Intracellular Calcium and Hydrogen Ions in Diabetes Mellitus*

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ABSTRACT

Diabetes mellitus is a multi-component syndrome that is often complicated by angiopathy which is partly due to enhanced platelet functions. Using fluorescent dyes 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and Fura-2 AM, changes were evaluated in the concentration of baseline and thrombin-stimulated increases in intracellular ionized calcium (Ca^{2+}) relative to hydrogen ions in the platelets from control, insulin-treated, and non-treated diabetic rats. The cytosol of platelets from the diabetic rats were more acidic compared to the insulin-treated and normal control rats. The increased intracellular hydrogen ion concentration [H^+] or decreased pH (pH) in the diabetic rat platelets is associated with an increased baseline [Ca^{2+}],. Upon stimulation with thrombin, the mean peak [Ca^{2+}], for the insulin-treated (309 ± 97 nmol/L) and untreated (339 ± 135 nmol/L) diabetic rats was significantly higher than the concentration for the normal rats (213 ± 101 nmol/L). Treatment with insulin attempts to correct the diabetes-induced elevation in the baseline of [Ca^{2+}], and intracellular H^+. These results suggest that the relationships between Ca^{2+} and H^+ relative to binding sites are similar in the intra- and extracellular compartments. It is our conclusion that the enhanced platelet activity and associated development of vascular diseases in diabetes may be due to an increased intracellular H^+ that caused an increased baseline [Ca^{2+}], in diabetes mellitus.

Introduction

Platelet hyperfunction has been reported in patients with diabetes mellitus. This enhanced platelet reactivity has also been implicated as one of the most important factors involved in the pathogenesis of both diabetic microangiopathy and macroangiopathy.1 The enhanced platelet functions in diabetes mellitus include increased platelet adhesiveness and aggregation, serotonin secretions, and prostaglandin synthesis in response to agonist stimulation.2,3 Some reports
from human as well as animal studies have suggested that blood glucose and insulin levels are important factors responsible for this altered platelet function. Platelet aggregability has been shown to correlate positively with fasting blood glucose levels. Results from in vivo and in vitro studies indicate that insulin may be important in the physiologic regulation of platelet functions. However, the exact mechanism(s) by which insulin and potentially blood glucose modulate platelet function is still mainly speculative.

Recently, thrombin-stimulated platelets from patients with type II (non-insulin dependent) diabetes mellitus were reported to have increased intracellular ionized calcium ($Ca^{2+}$) mobilization. This increase was suggested to be related to the enhanced platelet functions that occur in this disease condition. Other studies have evaluated changes in $[Ca^{2+}]_i$ relative to intracellular hydrogen ion concentration in several type of cells and disease conditions. Some of these studies reported a direct relationship between $[Ca^{2+}]_i$ and intracellular hydrogen ion concentration.

Based on these findings, Levy et al proposed a theoretical model indicating that alterations in $[Ca^{2+}]_i$ play a vital role in the onset and pathogenesis of diabetes and related disorders. The investigation of $Ca^{2+}$ relative to intracellular hydrogen ion is critical because of the well documented relationship between the two ions in serum. In serum, ionized calcium concentration is directly related to hydrogen ion concentration because of its competitive binding to proteins and other anions. This relationship in serum led to the use of a pH corrected serum calcium ($[Ca^{2+}]_{pH7.4}$) in the management of patients with abnormal calcium homeostasis. The equation used for the correction is: $[Ca^{2+}]_{pH 7.4} = [Ca^{2+}]_{measured} + [0.47(pH_{measured} - 7.40)].$ Using this equation, a serum pH decrease of $-0.01$ units will lead to a $4,700$ nmol/L increase in serum ionized calcium.

This electrochemical relationship between $H^+$ and $Ca^{2+}$ in serum may also be applicable to the intracellular components of the two ions. It is therefore hypothesized that the baseline intracellular $[H^+]$ in platelets from diabetes mellitus patients is higher than the normal baseline, with a resultant increase in $[Ca^{2+}]_i$. This proposed intracellular hydrogen ion-related increase in $[Ca^{2+}]_i$ may be responsible in part for the enhanced platelet activities in diabetes mellitus and the consequent vascular disorders. To evaluate the validity of this hypothesis, the changes in $[Ca^{2+}]_i$ and intracellular $[H^+]$ were studied in platelets from control, insulin-treated, and untreated diabetic rats. The rat with streptozotocin (STZ) induced diabetes mellitus has been shown to be a useful model for investigating the various pathological processes that occur in this disease.

Materials and Methods

STUDY POPULATION

Twenty-four male Sprague-Dawley, age-matched rats weighing 200 to 250 grams* were randomly assigned to three equal groups: A, B, and C. They were maintained under similar conditions and allowed to acclimatize to the new environment for a period of at least seven days before starting experimental procedures. All the rats were provided with certified Purina food and water ad libitum during the entire acclimatization and experimental period. Group A animals served as controls. Rats in groups B and C were made diabetic by a single intra-

* Harlan, Houston, TX.
peritoneal injection of STZ at a dose of 65 mg/Kg body weight. Group A rats (controls) were injected intraperitoneally with normal saline to make up for the volumes injected into groups B and C rats. The induction of diabetes was confirmed by the presence of polydipsia, polyuria, glycosuria and ketonuria 24 hours after the STZ injection. A day after injections of STZ, group C rats were treated with daily subcutaneous injections of 3 IU Neutral Protamine Hagedorn† for six consecutive days. Each rat was weighed before and during the experimental period at three-day intervals and also before the terminal blood collection.

**Biochemical Studies**

**Specimen Collection:** The rats were fasted overnight for 12 hours with access to drinking water on day 7. On the eighth day, they were anesthetized using methoxyflurane, and the abdomen was dissected to expose the dorsal aorta. Using a 21 gauge needle, blood samples for intracellular studies were collected from the dorsal aorta into 10 ml polypropylene syringes containing 2 ml acid citrate dextrose buffer (ACD), (85 mM citric acid, 110 mM dextrose; pH 4.9). The blood samples for extracellular measurement of glucose, ionized calcium, pH and ketone bodies were collected using heparinized capillary tubes§ and Microtainer brand tubes.§

**Extracellular Assays:** In order to minimize the effect of pH changes on serum ionized calcium, the capillary blood collectors were filled with blood and then placed in ice granules until the time of analysis. Whole blood ionized calcium and pH were measured within one hour after sampling using the AVL 987-S Ion Selective Electrode Analyzer.† Serum glucose was measured using a Kodak Ektachem 700XR Analyzer.¶ The presence of ketone bodies in the urine was assessed using the sodium nitroprusside qualitative method.

**Simultaneous Measurement of Intracellular pH and Free Calcium:** Platelets were isolated from the ACD anticoagulated blood by the method of Brass et al.‡ The isolated platelets were re-suspended in a platelet buffer (5.0 mM [HEPES], 137 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl₂, 5.5 mM glucose; pH 7.4) and incubated at 25°C until a characteristic pearlescent appearance indicating discoid unactivated platelets was observed. Platelet counts were done using a Technicon H-1 System* and the platelets were adjusted to a count of 2 × 10⁵/µL.

The simultaneous measurements of [Ca²⁺], and intracellular H⁺ were done using a combination of methods by Tsien et al.²⁴ and Ives et al.²⁵ Performed in duplicates, 2 mL of the platelet suspension was loaded with 6 µL of Fura-2 AM (3.0 µM) and 4 µL of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (2.0 µM), and then incubated for 30 minutes at 37°C. After the loading period, excess unloaded Fura-2 AM and BCECF were removed by washing with platelet buffer and centrifugation at 1500 × g for 10 minutes. The platelet count was then repeated and adjusted to a final count of 1 × 10⁵/µL.

For intracellular calcium measurement, excitation wavelengths were 340 nm and 370 nm at an emission wavelength of 510 nm, with a band-pass setting of 10 nm. The relative fluorescence intensity (RFI) of the platelet suspension

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† Iletin I from Eli Lilly and Company, Indianapolis, IN.
‡ Radiometer, Copenhagen, Denmark.
§ Becton Dickinson Vacutainer Systems, Rutherford, NJ.

† AVL Scientific, Atlanta, GA.
¶ Eastman Kodak Company, Rochester, NY.
* Miles Inc., Tarrytown, NY.
was then measured at 37°C using the Hitachi F-2000 Fluorescent Spectrophotometer. For the intracellular H⁺, intensities at excitation wavelengths 440 nm and 500 nm with an emission wavelength of 530 nm were measured. Using 1.5 mL of the platelets suspension in acrylic fluorescence cuvettes, the concentration of Ca²⁺ and fluorescence ratio for the intracellular H⁺ (excitation wavelength intensity ratio at: Intensity₄₄₀ nm/Intensity₅₀₀ nm × K_d, where K_d = effective dissociation constant or 250 nmol/L) were determined before and after stimulation with 7 international units (IU) of bovine plasma thrombin. The 7 IU of thrombin was introduced into the cuvette containing the platelet suspension at 50 seconds after the start of fluorescence measurement. The maximum and minimum fluorescence were measured after the addition of 5 µL 10% triton x 100 and 10 µL 0.5 molar ethyleneglycol-bis-(β-amino ethyl ether)N,N’-tetra acetic acid at 150 and 250 seconds after initiation of measurement, respectively. The [Ca²⁺]j and intracellular [H⁺] (as indicated by the fluorescence intensity ratio) were calculated automatically using the Hitachi F-2000 Fluorescence Spectrophotometer's built-in software. The intracellular free calcium concentrations were calculated using the following equation:

$$[\text{Ca}^{2+}]_j = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)$$

where

- F = fluorescence as a function of [Ca²⁺]j;
- F_{min} = fluorescence at very low [Ca²⁺]j;
- F_{max} = fluorescence at very high [Ca²⁺]j; and
- K_d = effective dissociation constant or 224 nmol/L.

Results

In Table I are shown the concentrations of serum fasting blood glucose, ionized calcium, pH, and ketone bodies for each experimental group eight days after the induction of diabetes mellitus. The increased serum glucose and ketosis that occurred in the diabetic groups confirms the successful induction of diabetes mellitus. The differences in the mean concentrations of the fasting blood glucose between the experimental groups are statistically significant (P < 0.05). These results show that insulin treatment did partially control the STZ-induced diabetes mellitus. This is further supported by

| TABLE I |
|---|---|---|---|
| **Fasting Plasma Concentrations of Glucose, Ionized Calcium, and Ketone Bodies** | **Analytes** | **Control** | **Insulin-treated Diabetic Group** | **Untreated Diabetic Group** |
| Glucose* (mg/dL) | 154.0 ± 38 | 232.0 ± 64 | 527.0 ± 178 |
| Ionized calcium (mmol/L) @ pH 7.4 | 1.42 ± 0.05 | 1.39 ± 0.04 | 1.42 ± 0.06 |
| pH | 7.39 ± 0.07 | 7.38 ± 0.06 | 7.37 ± 0.07 |
| Ketone (qualitative) | Negative | Negative | Small to Large |

*The differences in the mean fasting blood glucose level between the experimental groups are statistically significant (P < 0.05; df = 14). Values given as mean ± SD.
the absence of ketosis in the insulin-treated diabetic rats. The serum ionized calcium levels were statistically similar in all the experimental groups.

The results of the resting and thrombin-stimulated intracellular free calcium are shown in table II. The mean baseline concentration of Ca\(^{2+}\) (126 ± 33 nmol/L) in untreated diabetic rats was statistically higher than values in both control rats (51 ± 16 nmol/L) and insulin-treated diabetic rats (61.9 ± 13 nmol/L), (P < 0.05). This increased concentration of Ca\(^{2+}\) in the untreated diabetic rats is associated with a slightly higher intracellular H\(^+\) concentration as indicated by the means of fluorescence intensity ratios for hydrogen ions in table III. The intracellular [H\(^+\)] is expressed as the mean ± SD of the fluorescence intensity ratio for each experimental group of rats. The differences in fluorescence intensity ratios between the experimental groups are not statistically significant. Figure 1 and table III also show that the cytosolic hydrogen ions fluorescence intensity ratios are not affected (p < 0.05) by the thrombin-stimulation.

The peak intracellular ionized calcium concentrations in response to thrombin-stimulation are shown in table II. The mean of the peak responses in Ca\(^{2+}\) for platelets from both treated and untreated diabetic rats (309 ± 97 nmol/L and 339 ± 135 nmol/L, respectively) are significantly higher than in platelets from the control animals (213 ± 101 nmol/L). These observed differences in response to thrombin-stimulation are statistically

![Figure 1. Tracings of representative baseline and thrombin-induced changes in platelet [Ca\(^{2+}\)] and [H\(^+\)], (expressed as ratio of fluorescence intensity at 440 nm to that of 500 nm times K_d: K_d = 250 nmol/L).](image)

| TABLE II
| Intracellular Ionized Calcium Concentration* |
|---------------------------------------------|---------------------------------------------|
| Control Insulin Treated Diabetic Group Untreated Diabetic Group | | |
| Baseline 51 ± 16 61.9 ± 13 a126 ± 33 | Peak b 213 ± 101 309 ± 97 339 ± 135 |

*Expressed in nmol/L (mean ± SD); n = 8 per experimental group.
Mean baseline calcium concentration in untreated–diabetic rats is statistically different (a p < 0.05; df = 14) from baseline concentration in control and insulin treated, streptozotocin injected diabetic rats. Mean peak calcium concentration is statistically lower (b p < 0.05; df = 14) in control rats.
Intracellular Hydrogen Ion Concentration

TABLE III

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Insulin Treated Diabetic Group</th>
<th>Untreated Diabetic Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1935 ± 548</td>
<td>2072 ± 101</td>
<td>2085 ± 275</td>
</tr>
<tr>
<td>Peak</td>
<td>1969 ± 540</td>
<td>2143 ± 101</td>
<td>2104 ± 265</td>
</tr>
</tbody>
</table>

*Expressed as fluorescence intensity ratio; n = 8 in each experimental group.
Mean fluorescence intensity ratios are not statistically significant.

significant (p < 0.05) and clinically important relative to normal platelet function.

Discussion

Previous studies\(^{27,28}\) have suggested that the enhanced platelet aggregability frequently observed in diabetic patients may be due to an underlying insulin deficiency. In vitro addition of insulin to platelet suspension directly reduces their sensitivity to aggregating agents.\(^{27}\) In this study, treatment with insulin partially corrected the hyperglycemia and restored the diabetes-associated increase in baseline \([\text{Ca}^{2+}]_{i}\), and intracellular \([\text{H}^+]\) to within normal levels. The exact mechanism underlying these intracellular ionic changes with a resultant enhanced platelet activity is unknown. Some investigators have speculated that insulin probably regulates these changes by directly reducing platelet’s \([\text{Ca}^{2+}]_{i}\) via the phospholipase-controlled IP\(_3\) generation.\(^{29}\)

In this study, a direct relationships is reported between \([\text{Ca}^{2+}]_{i}\) and intracellular \([\text{H}^+]\). This finding is similar to the interrelationship between intracellular pH and \([\text{Ca}^{2+}]_{i}\) in other reports.\(^{11,12,13}\) Also, the extracellular relationship between the two ions has been attributed to their competition for binding on various molecules, e.g., proteins, sulfate, phosphate and other anions.\(^{15,16}\) Thus, it is proposed that cytosolic acidification leads to or is associated with a concomitant cytosolic increase in intracellular ionized calcium. Based on the calculated relationship in the extracellular environment, a pH change of −0.01 units leads to significant increase of +4,700 nmol/L in \([\text{Ca}^{2+}]\). Thus, a change as small as −0.001 pH unit will potentially cause an increase of +470 nmol/L in \([\text{Ca}^{2+}]\). This degree of increase in \([\text{Ca}^{2+}]_{i}\) will cause a significant disturbance in the maintenance of intracellular homeostasis. It is therefore important to evaluate the results of \([\text{Ca}^{2+}]_{i}\) relative to the theoretical effect of these small changes intracellular \([\text{H}^+]\). In the work of Noda et al.\(^{30}\) a difference of 0.01 pH unit between diabetic mellitus rat monocytes (7.06 ± 0.02) and control rat monocytes (7.07 ± 0.02) was reported. This magnitude of change in intracellular \([\text{H}^+]\) will theoretically result in a ±4,700 nmol/L change in the \([\text{Ca}^{2+}]\) and may produce significant disturbance in cellular homeostasis. Viewed in the light of this theoretical estimation, our results indicating very small changes in \([\text{H}^+]\) are in agreement with the findings of Noda et al.

An understanding of the electrochemistry between \([\text{Ca}^{2+}]\) and \([\text{H}^+]\) relative to the other ions and molecules in the complex intracellular environment will be required to define the mechanism responsible for the increased \([\text{Ca}^{2+}]_{i}\) in diabetes mellitus, hypertension, obesity,
and vascular disorders. Although our results support the findings of previous investigators, the current authors disagree with the hypothesis that alterations in the levels of insulin and glucose are directly responsible for the increased Ca
2+
 and resultant enhanced platelet reactivity in diabetic patients. Based on electrochemical principles, it is proposed by us that the increased intracellular [H+] or acidification of the cytosol leads to dissociation of Ca
2+ from its complexes with intracellular cations and other macromolecules. This displacement of Ca
2+ by H+ is explained by the higher electropositivity of H+ relative that of to Ca
2+. Thus, the total intracellular calcium may remain constant in these diseases while the ionized calcium increases. Therefore, it is proposed that the primary derangement in cellular ionic homeostasis is at the level of H+ with resultant cellular acidification which triggers the increase in Ca
2+.

Acknowledgement

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References


