Cyclic Guanosine Monophosphate-Enhanced Sequestration of Ca$^{2+}$ by Sarcoplasmic Reticulum in Vascular Smooth Muscle

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The purpose of this study was to investigate the effects of the intracellular messenger cyclic GMP (cGMP) on sequestration of cytosolic calcium (Ca$^{2+}$) into the intracellular Ca$^{2+}$ store (the sarcoplasmic reticulum) of vascular smooth muscle. Using saponin-skinned primary cultures of rat aortic smooth muscle, we investigated the effect of cGMP on $^{45}$Ca uptake in monolayers of cells. The intracellular store was loaded with Ca$^{2+}$ by exposing the skinned cells to a $^{45}$Ca-labeled 1-$\mu$M free Ca$^{2+}$-containing solution for varying durations (0-20 minutes). Addition of 10 $\mu$M cGMP to six monolayers increased both the initial Ca$^{2+}$ uptake at 2 minutes (control, 240 ±8 pmol Ca$^{2+}$/10$^6$ cells; +cGMP 295 ±7; mean ±SEM; n = 6, p<0.01) and the final steady-state uptake reached at 20 minutes (control, 0.96±0.03 nmol Ca$^{2+}$/10$^6$ cells; +cGMP 1.12±0.03, p<0.02). This stimulation of uptake was quantitatively similar to that caused by 10 $\mu$M cyclic AMP. It occurred at varying ambient cytosolic Ca$^{2+}$ concentrations (0.1-1.0 $\mu$M Ca$^{2+}$) and was not further enhanced by addition of 10 $\mu$M cGMP-dependent protein kinase. The dose-response of stimulation of Ca$^{2+}$ uptake with cGMP indicated an $ED_50$ of 5 nM cGMP. The release of Ca$^{2+}$ from the sarcoplasmic reticulum in response to inositol 1,4,5-trisphosphate or caffeine was unaffected by cGMP. We conclude that the relaxation of vascular smooth muscle with cGMP-producing vasodilators is mediated in part by sequestration of cytosolic Ca$^{2+}$ by the sarcoplasmic reticulum. (Circulation Research 1988;62:961-964)

Cyclic GMP (cGMP) is currently assuming physiological importance as an intracellular mediator of vascular smooth muscle relaxation. Stimulation of particulate and soluble guanylate cyclase elevates cytosolic cGMP during the relaxation produced both by endogenous vasodilators (endothelium-derived relaxing factor) and by the nitric oxide–producing drugs (nitroglycerine, nitroprusside). The relaxation occurring with cGMP results from a lowering of the cytosolic concentration of calcium ions (Ca$^{2+}$). This is thought to result predominantly from stimulation of the plasmaembral Ca$^{2+}$-extrusion ATPase. An additional means of reducing cytosolic Ca$^{2+}$ in vascular smooth muscle is the sequestration of Ca$^{2+}$ into an intracellular store, the sarcoplasmic reticulum (SR). Although stimulation of SR Ca$^{2+}$ uptake is recognized for cyclic AMP–dependent relaxation of vascular smooth muscle, a similar effect for cGMP has yet to be demonstrated. This results partly from the difficulty in experimentally isolating the effects of cGMP on Ca$^{2+}$ transport at the SR from those at the plasmalemma. However, the recently described technique of chemical skinning of the plasmalemma by saponin has enabled the investigation of the function of the SR in vascular smooth muscle. Saponin forms micellar complexes with cholesterol, a major component of the plasmalemma. This renders the plasmalemma permeable, while not affecting the membranes of the intracellular organelles, which have a lower cholesterol content. We have used this technique to investigate the role of cGMP on SR Ca$^{2+}$ transport in primary cultures of rat aortic smooth muscle. We have investigated the effect of cGMP on Ca$^{2+}$ uptake by the SR and also on the release of Ca$^{2+}$ by the SR in response to inositol 1,4,5-trisphosphate or caffeine.

Materials and Methods

Smooth Muscle Culture

Primary cultures of rat aortic smooth muscle were prepared by an established method. Aortae from male Wistar rats (200–250 g) were stripped of adventitia and endothelium after treatment with collagenase (type I) and elastase (type I). A second incubation in collagenase and elastase yielded single cells and cell clumps that were inoculated with equal density (2 x 10$^3$ cells/ml) on 35-mm diameter plastic dishes containing Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum. Between 8 and 10 days, the cells formed a confluent monolayer on the base of the dish suitable for skinning and $^{45}$Ca flux measurements.

Saponin Skinning

After discarding the culture medium, the monolayer of cells was washed and incubated for 15 minutes in a physiological salt solution of (mM) NaCl 140, KCl 5, MgCl$_2$ 1, CaCl$_2$ 1.5, glucose 10, HEPES 10, pH 7.4 at 37°C. The subsequent skinning procedure and all further procedures were performed at room temperature, 25°C; the cells were washed for 2 minutes in a
solution for skinned cells of (mM) KCl 130, MgCl₂ 5, Na₄ATP 3.15, Tris 20, pH 6.8 at 25°C containing in addition 4 mM EGTA. They were then skinned for 15 minutes in an identical solution containing saponin (50 µg/ml). The cells were washed free of saponin for 2 minutes with the solution for skinned cells containing 0.1 mM EGTA.

**Ca Uptake in Skinned Cells**

Before exposing the cells to a ³⁵Ca-labeled Ca²⁺-containing solution, they were preincubated for 5 minutes in 0.1 mM EGTA solution in the absence or presence of cGMP at varying doses (1 nM–10 µM). They were then incubated in a Ca²⁺-containing 0.1 mM EGTA solution labeled with ³⁵Ca (1 µCi/ml), also in the absence or presence of cGMP. This period of Ca²⁺ loading of the SR was performed for varying durations (0.5–20 minutes) and at varying ambient Ca²⁺ concentrations (0.1–1 µM CaCl₂). The varying amounts of CaCl₂ required to give each free Ca²⁺ concentration were calculated from the binding constant of 0.1 mM EGTA for Ca²⁺ at pH 6.8, 25°C (10⁶ M⁻¹). Calcium uptake was stopped by removing the ³⁵Ca-labeled Ca²⁺-containing 0.1 mM EGTA solution. In separate experiments, Ca²⁺ uptake was repeated in the presence of 10 µM cyclic AMP, also in the presence of 10 µM cGMP-dependent protein kinase in addition to 10 µM cGMP.

The Ca²⁺ content of the monolayer after the period of uptake included extracellular bound Ca²⁺ and cytosolic Ca²⁺ in addition to Ca²⁺ taken up by the SR. Previous study of the comparison of the efflux characteristics of Ca²⁺-loaded skinned monolayers with those of extracellular material alone has shown that washing the cells in Ca²⁺-free 0.1 mM EGTA solution for 7 minutes removes extracellular and cytosolic Ca²⁺, providing an estimate of the Ca²⁺ content of the SR. The cells were therefore washed for 7 minutes and then removed from the dish by treatment with collagenase and trypsin in physiological salt solution. The radioactive content of the cells together with aliquots from the ³⁵Ca-labeled Ca²⁺-loading solutions was measured by liquid scintillation counting. The number of cells per dish was counted with a hemocytometer after harvesting two separate dishes from a group of dishes cultured at the same time. The Ca²⁺ content of the cells was calculated and expressed per million cells.

**IP₃- and Caffeine-Induced Ca²⁺ Release**

After loading the SR with Ca²⁺ to steady state by a 20-minute incubation in ³⁵Ca-labeled 1 µM Ca²⁺-containing 0.1 mM EGTA solution, the cells were exposed for 1 minute further to identical solution (control) or to one containing in addition IP₃ (10 or 1 µM) or caffeine (25 mM). These procedures were performed in the absence or presence of 10 µM cGMP. The cells were then washed for 7 minutes in Ca²⁺-free 0.1 mM EGTA solution and harvested, as described above.

**Chemicals**

All chemicals were purchased from Sigma Chemical, St. Louis, Missouri, with the exception of saponin from ICN Pharmaceuticals, Cleveland, Ohio; Dulbecco's Modified Eagle Medium and fetal bovine serum from GIBCO, Grand Island, New York. cGMP-dependent protein kinase was the generous gift of Dr. T.M. Lincoln.

**Statistics**

The data collected were expressed as mean ± SEM. The difference between results was compared by Student's t test for unpaired values. The Bonferroni correction for multiple comparisons was applied.

**Results**

cGMP 10 µM increased both the initial Ca²⁺ uptake by the skinned cells and also the final steady state reached (Figure 1). The uptake of Ca²⁺ by the skinned cells was largely dependent on the presence of MgATP (Figure 1). Cyclic AMP (10 µM) also increased the Ca²⁺ content of the skinned cells (control uptake at 2 minutes, 230 ±11 pmol Ca²⁺/10⁶ cells; +10 µM cAMP, 275 ± 8; n = 6, p <0.025). This compared with the stimulation of Ca²⁺ uptake observed for 10 µM cGMP at 2 minutes.

The addition of 10 µM cGMP-dependent protein kinase did not enhance further the response of Ca²⁺ uptake to 10 µM cGMP (control uptake at 2 minutes, 240 ±19 pmol Ca²⁺/10⁶ cells; + cGMP, 334 ±10; + cGMP + cGMP-dependent protein kinase 357 ±9, n = 6).

**FIGURE 1.** Effect of cGMP on the time course of Ca²⁺ uptake by saponin-skinned primary cultures of rat aortic smooth muscle cells. The skinned cells were loaded for varying periods with a ³⁵Ca-labeled solution containing 1 µM free Ca²⁺ buffered with 0.1 mM EGTA. Ca²⁺ content was measured in the absence () and presence (C) of cGMP 10 µM during preincubation and Ca²⁺ loading periods. MgATP-independent Ca²⁺ uptake (in the absence of cGMP) was measured by preincubating and loading in the absence of Na₄ATP. Each point represents mean ± SEM of six to eight observations.
Figure 2. Dose-response of cGMP-induced Ca\(^{2+}\) uptake by skinned cells. Skinned cells were loaded with "Ca-labeled 1 \(\mu\)M free Ca\(^{2+}\)-containing solution for 2 minutes in the presence of varying concentrations of cGMP. Each point represents mean ± SEM of four to eight observations.

The stimulation of Ca\(^{2+}\) uptake occurred over a range of concentrations of cGMP with an ED\(_{50}\) of approximately 5 nM cGMP (Figure 2). The response to 10 \(\mu\)M cGMP also occurred over a range of ambient Ca\(^{2+}\) concentrations (Table 1). Both IP\(_3\) and caffeine released Ca\(^{2+}\) from the Ca\(^{2+}\)-loaded skinned cells (Figure 3). The Ca\(^{2+}\) content of the cells following IP\(_3\)- or caffeine-induced Ca\(^{2+}\) release was unaffected by the presence of 10 \(\mu\)M cGMP during the loading and release periods. Six separate t tests have been performed on the data presented. When multiple t tests are performed, there is a risk that "statistically significant" results may occur by chance. Applying Bonferroni's correction, each of the six tests should be regarded as statistically significant (giving an overall type I error of 0.05) if the result is nominally significant at the 1% level. Thus, three of the six results would be considered significant on this more stringent criterion.

Discussion

We have demonstrated that cGMP increases Ca\(^{2+}\) uptake in skinned primary cultured rat aortic smooth muscle cells in a time- and dose-dependent manner. This stimulation of uptake is not further enhanced by the presence of cGMP-dependent protein kinase and occurs over a range of cytosolic Ca\(^{2+}\) concentrations. The release of Ca\(^{2+}\) from the skinned cells is unaffected by cGMP.

The store into which Ca\(^{2+}\) uptake occurs in the skinned vascular smooth muscle is identified as SR by a number of criteria: 80% of the uptake is dependent on extracellularly supplied MgATP, indicating that the bulk of the uptake uses an energy-dependent process such as the Ca\(^{2+}\) pump of the SR; uptake was performed at ambient 1 \(\mu\)M Ca\(^{2+}\), a concentration too low for mitochondrial uptake to occur; and lastly, 97% of this store has been shown to be releasable by IP\(_3\). cGMP (10 \(\mu\)M) increased both the initial uptake and the final content of the store by 20%, indicating an increase in the rate of transport of Ca\(^{2+}\) by the SR Ca\(^{2+}\)-ATPase. The cellular effects of cGMP are mediated by a cGMP-dependent protein kinase. The addition of 10 \(\mu\)M cGMP-dependent protein kinase also produces a 20% increase in Ca\(^{2+}\) uptake and over a similar time course.

Table 1. Effect of cGMP on Ca\(^{2+}\) Uptake by Skinned Cells at Varying Ambient Ca\(^{2+}\) Concentrations

<table>
<thead>
<tr>
<th>Ca(^{2+}) concentration ((\mu)M)</th>
<th>Ca(^{2+}) content (pmol Ca(^{2+})/million cells)</th>
<th>Control</th>
<th>+ 10 (\mu)M cGMP</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>63 ± 2</td>
<td>73 ± 2</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>150 ± 5</td>
<td>177 ± 5</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>238 ± 7</td>
<td>291 ± 8</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

Skinned cells were loaded for 2 minutes with "Ca-labeled solutions of varying free Ca\(^{2+}\) concentration (buffered with 0.1 mM EGTA) in the absence (control) and presence of 10 \(\mu\)M cGMP. Values represent mean ± SEM of six observations.
The ED$_{50}$ of 5 nM cGMP for cGMP-stimulated Ca$^{2+}$ uptake in these skinned cultured cells is a concentration known to exist in vascular smooth muscle. This is a similar concentration to 10 nM cGMP, which activates the plasmalemmal Ca$^{2+}$-ATPase and is considerably lower than the 5 μM cGMP required for a direct inhibitory effect of cGMP on the myofilaments. The increase in Ca$^{2+}$ uptake by the SR with cGMP occurs both at a cytosolic Ca$^{2+}$ concentration encountered in the relaxed cells (0.1 μM) and also at concentrations occurring in the contracting cell (0.3–1 μM).

An additional mechanism whereby cGMP could modulate the Ca$^{2+}$ content of the SR is by interfering with the release of Ca$^{2+}$ from this store. Human atrial natriuretic factor inhibits the release of intracellularly stored Ca$^{2+}$ in intact (nonskinned) vascular smooth muscle, although this may result from a reduction in the synthesis of IP$_3$ rather than from an effect of cGMP on the SR release channel. We have demonstrated that the release of SR Ca$^{2+}$ in response to either IP$_3$ or caffeine is not inhibited by cGMP; indeed, on an absolute basis, more Ca$^{2+}$ may possibly be released in the presence of cGMP.

These observations suggest that the sequestration of cytosolic Ca$^{2+}$ by the SR is an additional mechanism by which cGMP-stimulating vasodilators relax vascular smooth muscle.

References

Key Words: calcium • cyclic GMP • vascular smooth muscle • sarcoplasmic reticulum
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