Effect of 8-Bromo-Cyclic Guanosine Monophosphate on Intracellular pH and Calcium in Vascular Smooth Muscle

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Endothelium-dependent vasodilators, nitrates, and atrial natriuretic factor relax blood vessels by increasing vascular cyclic guanosine monophosphate (cGMP). The mechanisms by which cGMP relaxes vascular smooth muscle (VSM) are not known. Since contraction of VSM is associated with increased intracellular calcium and pH, we hypothesized that cGMP may decrease vascular tone by lowering ionized, intracellular calcium ([Ca^{2+}]_i) and pH. We used microfluorometry to measure cGMP-induced changes in intracellular calcium and pH of cultured A7r5 VSM cells after stimulation with contractile agonists. A cGMP analogue, 8-Br-cGMP, blocked vasopressin- but not thrombin-stimulated increases in [Ca^{2+}]_i. High extracellular potassium concentrations ([K^+]_o) increased [Ca^{2+}]_i, but the attenuation of [Ca^{2+}]_i by 8-Br-cGMP was not statistically significant. 8-Br-cGMP also attenuated vasopressin- but not thrombin-stimulated alkalinization of VSM cells. cGMP may decrease vascular tone by decreasing [Ca^{2+}]_i and pH, but these changes are dependent on the contractile agonist studied.

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ethylcarboxy-fluorescein (BCECF-AM) (Molecular Probes, Inc., Eugene, Oregon) for 30 minutes and then washed. A calibration curve was constructed by incubating the A7r5 cells in a buffered salt solution in which 130 mM NaCl was isoosmotically replaced with 130 mM KCl (KSS) and to which 6.9 μM nigericin (Sigma) was added. The pH of the buffer was titrated between 6.5 and 7.5, and the fluorescence emitted at 525 nm was measured after alternating excitation at 500 and 445 nm. To minimize any contribution to pH by the Cl/HCO₃ exchanger in vascular smooth muscle, all experiments were done in HEPES-buffered media.⁶,⁷

We first determined the effect of 8-Br-cGMP on Na-H antiporter activity in the absence of contractile agonists. Cells were equilibrated in the KSS solution and then washed in a sodium-free PSS containing nigericin and 130 mM choline chloride at pH 6.5. The coverslips were then placed in the fluorometer cuvette and an isoosmotic HEPES-buffered salt solution containing 30 mM NaCl, 100 mM choline chloride, and 1.6 mM Ca²⁺, at 24°C, pH 7.3, was added. The initial change in fluorescence ratio (T₁) and at 8 minutes (T₂) were measured, and the changes in intracellular pH were calculated by fitting the fluorescence ratio to a value derived from regression analysis of the calibration curve.

The effect of 10⁻⁵ M AVP or 1 unit/ml thrombin on intracellular pH of VSMC was determined by adding these agonists to preacidified VSMC to which a 30 mM Na⁺ solution, with and without 10⁻³ M 8-Br-cGMP, was added. Again, fluorescence ratios were determined on the initial addition of the agonist and 8 minutes subsequently. Results obtained from the VSMC treated with and without 8-Br-cGMP were analyzed using a Student's unpaired t test with Bonferroni adjustments for multiple comparisons. A p value <0.05 was considered statistically significant.

**Results**

AVP, thrombin, and KCl induced immediate and significant increases in the ionized intracellular calcium concentrations of the cultured A7r5 cells. All values are expressed as mean±SEM in nanomolars before and after an agonist was added: AVP, from 74±13 to 859±244; thrombin, 30±6 to 331±7; and KCl, 94±34 to 432±153. The increase in Ca²⁺ induced by AVP and thrombin was maintained for at least 5 minutes; however, KCl-induced increases in Ca²⁺ were not maintained (Figure 1). An analogue of cGMP, 10⁻⁴ M 8-Br-cGMP, was added. Again, fluorescence ratios were determined on the initial addition of the agonist and 8 minutes subsequently. Results obtained from the VSMC treated with and without 8-Br-cGMP were analyzed using a Student's unpaired t test with Bonferroni adjustments for multiple comparisons. A p value <0.05 was considered statistically significant.

Since 8-Br-cGMP is able to relax blood vessels contracted with all of these contractile agonists, it is likely that 8-Br-cGMP is able to relax vascular...
smooth muscle through multiple mechanisms. Since agonist-induced alkalization may be linked to increased contractile tone, we studied the effect of 8-Br-cGMP on Na-H antiporter activity. Between pH values of 6.5 and 7.5, fluorescence signals obtained from the cells loaded with BCECF varied directly with pH (Figure 2). Since changes in intracellular pH are small under physiological conditions, we preacidified the cells to amplify any cGMP-mediated effects on intracellular pH. When cells were preacidified to pH 6.5 in a sodium-free salt solution, an immediate alkalinization (Figure 3) was observed upon the readdition of sodium and completed by 8 minutes. We found that cGMP decreases the rapid, initial alkalization of VSMC induced by activation of the Na-H antiporter.

AVP induced a rapid alkalization, and the initial, rapid alkalization was significantly attenuated by 10^{-3} M 8-Br-cGMP (Figure 4). Thrombin, unlike AVP, induced a transient acidification and subsequent alkalinization of the VSMC. However, this transient acidification induced by thrombin was blocked by 8-Br-cGMP, and this cyclic nucleotide had no effect on the final intracellular pH of thrombin-treated cells (Figure 5).

**Discussion**

The mechanisms by which elevations in cGMP by endothelium-derived vascular relaxing factors, nitrovasodilators, or ANF are able to relax blood vessels are not known. It is possible that cGMP may decrease receptor binding by contractile agonists, decrease phosphatidylinositol hydrolysis, or decrease the sensitivity of contractile proteins to Ca^{2+}. Using 8-Br-cGMP, we tested the hypothesis that cGMP decreases the ionized intracellular calcium concentration of vascular smooth muscle. It has been demonstrated that 8-Br-cGMP mimics the protein phosphorylation induced by cGMP-dependent vasodilators. We found that 8-Br-cGMP decreases AVP-induced increases in Ca^{2+} but not those calcium transients induced by thrombin or activation of voltage-dependent calcium channels with high concentrations of potassium.

It has been reported that blood vessels bathed in a calcium-free/EGTA solution contract in response to norepinephrine, but these contractions, presumably caused by intracellular calcium release, were...
inhibited by cGMP. These findings suggest that cGMP interferes with the release of intracellular calcium or activation of the contractile apparatus by the intracellular calcium. It has been reported that 8-Br-cGMP was ineffective in relaxing precontracted Triton-X skinned vessels, suggesting that only a small fraction of the relaxation of rat aorta with cyclic nucleotides is due to action of these agents at the level of the contractile proteins. The cGMP-dependent vasodilators nitroglycerine and atrial natriuretic factor have been shown to reduce basal intracellular calcium concentrations and angiotensin II-induced increases in Ca2+. Since cGMP did not completely inhibit vasopressin-induced increases in intracellular calcium, it is likely that cGMP acts on selective stores of intracellular calcium.

cGMP has been demonstrated to relax blood vessels contracted with most agonists; yet, cGMP significantly inhibited only the AVP-induced increases in intracellular Ca2+. It is suggested from this observation that cGMP must be able to relax blood vessels through other mechanisms.

When VSMC are placed in a sodium-free medium, Na-H exchange is inhibited; hydrogen ions accumulate within the cell, and intracellular pH falls. Redistribution of Na+ allows for the Na-H antiporter to alkalinize the intracellular milieu. We hypothesized that cGMP may decrease contractile tone by decreasing intracellular pH. The mechanisms by which intracellular alkalinization may be coupled to increases in vascular tone are not known.

We found that cGMP decreased AVP-induced alkalinization of VSMC. Thrombin, however, induced a transient acidification that was blocked by 8-Br-cGMP and a subsequent alkalinization. It has been reported by others that thrombin may induce a transient acidification, and this acidification is calcium dependent. It is possible that although cGMP does not inhibit thrombin-induced increases in intracellular calcium, cGMP may interfere with the step that links calcium with a transient acidification. In any event, it is clear that elevations in cGMP may decrease vascular tone by a number of mechanisms, and these mechanisms are dependent on the contractile agonist. There are conflicting reports concerning the relative role of Na-H exchange in the control of vascular tone. We have previously reported that "cGMP-dependent" vasodilation is significantly altered in hypertension. A decrease in vascular cGMP may contribute to the increased Na-H exchange and increased vascular resistance that has been reported to occur in hypertension.

References

**KEY WORDS** • vascular smooth muscle • rat studies • cyclic GMP • calcium
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