Activation of the Particulate and Not the Soluble Guanylate Cyclase Leads to the Inhibition of Ca\textsuperscript{2+} Extrusion through Localized Elevation of cGMP*

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We examined whether localized increases in cytosolic cGMP have distinct regulatory effects on the concentration of cytosolic free Ca\textsuperscript{2+} in ECV304 cells. Stimulation of the particulate guanylate cyclase by brain-type natriuretic peptide in fura-2-loaded cells caused a profound potentiation of the ATP-stimulated and thapsigargin-stimulated rise in cytosolic free Ca\textsuperscript{2+}. This effect is mediated by the inhibition of Ca\textsuperscript{2+} efflux via the plasma membrane Ca\textsuperscript{2+}-ATPase pump. Furthermore, the addition of brain-type natriuretic peptide caused the partial inhibition of cation influx in ATP-stimulated cells. In contrast, elevation of cytosolic cGMP by activation of the soluble guanylate cyclase induced by the addition of sodium nitroprusside causes an increased reuptake of Ca\textsuperscript{2+} into the intracellular stores without affecting cation influx or Ca\textsuperscript{2+} efflux. Thus, localized pools of cGMP play distinct regulatory roles in the regulation of Ca\textsuperscript{2+} homeostasis within individual cells. We define a new role for natriuretic peptides in the inhibition of Ca\textsuperscript{2+} efflux that leads to the potentiation of agonist-evoked increases in cytosolic free Ca\textsuperscript{2+}.

Cytosolic free Ca\textsuperscript{2+} and cGMP are fundamental intracellular second messengers that are often interdependent. Thus, cGMP is able to influence Ca\textsuperscript{2+} homeostasis (1–3), and likewise the concentration of cytosolic free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) may influence cGMP levels (4–6). Acting in concert, these second messengers mediate many signaling events from phototransduction to contractile activity in smooth muscle (7–10).

Ca\textsuperscript{2+} homeostasis is governed by the net flow of Ca\textsuperscript{2+} into and out of the cytosol. For Ca\textsuperscript{2+} to act as a signal, its cytosolic concentration is kept low through constant extrusion at the plasma membrane and the sequestration of Ca\textsuperscript{2+} into intracellular stores (11, 12). Initiation of a Ca\textsuperscript{2+} signal involves either the activation of influx or the release of Ca\textsuperscript{2+} from intracellular stores (13, 14). The key sites for control of Ca\textsuperscript{2+} homeostasis are therefore located on the plasma membrane and the endoplasmic reticulum (14). The means by which the [Ca\textsuperscript{2+}]\textsubscript{i} is regulated at the plasma membrane is through Ca\textsuperscript{2+} influx channels and extrusion through the plasma membrane Ca\textsuperscript{2+}-ATPase pump (PMCA) and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. The sarco/endoplasmic reticulum contains the Ca\textsuperscript{2+}-ATPase pump (SERCA) responsible for sequestration of Ca\textsuperscript{2+} along with inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) or ryanodine-sensitive Ca\textsuperscript{2+} release channels. There is a considerable amount of evidence for the nonuniformity of intracellular Ca\textsuperscript{2+} signals. These take the form of microdomains (15), Ca\textsuperscript{2+} waves (16), polarized responses (17), and effects limited to the plasma membrane (18, 19). This localization of the key processes involved in Ca\textsuperscript{2+} homeostasis supports the generation of complex spatial and temporal patterns of Ca\textsuperscript{2+} signaling. These are necessary if Ca\textsuperscript{2+} is to fulfill its role as a multitasking second messenger.

Likewise, cGMP formation is not spread uniformly within cells as revealed by its distinct particulate and cytosolic sources (20). The guanylate cyclase domain of the particulate enzyme is contained within natriuretic peptide receptors at the plasma membrane (21–24). The soluble guanylate cyclase is stimulated by NO (4, 25). Diffusion of cGMP will be limited by the presence of five different ubiquitously expressed phosphodiesterases (PDE1, -2, -3, -5, and -9) involved in the hydrolysis of cGMP (20, 21, 25, 26). Some phosphodiesterases and their isomers are soluble, while others are plasma membrane-bound (4, 27). The separate subcellular sources of cGMP offer the possibility of localized elevation of cGMP within a cell, putatively under the plasma membrane or within the cytosol.

The purpose of this study is to examine the influence of the cytosolic and particulate pools of cGMP on Ca\textsuperscript{2+} homeostasis in ECV304 human epithelial cells. We therefore examined the effects on [Ca\textsuperscript{2+}]\textsubscript{i}, of elevated cGMP using the NO donor sodium nitroprusside (SNP) and compared its actions with the effects of cGMP elevated by brain-type natriuretic peptide (BNP). BNP acts on both NP-A and NP-B receptors and is thus able to stimulate particulate guanylate cyclase activity (21). We also investigated how the decay of the [Ca\textsuperscript{2+}]\textsubscript{i} transients, cation influx, and extrusion of Ca\textsuperscript{2+} are affected by elevated cGMP.

We find not only that the particulate and soluble guanylate cyclases modulate [Ca\textsuperscript{2+}]\textsubscript{i}, through different Ca\textsuperscript{2+} homeostatic mechanisms but that activation of the particulate and not the soluble guanylate cyclase is coupled to the inhibition of Ca\textsuperscript{2+} efflux. We also find that NO-stimulated soluble guanylate cyclase and not the particulate guanylate cyclase is involved in the stimulation of Ca\textsuperscript{2+} sequestration into the intracellular Ca\textsuperscript{2+} stores. We therefore provide evidence for the control of Ca\textsuperscript{2+} homeostasis by localized pools of cGMP in ECV304 epithelial cells. In addition, we define a new role for natriuretic...
peptides as circulating modulators of Ca\(^{2+}\) efflux that act to potentiate existing Ca\(^{2+}\) signals.

**EXPERIMENTAL PROCEDURES**

**Fluorescence Ratio Imaging for \([\text{Ca}^{2+}]_c\)**. Measurements—Cells were grown on 22-mm glass coverslips for 48 h and then loaded with 2 \(\mu\)M fura-2/AM in the presence of 0.0125% pluronic F127 at 37 °C for 45 min. The bathing medium was Na\(^{-}\)-HEPES buffered physiological saline (HPS) composed of 145 mM NaCl, 5 mM KCl, 1 mM Na\(_2\)HPO\(_4\), 1 mM MgSO\(_4\)·7H\(_2\)O, 10 mM HEPES, 10 mM d-glucose, and 1 mM CaCl\(_2\). All reagents were purchased from Merck or Sigma. All cGMP-elevating agents were purchased from Calbiochem-Novabiochem.

Coverslips were transferred to a chamber warmed at 37 °C and held on the stage of a Nikon Diaphot inverted epifluorescence microscope. Cells were alternately excited at 340- and 380-nm wavelengths by means of a PTI D101 dual excitation light source. The 510-nm emission was detected by an ICCD camera (Photonic Science). Digital images were then analyzed by Image Master software for Windows (PTI). \([\text{Ca}^{2+}]_c\) was calculated by calibrating the photometric 340/380-nm ratio signal (28). Reagents and vehicle controls were added to cells by bolus addition to the sample chamber.

**Mn\(^{2+}\) Quench Protocols**—Cells grown on 22-mm coverslips were bathed in HPS and excited at 340 and 360 nm. Instead of ratio images, the fluorescence intensity images were collected for analysis. The addition of 1 mM Mn\(^{2+}\) in the form of MnCl\(_2\) is indicated by the solid bar above each trace (see Fig. 5).

**Ca\(^{2+}\) Extrusion Measurements**—Cells grown in 80-cm\(^2\) flasks were treated with trypsin/EDTA and resuspended in 5 ml of Dulbecco’s modified Eagle’s medium. They were then centrifuged for 2 min at 190 \(\times\) g, and the pellet was resuspended in Na\(^{-}\)-HEPES-buffered physiological saline made up in milliQ (Millipore, Ltd.) purified water where no Ca\(^{2+}\) had been added. Cells were then separated into 1-ml Eppendorf tubes, centrifuged at 1000 rpm for 15 s in a microcentrifuge, and resuspended in the sample buffer. The procedure was repeated three times. Cells were then transferred to a sample chamber at 37 °C. Fluorescence was measured using a PTI Deltascan dual excitation spectrofluorimeter exciting alternatively at 340 and 380 nm and collecting emission at 510 nm. \([\text{Ca}^{2+}]_c\) was calculated by calibrating the photometric 340/380-nm ratio signal (28).

**cGMP Measurements**—Cells were suspended in HPS. After centrifuging at 1000 rpm for 2 min, they were incubated periods of 2–10 min in the presence of the different reagents tested. Cyclic GMP levels were determined using the nonacetylation method in a cGMP enzyme immunoassay system as described in the manual provided by Amersham Pharmacia Biotech (Biotrak cellular communication assays).

**Cell Count**—The number of cells present in 1 ml of suspension for Ca\(^{2+}\) extrusion experiments was measured using a hemocytometer.

**Statistics**—Values presented are means ± S.E. Student’s t-test was used to compare data sets.

**RESULTS**

Effects of BNP, SNP, and Bt2cGMP on \([\text{Ca}^{2+}]_c\)—Direct stimulation of the particulate guanylate cyclase was induced by bolus addition of 100 nM BNP to fura-2-loaded cells. In contrast, stimulation of the soluble guanylate cyclase was achieved by the addition of the NO donor SNP. The addition of BNP prior to 1 \(\mu\)M ATP caused about a 4-fold potentiation of the ATP-stimulated rise in \([\text{Ca}^{2+}]_c\) (Fig. 1A). The mean ATP-stimulated

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Fig. 1. Comparison of Ca\(^{2+}\) responses to Bt2cGMP, BNP, and SNP. A, control ATP-evoked rise in \([\text{Ca}^{2+}]_c\), and application of 100 nM SNP followed by the addition of 1 \(\mu\)M ATP. The addition of reagents is indicated by the solid bars. Cells on coverslips were loaded with 2 \(\mu\)M fura-2/AM and placed in a chamber at 37 °C bathed with HPS containing 1 mM Ca\(^{2+}\). B, control ATP-evoked rise in \([\text{Ca}^{2+}]_c\), and application of 100 \(\mu\)M SNP followed by an ATP-stimulated increase in \([\text{Ca}^{2+}]_c\). Cells were prepared as described above. C, control ATP-evoked rise in \([\text{Ca}^{2+}]_c\), and application of 100 \(\mu\)M Bt2cGMP on the sustained \([\text{Ca}^{2+}]_c\) rise evoked by 1 \(\mu\)M ATP. Cells were treated as described for A, D, histogram of the mean \([\Delta[\text{Ca}^{2+}]_c]\) peak values for control 1 \(\mu\)M ATP-evoked rise in \([\text{Ca}^{2+}]_c\), and for 1 \(\mu\)M ATP-stimulated cells in the presence of BNP or SNP. These data summarize the mean \([\Delta[\text{Ca}^{2+}]_c]\), peak values obtained for the different cGMP-elevating agents on 1 \(\mu\)M ATP-stimulated cells indicated as shown in B for BNP and in C for SNP. The mean \([\Delta[\text{Ca}^{2+}]_c]\), peak for Bt2cGMP shown corresponds to the application of 100 \(\mu\)M Bt2cGMP prior to the ATP-induced rise in \([\text{Ca}^{2+}]_c\), and thus is not a reflection of A. Mean values shown above each corresponding column are expressed ± S.E. and were collected at the maximal \([\text{Ca}^{2+}]_c\), peaks obtained after the addition of ATP, n = number of experiments, shown under each corresponding column.
Δ[Ca²⁺] between basal [Ca²⁺] levels and peak [Ca²⁺] in the presence of BNP was approximately 3 μM. This was significantly greater (p < 0.05) than the control 1 μM ATP response of approximately 600 nM (as indicated in Fig. 1D).

In contrast, SNP was ineffective at enhancing the ATP-induced [Ca²⁺] rise (Fig. 1B). The mean Δ[Ca²⁺], peak for this response was about 500 nM. This response was not significantly different (p < 0.05) from control ATP-stimulated rise in [Ca²⁺], (Fig. 1D). These results suggest that an endogenous increase in cGMP levels induced by activation of the particulate guanylate cyclase leads to the large enhancement of the ATP-stimulated [Ca²⁺] response.

The addition of the cGMP analogue Bt₂cGMP to the sustained component of the ATP-evoked rise in [Ca²⁺], caused an additional elevation in [Ca²⁺], (Fig. 1C). This additional increase in [Ca²⁺], (approximately 300 nM) occurs only upon the addition of the compound to the sustained component of the ATP-evoked rise in [Ca²⁺], and not when added prior to stimulation by ATP (Fig. 1D). This effect is abolished by removal of Bt₂cGMP from the medium, causing a restoration of [Ca²⁺], levels to that of the sustained ATP-induced [Ca²⁺], rise. These experiments show that Bt₂cGMP only causes a moderate rise in [Ca²⁺], in cells previously stimulated by ATP.

The extent to which SNP and BNP were able to induce an increase in cGMP levels was measured by enzyme immunoassay and compared with the respective [Ca²⁺], increases obtained in the presence of 1 μM ATP. The effects of both compounds were measured for different concentrations (0.5, 1, 50, 500, and 1000 nM for BNP and 0.5, 1, 100, 500, and 1000 μM for SNP) and after 2-, 6-, and 10-min incubations. The resulting data were used to construct the cGMP/[Ca²⁺], curves for each of the incubation periods (Fig. 2. A, B, and C, respectively). Both SNP and BNP cause an increase in cGMP levels above that of control untreated cells. Despite its ability to raise cGMP levels, SNP did not induce the potentiation of the ATP-stimulated rise in [Ca²⁺],. The linear fit reveals that in the presence of SNP, cGMP increases are independent from [Ca²⁺],. There is no significance difference at the p < 0.05 level between control ATP-stimulated [Ca²⁺], rises and those in the presence of SNP for any concentration used or incubation time analyzed. Although at low concentrations BNP may cause a modest rise in cGMP (2–3 fmol/10⁶ cells), it is still able to potentiate the [Ca²⁺], rise induced by ATP, even at different incubation times. This effect is demonstrated by the nonlinear relationship between cGMP and [Ca²⁺], in the presence of BNP. In some cases, particularly for the higher concentrations of BNP, the fura-2 dye reached saturation levels. These results show that both SNP and BNP cause an increase in cGMP levels, but only BNP is able to potentiate the ATP-stimulated rise in [Ca²⁺],.

Enhancement of the Thapsigargin-induced Rise in [Ca²⁺], by BNP—Although BNP activates the particulate guanylate cyclase contained within the NP-A and NP-B receptors, we considered whether BNP may enhance G-protein-coupled signal transduction in ATP-stimulated cells. If so, this could lead to an enhanced release of Ca²⁺ from the InsP₃-sensitive stores, thereby causing the observed potentiation of the ATP-stimulated rise in [Ca²⁺],. We therefore investigated if InsP₃-independent elevation of [Ca²⁺], as induced by thapsigargin could also be potentiated by BNP. Thapsigargin is an inhibitor of the SERCA pump that causes a slow “leak” of Ca²⁺ from the stores independently of InsP₃ production (29). As predicted, the addition of 100 nM thapsigargin caused a gradual but sustained elevation in [Ca²⁺], (Fig. 3A). The mean peak Δ[Ca²⁺], value obtained for this control response was 889 ± 26 (n = 5). In the presence of BNP, the sustained increase in [Ca²⁺], evoked by thapsigargin was enhanced (Fig. 3B). The mean peak Δ[Ca²⁺], value for this response was 1611 ± 142 nM (n = 3). This response was significantly greater (p < 0.05) than the control response in the absence of BNP. The potentiation of the thapsigargin-induced rise in [Ca²⁺], by BNP was dose-dependent (Fig. 3C). Thus, BNP was able to potentiate the ATP-stimulated rise in [Ca²⁺],, thereby causing the observed potentiation of the ATP-stimulated rise in [Ca²⁺],. These results suggest that an endogenous increase in cGMP levels induced by activation of the particulate guanylate cyclase leads to the large enhancement of the ATP-stimulated [Ca²⁺], response.

In contrast, SNP was ineffective at enhancing the ATP-induced rise in [Ca²⁺],. As predicted, the addition of the cGMP analogue Bt₂cGMP to the sustained component of the ATP-evoked rise in [Ca²⁺], caused an additional elevation in [Ca²⁺],. This effect is abolished by removal of Bt₂cGMP from the medium, causing a restoration of [Ca²⁺], levels to that of the sustained ATP-induced [Ca²⁺], rise. These experiments show that Bt₂cGMP only causes a moderate rise in [Ca²⁺], in cells previously stimulated by ATP.

The extent to which SNP and BNP were able to induce an increase in cGMP levels was measured by enzyme immunoassay and compared with the respective [Ca²⁺], increases obtained in the presence of 1 μM ATP. The effects of both compounds were measured for different concentrations (0.5, 1, 50, 500, and 1000 nM for BNP and 0.5, 1, 100, 500, and 1000 μM for SNP) and after 2-, 6-, and 10-min incubations. The resulting data were used to construct the cGMP/[Ca²⁺], curves for each of the incubation periods (Fig. 2. A, B, and C, respectively). Both SNP and BNP cause an increase in cGMP levels above that of control untreated cells. Despite its ability to raise cGMP levels, SNP did not induce the potentiation of the ATP-stimulated rise in [Ca²⁺],. The linear fit reveals that in the presence of SNP, cGMP increases are independent from [Ca²⁺],. There is no significance difference at the p < 0.05 level between control ATP-stimulated [Ca²⁺], rises and those in the presence of SNP for any concentration used or incubation time analyzed. Although at low concentrations BNP may cause a modest rise in cGMP (2–3 fmol/10⁶ cells), it is still able to potentiate the [Ca²⁺], rise induced by ATP, even at different incubation times. This effect is demonstrated by the nonlinear relationship between cGMP and [Ca²⁺], in the presence of BNP. In some cases, particularly for the higher concentrations of BNP, the fura-2 dye reached saturation levels. These results show that both SNP and BNP cause an increase in cGMP levels, but only BNP is able to potentiate the ATP-stimulated rise in [Ca²⁺],.

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Ca\(^{2+}\) stores. Cells were stimulated with 1 mM ATP in a Ca\(^{2+}\)-free medium containing 500 \(\mu\)M Ca\(^{2+}\) at 37 °C. Since SNP did not potentiate the ATP-stimulated rise in [Ca\(^{2+}\)], we examined whether it could influence replenishing of the Ca\(^{2+}\) stores, since we show below that SNP does not affect Ca\(^{2+}\) extrusion.

**SNP Causes a Fast Reuptake of [Ca\(^{2+}\)]\(_i\) into the Stores**—The results presented so far show that BNP and not SNP may potentiate the [Ca\(^{2+}\)]\(_i\) rise in thapsigargin-stimulated cells independently from agonist activation.

We have shown that BNP may potentiate a [Ca\(^{2+}\)]\(_i\) rise induced by thapsigargin (Fig. 3A and B). We therefore examined the effects of SNP and BNP on ATP-stimulated cation influx by performing Mn\(^{2+}\) quenches. The 340- and 360-nm fluorescence were recorded in fura-2-loaded cells. The 340-nm trace reflects the changes in [Ca\(^{2+}\)]\(_i\), that occur upon agonist stimulation. The 360-nm fluorescence represents the isosbestic point on the fura-2 excitation spectrum and does not change with respect to the degree of Ca\(^{2+}\) binding to the dye (28) but is sensitive to the presence of Mn\(^{2+}\) (33). The 340-nm (dashed trace) and 360-nm (black trace) fluorescence intensities for a representative control 1 mM ATP-induced response in the absence of SNP (Fig. 4A). The mean maximum rate of decay in control cells was 1.0 ± 0.2 nM/s (n = 4) (Fig. 4A). The mean rate of decay obtained in the presence of SNP was 1.7 ± 0.3 nM/s (n = 8) (Fig. 4B). This was significantly faster than the control cells (p < 0.05). We conclude from these experiments that activation of the soluble guanylate cyclase by SNP induces a faster reuptake of [Ca\(^{2+}\)]\(_i\), into the intracellular stores, since we show below that SNP does not affect Ca\(^{2+}\) extrusion.

**Partial Inhibition of Cation Influx by BNP**—We have shown that BNP may potentiate a [Ca\(^{2+}\)]\(_i\) rise induced by thapsigargin (Fig. 3B). We therefore examined the effects of SNP and BNP on ATP-stimulated cation influx by performing Mn\(^{2+}\) quenches. The 340- and 360-nm fluorescence were recorded in fura-2-loaded cells. The 340-nm trace reflects the changes in [Ca\(^{2+}\)]\(_i\), that occur upon agonist stimulation. The 360-nm fluorescence represents the isosbestic point on the fura-2 excitation spectrum and does not change with respect to the degree of Ca\(^{2+}\) binding to the dye (28) but is sensitive to the presence of Mn\(^{2+}\) (33). The 340-nm (dashed trace) and 360-nm (black trace) fluorescence intensities for a representative control 1 mM ATP response in the presence of 1 mM Mn\(^{2+}\) are shown in Fig. 5A.
The rate of quench of the 360-nm signal increases (fluorescence decreases) after the addition of the agonist. This occurs due to the activation of store-operated cation influx channels. The rate of Mn\(^{2+}\) quench after stimulation of cation influx was shown by FIG. 5.

**FIG. 5. Comparison of ATP-stimulated Mn\(^{2+}\) quenches to BNP and SNP.** A, control ATP-induced cation influx (n = 17). Cells were loaded with fura-2, and the application of 1 mM extracellular Mn\(^{2+}\) was as shown by the solid bar. Changes in fluorescence were recorded at 340 nm (dashed trace) and 380 nm (solid trace). Measurements were carried out at 37 °C. Fluorescence was normalized to the maximum fluorescence units. B, comparison of Mn\(^{2+}\) extrusion in response to ATP, BNP, SNP, SNAP, and Zaprinast. A, the initial rate of Ca\(^{2+}\) extrusion and the effects of adding 1 μM ATP in either the absence (black trace; n = 9) or the presence (gray trace) of 100 nM BNP (n = 8). ECV304 cells were incubated for 10 min at room temperature with (gray trace) or without (black trace) BNP. Cells were suspended in 1 ml of HPS buffer containing about 60 nM free Ca\(^{2+}\) and 5 μM fura-2 free acid. Application of 1 μM ATP is shown by the arrows above each corresponding trace. Ca\(^{2+}\) extrusion measurements were measured at 37 °C. B, comparison of control initial and 1 μM ATP-stimulated efflux (black trace) (n = 8) with initial and 1 μM ATP-stimulated efflux in the presence of 100 μM SNP (gray trace) (n = 8). Each respective arrow represents the addition of 1 μM ATP to either control or SNP-treated cells. Cells were treated as explained in A. C, comparison of mean efflux values corresponding to control intrinsic and ATP-stimulated rates alone or in the presence of BNP, SNP, SNAP, and Zaprinast. Efflux values are located above each corresponding column ± S.E., and n numbers for each experiment are shown below each column.

The rate of quench of the 360-nm signal increases (fluorescence decreases) after the addition of the agonist. This occurs due to the activation of store-operated cation influx channels. The rate of Mn\(^{2+}\) quench after stimulation of cation influx was carried out at 37 °C. Fluorescence was normalized to the maximum fluorescence units. B, application of 100 nM BNP followed by 1 μM ATP (n = 18). Experiments were carried out as in A. C, application of 100 μM SNP followed by 1 μM ATP (n = 15). Experiments were carried out as in A. D, comparison of cation influx stimulated by application of 1 μM ATP in response to either 100 nM BNP, 100 μM SNP, or 20 μM SKF96365. Rates were measured as the maximum peak rate for each experiment. The mean influx values are shown above each corresponding column and are expressed as mean ± S.E. The total number of experiments (n) is shown below each column.
BNP (trace is the response in the presence of and unstimulated cells (represented by the black trace). Glucamine (NMDG) in the presence of ATP-stimulated efflux (Fig. 6) was inhibited by 91%. In contrast to the effects of BNP, a 90% inhibition of ATP-stimulated efflux was observed. The ATP-stimulated efflux was inhibited by 88%, and the ATP-stimulated efflux was inhibited by 91%. In contrast to the effects of BNP, a 10-min incubation with SNP did not affect either initial or ATP-stimulated efflux (Fig. 6B, gray trace). The same experimental procedure was used for another NO donor, SNAP, for comparison. SNAP (100 μM) did not affect either the initial or ATP-stimulated efflux, thereby confirming our results obtained with SNP (Fig. 6C). In order to verify that the effects of BNP on the inhibition of efflux occur as a consequence of elevated cGMP, we used the cGMP-specific phosphodiesterase inhibitor Zaprinast (10 μM), like BNP, inhibited the initial and ATP-stimulated efflux. This suggests that the inhibition of efflux by BNP is mediated by a cGMP-dependent pathway. The data including efflux values for Zaprinast are summarized in Fig. 6C.

These results indicate that BNP inhibits both initial and ATP-stimulated Ca²⁺ extrusion in a cGMP-dependent manner. Neither SNAP nor SNP shows any effect on efflux. The potentiation of the ATP or thapsigargin-stimulated rise in [Ca²⁺]c evoked by the addition of BNP (Figs. 1A, 2B, and 3B) may therefore be due to the inhibition of efflux.

BNP Causes the Inhibition of the PMCA—It has been established that most cells have two mechanisms by which Ca²⁺ may be extruded: the PMCA and the Na+/Ca²⁺ exchanger (11, 12). Ca²⁺ extrusion by the Na+/Ca²⁺ exchanger is inhibited when cells are bathed in a low Na⁺-containing medium (12, 18). We investigated the effects of BNP on [Ca²⁺]c in cells bathed in a saline solution where N-methyl-D-glucamine was the iso-osmotic substitute for Na⁺. Experiments were performed in 1 mM extracellular Ca²⁺. In the absence of Na⁺, the addition of 100 mM BNP caused an increase in resting [Ca²⁺]c of 592 ± 41 nM (n = 14) (Fig. 7). These results contrast those obtained for cells washed in a normal physiological saline containing 145 mM Na⁺ and 1 mM Ca²⁺, where the addition of 100 mM BNP caused no apparent increase in resting [Ca²⁺]c (Fig. 1A). SNP had no effect on basal [Ca²⁺]c in cells bathed in a medium lacking Na⁺ (Fig. 7, dashed trace). When similar experiments were performed in the absence of Na⁺, the addition of ATP to BNP-treated cells produced a response so large that the Ca²⁺ indicator saturated (data not shown), producing off scale data sets in the analysis software.

DISCUSSION

The concept of spatial control of [Ca²⁺]c signals is well established, but comparable evidence for cGMP is lacking. This study reveals how cGMP synthesized at the plasma membrane inhibits Ca²⁺ extrusion via the PMCA, while cGMP generated in the cytosol increases Ca²⁺ uptake into the intracellular stores (data summarized in Fig. 8).

Cyclic GMP has previously been shown to increase Ca²⁺ sequestration into intracellular stores (1). In muscle cells, this effect is mediated via phosphorylation of phospholamban (35). At this stage, we cannot be certain of how cGMP elevated by SNP activates Ca²⁺ reuptake in ECV304 cells. Nonetheless,
the specific source of cGMP appears to be important to this effect. SNP is effective at increasing Ca\(^{2+}\) reuptake (Fig. 4, A and B) but not at modulating efflux (Fig. 6B). The lack of effect of SNP on efflux is corroborated by our results with another NO donor, SNAP (Fig. 6C). It is possible that activating the particulate guanylate cyclase has some influence on Ca\(^{2+}\) sequestration, but any such effects are masked by the predominant actions of BNP on plasma membrane Ca\(^{2+}\) fluxes.

The addition of BNP dramatically potentiates the response to ATP (Figs. 1A and 2B), reduces influx (Fig. 5, B and D), and inhibits efflux (Fig. 6, A and D). We therefore examined the relationship between the elevation of cGMP stimulated by either BNP or SNP and the potentiation of the ATP-evoked elevation of [Ca\(^{2+}\)]. Fig. 2 reveals that after incubations of 2, 6, or 10 min with a guanylate cyclase activator, cGMP elevated by BNP causes a potentiation of the [Ca\(^{2+}\)] response, whereas when cGMP is elevated by SNP the effect on the [Ca\(^{2+}\)] response is negligible. When cGMP is elevated by BNP, only very modest increases in cGMP are needed in order to have a substantial effect on [Ca\(^{2+}\)]. Thus, it appears that activation of the soluble guanylate cyclase is disassociated from the potentiation of the [Ca\(^{2+}\)] signal, whereas elevation of cGMP by BNP is tightly coupled to the potentiation of the [Ca\(^{2+}\)] response. The data suggest that the two cGMP pools can act independently.

We believe that the BNP-mediated reduction in influx is a consequence of the increased elevation of [Ca\(^{2+}\)], especially adjacent to the plasma membrane. Both store-operated (36, 37) and voltage-operated Ca\(^{2+}\) channels (38) show Ca\(^{2+}\)-dependent inactivation. Evidence from luminescent and fluorescent indicators suggests that the subplasmalemmal Ca\(^{2+}\) can be greater than in the bulk cytosol (18, 19, 39, 40). Elevated subplasmalemmal Ca\(^{2+}\) has even been postulated as the control mechanism for regulating Ca\(^{2+}\) entry (41, 42). It is plausible that the cGMP-mediated inhibition of Ca\(^{2+}\) efflux leads to an elevation of Ca\(^{2+}\) adjacent to the influx channels, hence causing some degree of inactivation. Inhibition of efflux and the resulting inhibition of influx may be energetically favorable during the maintained elevation of [Ca\(^{2+}\)] in stimulated cells. Lower overall fluxes would also help the termination of [Ca\(^{2+}\)] signals.

The BNP-mediated increase in resting [Ca\(^{2+}\)], observed in the absence of extracellular Na\(^{+}\) (Fig. 7) suggests that the BNP-induced inhibition of efflux occurs via the PMCA rather than the Na\(^+/\)Ca\(^{2+}\) exchanger. This argument also applies to stimulated cells (Fig. 1A). The ∼90% inhibition of efflux observed with BNP and Zaprinast applies to cells where efflux has been stimulated by either warming or the addition of ATP (Fig. 6C). In unstimulated cells, BNP-sensitive efflux exerts a proportionally smaller effect on resting [Ca\(^{2+}\)], (before the ATP addition; Fig. 1A). The combination of other compensatory factors, such as Ca\(^{2+}\) buffering, Ca\(^{2+}\) sequestration, and Ca\(^{2+}\) efflux via the Na\(^+/\)Ca\(^{2+}\) exchanger (18, 43–45), may all influence resting [Ca\(^{2+}\)]. Only when the two efflux pathways are both impaired, as in the absence of extracellular Na\(^{+}\) (Fig. 7), does resting [Ca\(^{2+}\)] increase.

The ability of BNP to potentiate the response to thapsigargin shows that BNP potentiates an elevation of [Ca\(^{2+}\)], and not signal transduction events prior to Ca\(^{2+}\) release. Since the cGMP-specific PDE5 inhibitor Zaprinast also inhibits Ca\(^{2+}\) efflux, this further substantiates our argument that BNP-induced inhibition of Ca\(^{2+}\) efflux is mediated via cGMP. Although we have established that cGMP-mediated inhibition of Ca\(^{2+}\) extrusion occurs via the PMCA, we have not determined if its
action is direct or as a result of cGMP-dependent protein kinase. Results from earlier work on the heart (46), on smooth muscle (47, 48), and in hepatocytes (49) indicate that cGMP can also stimulate Ca$^{2+}$ efflux. Whether efflux is stimulated or inhibited may depend on how the cGMP signal is transduced downstream of cGMP synthesis in a particular cell type. Further experimentation is needed to determine how cGMP modulates PMCA activity.

The differential effects of cGMP elevation on Ca$^{2+}$ homeostasis and the importance of its location may serve as an explanation for the results obtained with Bt$_2$cGMP. We have shown that the addition of Bt$_2$cGMP only caused a moderate additional increase in the ATP-induced sustained rise in [Ca$^{2+}$]$_i$ (Fig. 1C). The potentiation observed with the analogue is not as prominent as that of BNP (Fig. 1A). This is probably because this agent acts uniformly throughout the cells, influencing both reuptake and efflux, thereby losing the more specific effects of localized cGMP.

Natriuretic peptides are potent circulating vasodilators generated in the right atrium, brain, kidney, and endothelium (23). The mode of action we describe here represents a novel mechanism by which these hormones can exert their physiological actions. However, the NO-mediated effects on Ca$^{2+}$ reuptake into the intracellular stores are quite distinct from the modulation of Ca$^{2+}$ efflux seen with BNP. These differential effects may be particularly important in cells continually exposed to either NO or natriuretic peptides (50, 51).

In summary, we find that activation of the particulate guanylate cyclase leads to inhibition of Ca$^{2+}$ efflux by the PMCA, a reduction in cation influx, and a marked potentiation of agonist-evoked elevation of [Ca$^{2+}$]$_i$. Activation of the soluble guanylate cyclase did not influence plasma membrane Ca$^{2+}$ fluxes but did increase Ca$^{2+}$ reuptake into the intracellular stores. We have shown in this report that differential production of cGMP within a cell can cause distinct effects on [Ca$^{2+}$]$_i$, thus somewhat clarifying the complex interrelation between these two intracellular signaling molecules. The distinction between the different sources of cGMP is therefore necessary to evaluate the physiological and intracellular effects of this cyclic nucleotide in different cell types.

Acknowledgments—We acknowledge Dr. José Cancela, Dr. Anne Green, Professor Tuilio Pozzan, and Professor Peter Cobbold for valuable comments and discussion of the research.

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