes of 2 and 4 μl, respectively; and platelet-activating factor (PAF) (0.5 and 1.0 mM) was applied in volumes of 3 and 6 μl.

Aequorin Signal Calibration

Calibration of the signal indicated by aequorin was carried out as previously described.₅,₆,₁₀,₁¹ The binding of aequorin to Ca²⁺ is competitively inhibited by Mg²⁺; therefore, a stable intracellular Mg²⁺ concentration ([Mg²⁺]) must be present.₁₀,₁¹ Briefly, the luminescence obtained after Triton X-100 lysis in the presence of a saturating concentration of Ca²⁺ (Lₘₐₓ) was divided by the luminescence obtained in the basal state or after stimulation (L). The logarithm of L/Lₘₐₓ was compared with a calibration curve for a [Mg²⁺] of 1.25 mM (by analogy to lymphocytes since the exact [Mg²⁺] in platelets is not known). More recently by use of nuclear magnetic resonance spectroscopy and atomic absorption, the platelet [Mg²⁺] has been measured to be between 0.05 and 0.35 mM, which is substantially lower than in lymphocytes.₁₂ If these results are substantiated, then reconstruction of the [Ca²⁺] calibration curve for aequorin at the lower [Mg²⁺] will indicate resting and stimulated [Ca²⁺] values about a magnitude lower than those obtained with current calibration curves.

Inducing Agents

Thrombin, collagen, and PAF are potent inducers of platelet activation and may play an important role in the pathogenesis of thrombosis.₁₃ Thrombin interacts with glycoprotein receptors GPIb and GPV on the platelet membrane and activates platelets by ADP-dependent (in low doses) and ADP-independent (in high doses) mechanisms. We applied thrombin of bovine origin (Parke Davis, Morris Plains, New Jersey) in final concentrations of 0.5 and 1.0 unit/ml. The quaternary structure of collagen is considered critical for the interaction of collagen with platelets. The effects of collagen are believed to be mediated both by the release of ADP and the oxygenation of arachidonic acid.₁₃ We used native collagen derived from equine tendon (Chrono-Log) in final concentrations of 2 and 4 μg/ml. PAF (1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphocholine), a naturally occurring phospholipid, is a potent in vivo inducer of thrombosis and is present in vascular endothelial cells and leukocytes; therefore, it may be expected to be present in high concentrations in regions of vascular injury.₁₄,₁₅ PAF (Avanti Polar Lipids, Birmingham, Alabama) 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-albumin buffer. It was our intention to study maximal responses to PAF; therefore, final concentrations of 0.5 and 1.0 mM were chosen arbitrarily.₁₆
Cross-Incubation Studies

Cross incubation of platelets from healthy controls with plasma from other healthy controls and acute ischemic stroke patients was carried out to test the hypothesis that a plasmatic factor caused platelet activation in acute ischemic stroke. Plasma obtained by centrifuging blood (1,660g × 15 minutes) from patients or controls was kept frozen (−70° C) for up to 30 days before study until platelets from healthy controls for these experiments became available. Seven experiments were done to measure changes in platelet basal [Ca\textsuperscript{2+}], and six experiments were performed to measure the release of serotonin from platelets.

Sixty milliliters of blood was used to obtain platelet-rich plasma from each control subject. PGE\textsubscript{1} was added, and the platelet suspension was divided into three equal portions: samples a, b, and c. The samples were centrifuged at 1,660g for 15 minutes, and supernatant was removed. The platelet pellet was resuspended as follows: sample a in 4 ml homologous plasma obtained by centrifuging blood at 1,660g for 15 minutes, sample b in 4 ml plasma from another control, and sample c in 4 ml plasma from an acute ischemic stroke patient (collected within 8 days after onset of neurological dysfunction). The samples were then incubated in a water bath (at 37° C for 45 minutes), loaded with aequorin, and processed by gel-filtration; platelet count was adjusted to 7.5 × 10\textsuperscript{9/ml} as described above. Platelet basal [Ca\textsuperscript{2+}] was measured as previously described. Subsequently, samples were centrifuged at 31,000g for 15 seconds, and the supernatant was removed and passed through a 0.22-μm syringe filter. Serotonin levels were determined by high-performance liquid chromatography with an amperometric detector (0.65 V).\textsuperscript{17,18} The system consisted of an injector (model 7125, Rheodyne, Cotati, California), a pump (model M-45, Waters Chromatography, Milford, Massachusetts), and a BAS detector (model LC-4A, Bioanalytical Systems, Lafayette, Indiana). The mobile phase, adjusted to a pH 4.0, consisted of 0.1 M sodium acetate, 0.7 M citrate, 0.4 mM sodium octyl sulfate, 0.7 mM EDTA, and 10% methanol. The retention time of serotonin was 10 minutes. The level of serotonin was determined from a standard calibration curve in ng/7.5 × 10\textsuperscript{9} platelets.

Reagents

Aequorin was purchased from Dr. John Blinks, Mayo Clinic, Rochester, Minnesota; PAF, from Avanti Polar Lipids; thrombin, from Parke Davis; and collagen, from Chrono-Log. Sepharose 2B was obtained from Pharmacia Fine Chemicals and Triton X-100 from Kodak. The remainder of the chemicals was obtained from either Sigma Chemical, St. Louis, Missouri, or Fisher Scientific, Pittsburgh, Pennsylvania.

Statistical Analyses

Student's t test was used to test differences between controls and stroke patients for basal [Ca\textsuperscript{2+}] and to test differences in basal [Ca\textsuperscript{2+}] and serotonin release after cross incubation. Profile analysis was done to answer the following questions: First, do the groups (strokes vs. controls) follow the same trend over the three different levels (basal and two activated states) measured separately for each variable (thrombin, collagen, and PAF) (i.e., are the profiles parallel)? Second, if they follow the same trend, do the groups equal each other (i.e., does one group have higher readings than the other group)? Analysis of variance (ANOVA) was used to compare basal [Ca\textsuperscript{2+}] between controls and stroke patients at different time periods after the onset of neurological dysfunction.

Results

Effect of Gender and Age

The age for the male controls was 47±15 years (mean±SD) compared with 44±14 years for the female controls (p>0.66, two-sample t test). No statistically significant gender difference was observed for any of the measures in controls. The age for the controls was 45±14 years compared with 55±17 years for the stroke patients (p<0.03, two-sample t test). An analysis was done to determine if age had a significant effect on the variables of interest. ANOVA with repeated measures using age and group as the independent variables was done. In all variables (thrombin, collagen, and PAF), each including basal [Ca\textsuperscript{2+}], the effect of age was not statistically significant (p>0.37, p>0.15, and p>0.16, respectively).

Platelet Basal [Ca\textsuperscript{2+}]

Platelet basal [Ca\textsuperscript{2+}] was higher in stroke patients compared with controls (p<0.002; Table 1). Basal [Ca\textsuperscript{2+}] was maximally increased at 36–72 hours (p<0.0001) and then began to decrease by 96 hours after onset of neurological dysfunction (Figure 1).

Platelet Activated [Ca\textsuperscript{2+}]

Thrombin-induced (final concentration, 0.5 and 1.0 units/ml; Figures 2 and 3), collagen-induced (final concentration, 2 and 4 μg/ml; Figures 4 and 5), and PAF-induced (final concentration, 0.5 and 1.0 units/ml) increase in [Ca\textsuperscript{2+}] from basal levels were greater in stroke patients (Table 1, profile analysis). The profiles of these responses paralleled each other: thrombin (p<0.23, Figures 2 and 3), collagen (p>0.56, Figures 4 and 5), and PAF (p>0.11) (Table 1).

Patients with and without hypertension had comparable levels of basal and activated platelet [Ca\textsuperscript{2+}] (Table 2). No differences were observed relative to size of infarction.
Table 1. Platelet Ionized Calcium Concentration in Ischemic Stroke: Control Versus Stroke

<table>
<thead>
<tr>
<th></th>
<th>Control (n=25)</th>
<th>Stroke (n=26)</th>
<th>p</th>
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<tbody>
<tr>
<td><strong>Basal [Ca^{2+}]</strong></td>
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<tr>
<td></td>
<td>3.78±0.71×10^{-6} M</td>
<td>4.95±1.60×10^{-4} M</td>
<td>&lt;0.002</td>
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<tr>
<td><strong>Thrombin-induced [Ca^{2+}]</strong></td>
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<td></td>
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<tr>
<td>0.5 unit/ml</td>
<td>6.49±2.89×10^{-6} M</td>
<td>7.60±2.44×10^{-4} M</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1.0 unit/ml</td>
<td>7.28±3.51×10^{-6} M</td>
<td>9.33±2.80×10^{-4} M</td>
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</tr>
<tr>
<td><strong>Collagen-induced [Ca^{2+}]</strong></td>
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<tr>
<td>2.0 µg/ml</td>
<td>4.69±2.40×10^{-6} M</td>
<td>5.85±1.62×10^{-4} M</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>4.0 µg/ml</td>
<td>5.15±2.62×10^{-6} M</td>
<td>6.76±1.89×10^{-4} M</td>
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<tr>
<td><strong>PAF-induced [Ca^{2+}]</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0.5 mM</td>
<td>4.70±3.56×10^{-5} M</td>
<td>12.53±18.33×10^{-5} M</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>7.67±8.11×10^{-5} M</td>
<td>44.68±90.53×10^{-5} M</td>
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</tr>
</tbody>
</table>

Values are mean±SD. [Ca^{2+}], ionized calcium concentration; PAF, platelet activating factor. Platelet [Ca^{2+}] in controls and acute ischemic stroke patients is shown. Basal [Ca^{2+}] was greater in stroke patients (Student's t test). Profile analysis was used to compare across concentrations. Thrombin-, collagen-, and PAF-induced [Ca^{2+}] increases were greater in stroke patients; however, the profiles of their responses were parallel to control (p>0.23, p>0.56, and p>0.11, respectively). All values are in M/7.5×10^7 platelets.

Cross-Incubation Studies

Changes in platelet basal [Ca^{2+}] after cross incubation of control platelets with homologous plasma was 3.81±1.44 nM/7.5×10^7 platelets; with plasma from other healthy controls, 4.1±1.67 nM/7.5×10^7 platelets; and with plasma from stroke patients, 5.68±2.53 nM/7.5×10^7 platelets (mean±SD, p<0.02; Figure 6). Serotonin release after cross incubation of control platelets with homologous plasma was 29.5±24.4 ng/7.5×10^7 platelets; with plasma from other healthy controls, 26.2±25.7 ng/7.5×10^7 platelets; and with plasma from stroke patients, 64.4±51 ng/7.5×10^7 platelets (mean±SD, p<0.01; Figure 7). Figure 8 gives a diagrammatic representation of the cross-incubation experiments.

Discussion

Platelet [Ca^{2+}] is the common pivotal second messenger governing the extent of platelet activation following agonist stimulation. Accordingly, changes in [Ca^{2+}] may precede changes in platelet...
**Figure 3.** Representative figures of thrombin-induced platelet cytosolic ionized calcium concentration ([Ca\(^{2+}\)]) responses. On the left are the control responses, and on the right are responses seen in stroke patients. The top recordings were obtained with a final thrombin concentration of 0.5 unit/ml, and the bottom recordings were at 1.0 unit/ml. L Max, maximum luminescence; L, luminescence.

**Figure 4.** Graph showing effect of collagen on cytosolic ionized calcium concentration ([Ca\(^{2+}\)]). Basal and collagen-stimulated changes (4 and 2 µg/ml) in platelet [Ca\(^{2+}\)] were greater in stroke patients (p<0.02, profile analysis) compared with control. The control data are shown on the left side of the figure, and the stroke data are on the right. However, the profiles of the responses were parallel in both groups (p>0.56, profile analysis), suggesting that their platelets responded similarly to activation by collagen. The higher [Ca\(^{2+}\)] levels after collagen stimulation noted in stroke patients appears to be due to a higher basal [Ca\(^{2+}\)] in their platelets. All values are in µM/7.5×10⁷ platelets.
function and morphology such as shape change, adhesion, aggregation, and secretion. Until recently, the direct measurement of platelet \([Ca^{2+}]\) was impossible because the small size of the platelet did not permit microinjection of \(Ca^{2+}\)-sensitive indicators into the cell. The synthesis in 1980 of quin 2, a \(Ca^{2+}\)-sensitive fluorophore that diffuses across the plasma membrane, has led to a better understanding of platelet \(Ca^{2+}\) homeostasis. However, there are significant limitations with the use of this indicator including the high intracellular concentrations required, a low sensitivity to changes in \([Ca^{2+}]\), and alteration in platelet function caused by the loading process. Aequorin, a photoprotein isolated from the photocytes of the jellyfish Aequorea aequorea, binds 3 moles of \(Ca^{2+}\) per molecule and is simultaneously converted to apoaequorin, which emits a quantifiable blue luminescence. In 1985, Johnson et al. loaded platelets nondestructively with aequorin \((M_r 20,000)\) by modifying a technique introduced by McClellan and Winegard: EGTA and ATP were used to induce selective permeability of

| Table 2. Platelet Ionized Calcium Concentration in Stroke: Effect of Hypertension |
|---------------------------------|---------------------------------|----------------|
|                                | No hypertension \((n=9)\) | Hypertension \((n=17)\) |
| Basal \([Ca^{2+}]\)             | 5.60±2.26\times10^{-8} M     | 4.61±1.03\times10^{-8} M | NS          |
| Thrombin-induced \([Ca^{2+}]\)  |                                |                    |
| 0.5 unit/ml                    | 8.16±1.56\times10^{-8} M     | 7.30±2.79\times10^{-8} M | NS          |
| 1.0 unit/ml                    | 9.97±2.32\times10^{-8} M     | 9.02±3.04\times10^{-8} M | NS          |
| Collagen-induced \([Ca^{2+}]\) |                                |                    |
| 2.0 \(\mu\)g/ml               | 6.26±1.27\times10^{-8} M     | 5.64±1.77\times10^{-8} M | NS          |
| 4.0 \(\mu\)g/ml               | 7.07±1.55\times10^{-8} M     | 6.60±2.07\times10^{-8} M | NS          |
| PAF-induced \([Ca^{2+}]\)     |                                |                    |
| 0.5 mM                        | 13.8±17.8\times10^{-5} M     | 11.8±19.1\times10^{-5} M | NS          |
| 1.0 mM                        | 85.4±14.3\times10^{-5} M     | 23.1±33.2\times10^{-5} M | NS          |

Values are mean±SD. \([Ca^{2+}]\), ionized calcium concentration; NS, not significant; PAF, platelet-activating factor. Platelet \([Ca^{2+}]\) in acute ischemic stroke is shown. No significant differences were observed based on the presence or absence of hypertension. All values are in M/7.5\times10^7 platelets.
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Figure 6. Graph showing changes in platelet basal ionized calcium concentration ([Ca$^{2+}$]) after cross incubation of control platelets with plasma from other controls (left) and with plasma from stroke patients (right). Basal [Ca$^{2+}$] increased after incubation with stroke plasma (p<0.02). All values are in µM/7.5×10^7 platelets.

Figure 7. Graph showing changes in serotonin release after cross incubation of control platelets with plasma from other controls (left) and with stroke plasma (right). Serotonin release was greater after incubation with plasma from stroke patients (p<0.01). All values are in ng/7.5×10^7 platelets.

the platelet membrane to aequorin with the subsequent addition of Mg$^{2+}$ to repair the membrane. The platelet is not “skinned” or made grossly permeable to other molecules during this procedure, and a gradient to Ca$^{2+}$ is maintained across the platelet membrane. Platelet cytoplasmic ([H]adenine and lactate dehydrogenase), dense granule ([14C]serotonin), and α-granule (β-thromboglobulin) markers were retained, and serial electron micrographs confirmed the structural integrity of the platelets at all times during the aequorin-loading procedure. Furthermore, platelet function as studied by measurement of ATP secretion and aggregation was unaltered by the presence of aequorin in the platelet.

Though the exact mechanism for the uptake of aequorin into platelets is unknown, it is believed to be located in the cytoplasm outside the granules. Aequorin-loaded gel-filtered washed platelets in a media containing 1 mM Ca$^{2+}$ were used to study [Ca$^{2+}$] homeostasis after onset of cerebral ischemia. Compared with healthy controls, basal and stimulated platelet [Ca$^{2+}$] were both increased in acute ischemic stroke patients. These results are consistent with other observations of increased platelet activation during cerebral ischemia. Even though thrombin-, collagen-, and PAF-stimulated [Ca$^{2+}$] were greater in stroke patients, the profiles of these [Ca$^{2+}$] changes were similar to controls. Accordingly, the higher basal [Ca$^{2+}$] in acute stroke patients probably represents a lowered threshold for platelet

Figure 8. Diagrammatic representation of cross-incubation experiment. [Ca$^{2+}$], ionized calcium concentration; 5HT, 5-hydroxytryptamine; plts., platelets. For details see text. All values are mean±SD.
activation and can explain the increased platelet activation after agonist stimulation. Therefore, the platelets of acute ischemic stroke patients appear to be functionally similar to the platelets of control subjects but are more sensitive to activation. The incubation of platelets from healthy control subjects with plasma from the acute ischemic stroke patient resulted in raised basal \([Ca^{2+}]\) and also caused the release of serotonin. This strongly suggests that there is a plasmatic factor released during acute cerebral ischemia that activates platelets rather than the platelets being primarily abnormal. The secondary nature of platelet involvement in cerebral ischemia is also supported by the fact that platelet basal \([Ca^{2+}]\) was maximally increased only after 36–72 hours had elapsed from stroke onset. The identity of this plasmatic factor can only be speculative. One possibility is PAF, a naturally occurring phospholipid that is primarily derived from stimulated leukocytes and is present in plasma.\(^{14,25-27}\) PAF is also present in endothelial cells\(^{28,29}\) and is a potent inducer of platelet activation, thrombosis,\(^{30}\) and ischemia\(^{31,32}\) and has been postulated to play an important role in the pathogenesis of microangiopathy.\(^{33,34}\) Measurement of plasma concentrations of PAF and its inhibiting enzyme, acetylhydrolase, may further clarify this hypothesis.\(^{35-37}\)

A plasmatic factor has also been implicated in the platelet basal \([Ca^{2+}]\) increase measured in subjects with essential hypertension.\(^{38-40}\) Although essential hypertension is a major risk factor for thrombotic complications in various end organs, the pathophysiological mechanisms for this association have not been clearly identified. Perhaps increased platelet basal \([Ca^{2+}]\) may be an important mechanism by which hypertensives are predisposed to thrombotic complications such as stroke and myocardial infarction. In this study, platelet basal \([Ca^{2+}]\) was increased in stroke patients both with and without associated hypertension, so the same factor is unlikely to account for our findings. Moreover, basal \([Ca^{2+}]\) continued to increase for 36–72 hours after onset and supports the concept that this plasmatic factor was released after the onset of cerebral ischemia. Thus, the increase of platelet basal \([Ca^{2+}]\) in the days immediately after onset of stroke, not uncommonly coinciding with clinical deterioration,\(^{41-43}\) may be an independent risk factor for thrombus formation and its propagation. The results observed in these acute ischemic stroke patients may not be specific for this condition; similar changes may occur when thrombosis and infarction occur in other end organs such as the heart.

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References


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23. Miller DJ: Are cardiac muscle cells 'skinned' by EGTA or EDTA? *Nature* 1979;277:142-143

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