Inhibition of Human Platelet Aggregation and Cytoplasmic Calcium Response by Calcium Antagonists: Studies with Aequorin and Quin2

J. ANTHONY WARE, PETER C. JOHNSON, MARIANNE SMITH, AND EDWIN W. SALZMAN

SUMMARY Calcium antagonists inhibit platelet aggregation, but whether this action is due to inhibition of the effect of agonists on cytoplasmic ionized calcium concentration is unknown. We studied this problem by loading gel-filtered platelets with either quin2 or aequorin and stimulating them with epinephrine, arachidonate, thrombin, the calcium ionophore A23187, 1-oleoyl-2-acetyl glycerol, or adenosine diphosphate in media with or without extracellular calcium. In response to all of these agonists, aequorin indicated an increase in cytoplasmic calcium that accompanied or preceded platelet aggregation. In calcium-containing media, verapamil, nifedipine, and diltiazem inhibited these effects in a concentration-dependent fashion, except for those produced by thrombin and A23187. Removal of extracellular calcium with EGTA reduced the calcium response to arachidonate, adenosine diphosphate, and 1-oleoyl-2-acetyl glycerol, and the calcium response and aggregation were further inhibited by the calcium antagonists. In general, strong inhibition of the aequorin cytoplasmic calcium signal by approximately 100 μM concentrations of nifedipine, verapamil, and diltiazem was correlated with inhibition of platelet aggregation, but high concentrations of the inhibitors were required. Since inhibition by the calcium antagonists of the cytoplasmic calcium response and aggregation exceeded the effect of simple removal of extracellular calcium, these drugs may affect internal redistribution of calcium in human platelets. (Circulation Research 1986;59:39-42)

KEY WORDS  • calcium antagonists  • platelet calcium  • aequorin  • quin2

ALTHOUGH the Ca²⁺ antagonists verapamil, diltiazem, and nifedipine selectively inhibit the inward Ca²⁺ current in cardiac muscle cells and are known to inhibit platelet aggregation in vitro in response to several agonists, it is not established that this effect on platelet aggregation is due to limitation of influx of extracellular Ca²⁺ following stimulation. Other actions of the Ca²⁺ antagonists that might interfere with platelet function include competition for the α₂-adrenergic receptor and limitation of mobilization of Ca²⁺ from intracellular pools, as has been suggested in cardiac muscle. The present study compared inhibition of platelet aggregation by Ca²⁺ antagonists and inhibition of changes in cytoplasmic ionized Ca²⁺ concentration ([Ca²⁺]), as measured by the Ca²⁺-dependent fluorescent compound quin2, and the luminescent photoprotein aequorin, loaded into platelets by a nondestructive permeabilization technique.

Materials and Methods

Blood was collected and prepared as described previously. Quin2 (2-methyl-6+ methoxy-8-nitroquinoline) acetoxymethyl ester dissolved in dimethylsulfoxide was incubated with platelet-rich plasma for 20 minutes, which produced an average intracellular quin2 of 0.85 mM. The platelets were washed, gel-filtered, and suspended in a modified Hepes-Tyrode's buffer with 1 mM Ca²⁺ added, as previously reported. Calibration and determination of [Ca²⁺] were done as described. Platelets were loaded with aequorin by incubation at 0°C with solutions containing EGTA and adenosine triphosphate, according to our published method. After loading, the platelets were warmed to room temperature and gel-filtered in Hepes-Tyrode’s buffer containing 1 mM Ca²⁺. Calibration of light signals and determination of aequorin-indicated [Ca²⁺] were performed as described previously. For these experiments, a modified photomultiplier tube-aggregometer ("Whole Blood“ Lumiaggregometer, Chronolog, Havertown, Pa.) was used for simultaneous recording of aggregation and aequorin-indicated [Ca²⁺].

In studies "without extracellular Ca²⁺", 2 mM EGTA was added 30 seconds before the agonist. Diltiazem, verapamil, or nifedipine was added to the platelet suspension and stirred 2 minutes before testing. None of the three drugs quenched luminescence of aequorin added to a cell-free suspension. Nifedipine's effect on platelet [Ca²⁺] as measured by quin2 could not be assessed because we found, in preliminary experiments, that nifedipine markedly quenched both background and quin2-dependent fluorescence in a concentration-dependent manner. In some experi-
ments, the effect of the Ca\(^{2+}\) antagonists on changes in cyclic adenosine monophosphate (cAMP) was measured using a radioimmunoassay kit (Amersham, Arlington Heights, Ill.). Changes in malondialdehyde (MDA) were measured using a fluorescence assay as previously described.\(^{11}\) Both cAMP and MDA were measured 3 minutes after addition of arachidonate.

Aequorin was purchased from Dr. John Blinks (Mayo Clinic, Rochester, Minn.); 1-oleoyl-2-acetyl glycerol (OAG) was the kind gift of Drs. Yasutomi Nishizuka and Susan Rittenhouse. Quin2 was purchased from Amersham. The remainder of the reagents were obtained from commercial sources, and were agent grade or better.

Platelet agonists were studied at concentrations that caused complete platelet aggregation and elevations of aequorin-indicated [Ca\(^{2+}\)]; concentrations of up to 200 \(\mu M\) of each of the Ca\(^{2+}\) antagonists were tested. The data are presented as either the percentage of the agonist-induced values without Ca\(^{2+}\) antagonists (control), in Figure 1, or as the concentration of diltiazem, verapamil, or nifedipine necessary to inhibit control values by 50\% (I\(_{50}\), Table 1). Each value in Figure 1 or Table 1 represents the mean of at least three separate determinations.

**Results**

Addition of diltiazem, nifedipine, or verapamil did not lower the basal [Ca\(^{2+}\)] as indicated by either aequorin or quin2. Inhibition by Ca\(^{2+}\) antagonists of aequorin- and quin2-induced rises in [Ca\(^{2+}\)] and aggregation in response to arachidonate are seen in Figure 1. Similar determinations were made using adenosine diphosphate (ADP), the Ca\(^{2+}\) ionophore A23187, thrombin, epinephrine, and OAG and are summarized in Table 1. As noted previously,\(^{4, 12}\) no rise in quin2-induced [Ca\(^{2+}\)] was seen with epinephrine or OAG in quin2-loaded platelets, although these agonists caused concentration-dependent increases in aequorin-indicated [Ca\(^{2+}\)] and aggregation. Verapamil, nifedipine, and diltiazem inhibited the rise in aequorin-induced [Ca\(^{2+}\)] in response to arachidonate, ADP, epinephrine, and OAG in a concentration-dependent fashion.

No inhibition of either aggregation or peak [Ca\(^{2+}\)] occurred at submicromolar concentrations of Ca\(^{2+}\) antagonists. When a rise in [Ca\(^{2+}\)] was inhibited by more than 75\%, aggregation was reduced to the same extent; total inhibition of the [Ca\(^{2+}\)] rises and aggregation were also correlated. Pretreatment of the platelets with aspirin eliminated the rise in [Ca\(^{2+}\)] and aggregation induced by arachidonate. Although other agonists induced both aggregation and [Ca\(^{2+}\)] rises despite aspirin, the inhibitory effect of the Ca\(^{2+}\) antagonists on aspirin-treated platelets was not significantly different from that seen in non-aspirin-treated platelets. Verapamil had a greater effect on epinephrine-induced rise in [Ca\(^{2+}\)] and aggregation than did diltiazem or nifedipine. However, differences among the Ca\(^{2+}\) antagonists in platelets stimulated by agonists other than epinephrine were much less marked, and none of the drugs in concentrations less than 200 \(\mu M\) inhibited the rise in [Ca\(^{2+}\)] or aggregation induced by thrombin or A23187.

Unlike the ADP-induced aequorin signal, the quin2-induced [Ca\(^{2+}\)] signal produced by ADP was not inhibited by either diltiazem or verapamil, although they strongly inhibited aggregation at high concentrations. This suggests that the effect of the Ca\(^{2+}\) antagonists on ADP-induced aggregation involves a mechanism that is not reflected in the [Ca\(^{2+}\)] reported by quin2, in contrast to its correlation with the aequorin-induced peak [Ca\(^{2+}\)].

Chelation of extracellular Ca\(^{2+}\) with EGTA eliminated both the aequorin-induced rise in [Ca\(^{2+}\)] and aggregation in response to epinephrine, as we have previously reported.\(^{8}\) Addition of EGTA reduced but did not eliminate rises in aequorin-induced [Ca\(^{2+}\)] and aggregation in response to the other agonists. In higher concentrations, diltiazem, nifedipine, and verapamil were more inhibitory of both the aequorin-induced [Ca\(^{2+}\)] rise and aggregation in response to arachidonate, OAG, or ADP than was EGTA alone, suggesting that high concentrations of Ca\(^{2+}\) antago-
nists affected intracellular mobilization of Ca\(^{2+}\), perhaps indirectly.

To investigate the possible mechanisms for additional inhibition of arachidonate-induced [Ca\(^{2+}\)] signals and aggregation by Ca\(^{2+}\) antagonists beyond that produced by EGTA, cAMP and MDA levels of arachidonate-stimulated platelets were measured. Neither the cAMP concentration of the platelet suspension after arachidonate nor the MDA level was significantly altered by diltiazem, nifedipine, or verapamil, in the absence or presence of EGTA. Thus, we found no support for the hypothesis that the inhibitory effects of these Ca\(^{2+}\) antagonists are mediated by an increase in cAMP or by interference with prostaglandin metabolism.

## Discussion

In this study, the inhibitory effects of Ca\(^{2+}\) antagonists on aggregation of gel-filtered platelets were compared with their effect on cytoplasmic Ca\(^{2+}\) as determined by both the fluorophore quin2 and the Ca\(^{2+}\)-sensitive photoprotein aequorin. These indicators appear to reflect different aspects of Ca\(^{2+}\) homeostasis in stimulated platelets; aequorin may be more sensitive to local Ca\(^{2+}\) transients and quin2 to the mean cytosolic [Ca\(^{2+}\)]. In no instance did platelet aggregation occur without an increase in the peak [Ca\(^{2+}\)] as indicated by aequorin, whereas that was not always the case with quin2 (eg, with epinephrine or OAG, a synthetic diacylglycerol that has been proposed to activate platelets via protein kinase C in the absence of a rise in quin2-indicated [Ca\(^{2+}\)]). That this difference between the indicators is not due simply to aequorin’s greater sensitivity to low levels of [Ca\(^{2+}\)] is shown in the present study, in which inhibition of ADP-induced aggregation by Ca\(^{2+}\) antagonists was not accompanied by changes in [Ca\(^{2+}\)] as indicated by quin2, but was associated with decreases in the peak [Ca\(^{2+}\)], as seen with aequorin. Thus, aequorin-indicated [Ca\(^{2+}\)] appears to be more closely related to both stimulatory or inhibitory changes in aggregation than are rises in quin2-indicated [Ca\(^{2+}\)].

Three general observations regarding the mechanism of platelet inhibition by Ca\(^{2+}\) antagonists are suggested by this study. First, the similar abilities of diltiazem, verapamil, or nifedipine to reduce peak [Ca\(^{2+}\)] are compatible with these drugs acting via a common mechanism. An exception to this finding was seen with epinephrine-treated platelets, which were more strongly inhibited by verapamil than by nifedipine or diltiazem; this effect has been ascribed to verapamil’s competition with epinephrine for platelet \(\alpha\)-adrenergic receptors. Second, extracellular Ca\(^{2+}\), while not enhancing both aggregation and peak [Ca\(^{2+}\)], does not enhance platelets via voltage-dependent Ca\(^{2+}\) channels analogous to those in smooth muscle, since the influx of Ca\(^{2+}\) through such channels is inhibited by submicromolar concentrations of Ca\(^{2+}\) antagonists. Third, Ca\(^{2+}\) antagonists limit intracellular redistribution of Ca\(^{2+}\) in platelets, as shown by greater inhibition of the rise in [Ca\(^{2+}\)] and aggregation induced by arachidonate, ADP, or OAG than was seen with EGTA alone. Although inhibition of intracellular Ca\(^{2+}\) mobilization by Ca\(^{2+}\) antagonists has also been observed in cardiac muscle, the mechanism by which this occurs is unknown. The present study confirms the findings that the Ca\(^{2+}\) antagonists do not elevate cAMP levels in platelets or interfere with prostaglandin metabolism, as assessed by MDA assay. Diltiazem, verapamil, and nifedipine are not among the Ca\(^{2+}\) antagonists that...

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### Table 1. Inhibition of Platelet Aggregation and Peak [Ca\(^{2+}\)] by Calcium Antagonists*

<table>
<thead>
<tr>
<th>Agonist</th>
<th>(I_{50}, [\text{Ca aequ}])</th>
<th>(I_{50}, [\text{Ca quin}])</th>
<th>(I_{50}, \text{aggregation}^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>V</td>
<td>D</td>
</tr>
<tr>
<td>ADP 10 (\mu)M</td>
<td>35</td>
<td>45</td>
<td>63</td>
</tr>
<tr>
<td>Arachidonate 5 (\mu)M</td>
<td>149</td>
<td>173</td>
<td>44</td>
</tr>
<tr>
<td>Arachidonate EGTA (53/53)§</td>
<td>33</td>
<td>44</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Thrombin, 0.1 Units/ml</td>
<td>13</td>
<td>33</td>
<td>44</td>
</tr>
<tr>
<td>Thrombin, EGTA (49/57)</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>A23187, 100 nM</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>A23187, EGTA (33/37)§</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>OAG, 30 (\mu)g/ml</td>
<td>28</td>
<td>28</td>
<td>28</td>
</tr>
<tr>
<td>OAG, EGTA (67/50)</td>
<td>100</td>
<td>131</td>
<td>190</td>
</tr>
<tr>
<td>Epinephrine 10 (\mu)M</td>
<td>10</td>
<td>10</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

*Inhibition (\(I_{50}\)) is expressed as the micromolar concentration necessary to inhibit the height of the Ca\(^{2+}\) response (peak [Ca\(^{2+}\)]) or aggregation by 50%.

†[Ca aequ], [Ca quin] = peak [Ca\(^{2+}\)] as determined by aequorin or quin2, respectively; N = nifedipine; V = verapamil; D = diltiazem.

§Aggregation was measured as the change in optical density (mm from baseline at 90 sec — mm from baseline at 30 sec) following agonist addition.

\(^\dagger\)Numbers in parentheses refer to the inhibition of [Ca aequ]/aggregation by EGTA without calcium antagonists and are expressed as the percent of values obtained with the same agonist in media without EGTA. Values under N, V, or D in these rows indicate concentration of calcium antagonist necessary to reduce [Ca\(^{2+}\)] or aggregation to 50% of the EGTA-inhibited level.

\(^\ddagger\)No rise in [Ca quin] was seen in response to OAG or epinephrine.
Franson et al.\textsuperscript{16} found to inhibit platelet-associated phospholipases. Although supramicromolar concentrations of diltiazem and verapamil have been found to have a "membrane-stabilizing" effect similar to that seen with local anesthetics, this effect is not shared by nifedipine\textsuperscript{14} and thus could not account for the inhibition of aggregation or [Ca\textsuperscript{2+}] seen with this drug.

The agonist-induced [Ca\textsuperscript{2+}] changes reported by quin2 are resistant to inhibition by the Ca\textsuperscript{2+} antagonists, except with thrombin and A23187, are inhibited by these drugs, but the concentrations required are still higher than those obtainable \textit{in vivo}.\textsuperscript{17} The processes that account for the effect of the Ca\textsuperscript{2+} antagonists on the intracellular mobilization of Ca\textsuperscript{2+}, which may be reflected in the different responses of [Ca\textsuperscript{2+}] indicated by aequorin and quin2, remain to be identified.

\textbf{References}

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