

Modulation of Rabbit Ventricular Cell Volume and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ Cotransport by cGMP and Atrial Natriuretic Factor

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ABSTRACT Previously we showed that atrial natriuretic factor (ANF) decreases cardiac cell volume by inhibiting ion uptake by $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Digital video microscopy was used to study the role of guanosine 3',5'-monophosphate (cGMP) in this process in rabbit ventricular myocytes. Each cell served as its own control, and relative cell volumes ($\text{volume}_{\text{test}}/\text{volume}_{\text{control}}$) were determined. Exposure to 10 μM 8-bromo-cGMP (8-Br-cGMP) reversibly decreased cell volume to 0.892 ± 0.007 ; the ED_{50} was $0.77 \pm 0.33 \mu\text{M}$. Activating guanylate cyclase with 100 μM sodium nitroprusside also decreased cell volume to 0.889 ± 0.009 . In contrast, 8-bromo-adenosine 3',5'-monophosphate (8-Br-AMP; 0.01–100 μM) neither altered cell volume directly nor modified the response to 8-Br-cGMP. The idea that cGMP decreases cell volume by inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport was tested by blocking the cotransporter with 10 μM bumetanide (BUM) and removing the transported ions. After BUM treatment, 10 μM 8-Br-cGMP failed to decrease cell volume. Replacement of Na^+ with *N*-methyl-D-glucamine or Cl^- with methanesulfonate also prevented 8-Br-cGMP from shrinking cells. The data suggest that 8-Br-cGMP, like ANF, decreases ventricular cell volume by inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Evidence that ANF modulates cell volume via cGMP was also obtained. Pretreatment with 10 μM 8-Br-cGMP prevented the effect of 1 μM ANF on cell volume, and ANF suppressed 8-Br-cGMP-induced cell shrinkage. Inhibiting guanylate cyclase with the quinolinedione LY83583 (10 μM) diminished ANF-induced cell shrinkage, and inhibiting cGMP-specific phosphodiesterase with M&B22948 (Zaprinast; 100 μM) amplified the volume decrease caused by a low dose of ANF (0.01 μM) approximately fivefold. In contrast, neither 100 μM 8-Br-cAMP nor 50 μM forskolin affected the response to ANF. The effects of ANF, LY83583, and M&B22948 on cGMP levels in isolated ventricular myocytes were confirmed by ^{125}I -cGMP radioimmunoassay. These data argue that ANF shrinks cardiac cells by increasing intracellular cGMP, thereby inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Basal cGMP levels also appear to modulate cell volume.

INTRODUCTION

By moving ions inward across the plasma membrane, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport plays an important role in regulating cell volume in a number of tissues (Eveloff and

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Warnock, 1987; Larson and Spring, 1987; MacKnight, 1987; O'Grady, Palfrey, and Field, 1987; Hoffman and Simonsen, 1989). $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport has not been characterized fully in the heart. Nevertheless, intracellular ion activity and isotopic flux studies suggest that the cotransporter is present in rabbit ventricle (Baumgarten and Duncan, 1987) and cultured chick myocytes (Aiton, Chipperfield, Lamb, Ogden, and Simmons, 1981; Frelin, Chassande, and Lazdunski, 1986; Liu, Jacob, Piwnicka-Worms, and Lieberman, 1987), and measurements of cardiac cell volume by video microscopy suggest that $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport contributes to both the maintenance of cell volume under isotonic conditions and the response to osmotic stress (Clemo and Baumgarten, 1991a; Drewnowska and Baumgarten, 1991).

Rather than functioning at a static rate, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport is modulated by several hormones including atrial natriuretic factor (ANF), a peptide produced by the heart that has vasodilatory, natriuretic, and diuretic properties. ANF stimulates $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in rat aortic vascular smooth muscle (O'Donnell and Owen, 1986), but inhibits the cotransporter in flounder intestinal epithelium (O'Grady, Field, Nash, and Rao, 1985) and cultured bovine aortic endothelial cells (O'Donnell, 1989). Both the inhibition and stimulation of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in these systems is attributed to an ANF-induced increase in the cytosolic level of guanosine 3',5'-monophosphate (cGMP). $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport is not regulated by cGMP in some other tissues, however. Instead, adenosine 3',5'-monophosphate (cAMP) stimulates cotransport in avian erythrocytes and epithelial cells (Palfrey and Rao, 1983), but inhibits it in human erythrocytes (Garay and Ciccone, 1982). Modulation of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport by vasopressin and bradykinin has also been noted (Brock, Brugnara, Canessa, and Gimbrone, 1986).

We recently provided evidence that ANF inhibits $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in both atrial and ventricular myocytes and thereby decreases cardiac cell volume (Clemo and Baumgarten, 1991a). Although ANF acts by stimulating particulate guanylate cyclase and elevating the intracellular cGMP concentration in many systems (Waldman and Murad, 1987; Tremblay, Gerzer, and Hamet, 1988; Wildey, Misono, and Graham, 1992), either cAMP or cGMP could be involved in the control of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in heart. ANF stimulates particulate guanylate cyclase and increases cGMP in rabbit (Cramb, Banks, Rugg, and Aiton, 1987) and rat (Rugg, Aiton, and Cramb, 1989; McCall and Fried, 1990) myocytes as well as rabbit Purkinje fibers (Anand-Srivastava, Thibault, Sola, Fon, Ballak, Charbonneau, Haile-Meskel, Garcia, Genest, and Cantin, 1989). On the other hand, ANF is reported to decrease cAMP in atrial and ventricular myocytes (Anand-Srivastava and Cantin, 1986) and Purkinje fibers (Anand-Srivastava et al., 1989). Cramb et al. (1987) found that cAMP in ventricular myocytes was unaffected by ANF, however.

The present experiments suggest that ANF decreases ventricular cell volume by stimulating guanylate cyclase, increasing cytoplasmic cGMP, and thereby slowing $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Moreover, changes in cell volume observed on inhibiting guanylate cyclase or cGMP-specific phosphodiesterase in the absence of ANF imply that basal cGMP levels are a determinant of cell volume under isotonic conditions.

Some of these studies were reported previously in abstract form (Clemo and Baumgarten, 1991b).

METHODS

Cell Isolation Procedure

Ventricular myocytes were isolated from adult rabbits (New Zealand White, 1.5–2.5 kg) with collagenase and pronase as previously described (Poole, Halestrap, Price, and Levi, 1989; Clemo and Baumgarten, 1991a). For cell isolation, the Ca^{2+} -free modified Tyrode's solution was supplemented with (mM): 20 taurine, 10 creatine, 0.4 NaH_2PO_4 , and 3.5 MgCl_2 instead of MgSO_4 (pH 7.25, bubbled with 100% O_2). Myocytes were temporarily stored in KB solution and were used within 6 h of harvesting. This procedure typically gave >60% rod-shaped, Ca^{2+} -tolerant, viable cells. Only nonbranching, quiescent cells without membrane blebs were chosen for volume studies.

Solutions and Drugs

Modified Tyrode's solution contained (mM): 130 NaCl, 5 KCl, 2.5 CaCl_2 , 10 glucose, 1.2 MgSO_4 , and 5 HEPES (pH 7.4, bubbled with 100% O_2). *N*-Methyl-D-glucamine chloride (NMDG; Sigma Chemical Co., St. Louis, MO) was substituted for NaCl to make Na^+ -free media, and the corresponding salts of methanesulfonic acid ($\text{CH}_3\text{SO}_3\text{H}$; Aldrich Chemical Co., Milwaukee, WI) were substituted for NaCl, KCl, and CaCl_2 to make Cl^- -free media. Cells also were studied after swelling in hypotonic (0.8T) solutions as done previously (Cleml and Baumgarten, 1991a). NaCl was held constant at 65 mM, and the osmolarity was adjusted by adding mannitol to make either a hypotonic (0.8T; 60 mM) or isotonic (1T; 138 mM) medium. We have shown that partial substitution of mannitol for NaCl in isotonic solution does not affect cell length, width, or volume (Drewnowska and Baumgarten, 1991). Measurements were made in flowing solutions (~ 5 ml/min), except when ANF was used. Because of ANF's expense, bath flow was stopped after 1 min, a time sufficient to change bath volume >10 times. In control studies, cells exposed to Tyrode's solution with flow stopped for 10 min showed no change in morphometric parameters. All experiments were done at room temperature (21–22°C), and a freezing point depression osmometer (Osmette S; Precision Systems Inc., Natick, MA) was used to verify bathing solution osmolarity.

[Ser¹⁰³-Tyr¹²⁶]ANF (rat atriopeptin III; Calbiochem Corp., La Jolla, CA), 8-bromo-cGMP (8-Br-cGMP; Sigma Chemical Co.) and 8-Br-cAMP (Sigma Chemical Co.) were added to bathing media immediately before use. The rat and rabbit atrial peptides are identical (Willey et al., 1992). Stock solutions of bumetanide (BUM; Hoffmann-La Roche, Nutley, NJ), forskolin (Sigma Chemical Co.), 6-anilino-5,8-quinolinedione (LY83583; Eli Lilly, Indianapolis, IN), and 2-*o*-propoxyphenyl-8-azapurin-6-one (M&B22948, Zaprinast®; Rhône-Poulenc Rorer, Dagenham, Essex, UK) were made in dimethylsulfoxide (DMSO; Fluka Chemical Corp., Ronkonkoma, NY). DMSO did not affect cell volume at the final concentrations in the tissue bath (0.05–0.10%, vol/vol) (Drewnowska and Baumgarten, 1991; present results).

Determination of Myocyte Dimensions

The determination of cell volume was previously described in detail (Cleml and Baumgarten, 1991a). In brief, a high-resolution TV camera was mounted on an inverted microscope (E. Leitz GmbH, Wetzlar, Germany) equipped with Hoffman modulation optics (Nikon $\times 40$, 0.55 NA) and images were captured on-line by a video frame-grabber (Targa-M8; Truevision, Indianapolis, IN). The resolution of the digitization was ~ 0.25 μm /pixel. A commercial image analysis program (JAVA; Jandel Scientific, Corte Madera, CA) providing contrast enhancement and image magnification in software, and a custom program written in ASYST (Keithley Asyst, Rochester, NY) were used to determine the area of the cell's image, cell width at 1- μm intervals, and cell length.

Assuming that changes in cell width and thickness in test solutions are proportional, relative cell volume was determined as:

$$\text{volume}_t/\text{volume}_c = (\text{area}_t \times \text{width}_t)/(\text{area}_c \times \text{width}_c)$$

where t and c refer to test (e.g., ANF) and control solutions, respectively. We previously showed that changes in cell width and thickness are proportional with osmotic stress (Drewnowska and Baumgarten, 1991). Based on repeated measurements of single images and measurements of multiple images of a cell, the estimates of cell volume (i.e., area \times width) were reproducible to <1% (Clemo and Baumgarten, 1991a; Drewnowska and Baumgarten, 1991).

In the present studies, virtually all of the changes in relative cell volume resulted from changes in cell width (and thickness) rather than cell length. For an exemplar 25 $\mu\text{m} \times 25 \mu\text{m} \times 150 \mu\text{m}$ cell, a 10% decrease in relative cell volume at a constant length corresponds to a $\sim 1.3\text{-}\mu\text{m}$ decrease in cell width, and under optimal conditions, a 2–3% decrease in relative cell volume (i.e., $\sim 0.3\text{--}0.4 \mu\text{m}$ decrease in cell width) was shown to be statistically significant. This resolution depends in part on the fact that cell width represents an average calculated from ~ 150 measurements. For purposes of determining the lateral position of an image, the lowest resolution component of the system was the frame-grabber. According to Inoué (1989), microscope optics readily permit lateral position measurements to within <10% of the Airy disk radius. For the present optics, the Airy disk radius was calculated as $\sim 0.5 \mu\text{m}$.

Determination of cGMP

To verify the effects of ANF, LY83583, and M&B22948 under the present experimental conditions, cGMP levels in myocytes were determined by ^{125}I -radioimmunoassay using a commercially available kit (NEN Research, E.I. Dupont de Nemours & Co., Boston, MA) adapted from the protocols of Steiner, Parker, and Kipnis (1972). Myocytes were washed three times in ~ 40 times their volume of Tyrode's solution and allowed to settle by gravity. Aliquots (0.1 ml; $4.30\text{--}6.48 \times 10^6$ cells/ml) were resuspended in Tyrode's solution with or without drug to give a final volume of 0.5 ml. At selected times, the reaction was stopped with 0.5 ml of 10% trichloroacetic acid, and the myocytes were homogenized for 15 s four times (model SDT1810, 10 mm shaft, maximum speed; Tekmar Co., Cincinnati, OH). After centrifugation ($\sim 1,200g$, 10 min), 0.8 ml of the supernatant was extracted six times with 2-ml portions of water-saturated diethyl ether to remove the trichloroacetic acid, and the aqueous phase was evaporated to dryness at 60°C under a stream of dry N_2 . The residue was dissolved in 0.8 or 1.0 ml of 50 mM sodium acetate buffer (pH 6.2), and 0.1-ml aliquots were acetylated and assayed for cGMP in duplicate according to kit instructions. The duplicate assays were averaged and corrected for cGMP recovery based on the recovery of $91 \pm 2\%$ ($n = 3$) of marker [^3H]cGMP.

Statistics

Data are reported as mean \pm standard error; n represents the number of cells. After analysis of variance, multiple comparisons were made by the Bonferroni method. When only a single comparison was planned, Student's t test was used. The null hypothesis was rejected for $P < 0.05$.

RESULTS

Width, length, and volume of ventricular myocytes were initially measured in Tyrode's solution. The dimensions of ventricular cells were: width, $25.0 \pm 1.5 \mu\text{m}$; length, $147.5 \pm 4.5 \mu\text{m}$ ($n = 17$). Calculation of cell volume as area \times width,

assuming cells are brick-shaped with equal width and thickness, gave a volume of 97.0 ± 12.5 pl. If a cylindrical rather than a brick shape is assumed, cell volume was 76.2 ± 9.8 pl (i.e., in a ratio of $4/\pi$). Uncertainty regarding absolute volume also arises because cell width and thickness may not be equal. For rabbit ventricular cells, however, the ratio of minor-to-major cross-sectional axis is ~ 0.8 , and cells settle on either axis (Nassar, Reedy, and Anderson, 1987; Drewnowska and Baumgarten, 1991). To avoid these uncertainties, each cell was used as its own control and subsequent experimental values are presented relative to control values.

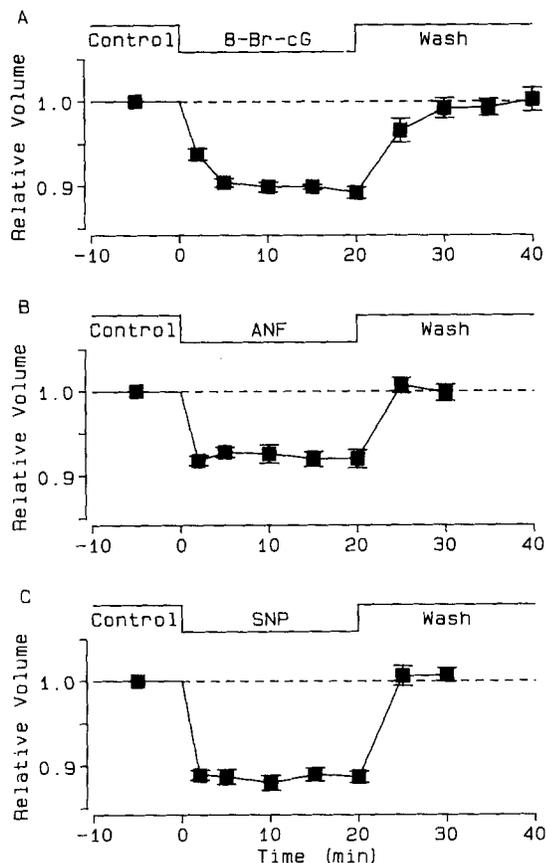


FIGURE 1. Time course of changes in ventricular cell volume during a 20-min exposure to $10 \mu\text{M}$ 8-Br-cGMP (8-Br-cG), $1 \mu\text{M}$ ANF, or $100 \mu\text{M}$ SNP and washout. Relative cell volume was calculated as a fraction of the volume of the same cell in control solution. Measurements were made at 2 and 5 min and then at 5-min intervals. 8-Br-cGMP (A) decreased cell volume to 0.938 ± 0.007 at 2 min, 0.904 ± 0.005 at 5 min, and 0.892 ± 0.007 at 20 min ($n = 5$). The small decrease between 5 and 20 min was not significant. Cell volume returned to its initial value after 10 min of washout of 8-Br-cGMP. ANF (B) and SNP (C) decreased cell volume to 0.918 ± 0.006 ($n = 5$) and 0.889 ± 0.009 ($n = 5$), respectively, within 2 min, and volume remained stable thereafter. In both cases, cell volume returned to its initial value after 5 min of washout.

8-Br-cGMP Reduces Ventricular Cell Volume

If cGMP is a second messenger for ANF in heart, 8-Br-cGMP, a nonmetabolizable, membrane-permeant analogue of cGMP, should mimic the effect of ANF on cell volume. Fig. 1 A shows that exposure to $10 \mu\text{M}$ 8-Br-cGMP for 20 min decreased the volume of ventricular myocytes. Cell volume was significantly less than control after 2 min in 8-Br-cGMP-containing Tyrode's solution, and decreased to 0.904 ± 0.005 ($n = 5$) after 5 min. Thereafter, cell volume did not significantly change, although the

minimum volume, 0.892 ± 0.007 , was attained at 20 min. Cell shrinkage occurred because of a decrease in cell width; 8-Br-cGMP did not significantly affect cell length. Upon washout of 8-Br-cGMP, cell volume returned to its initial value within 10 min.

For comparison, the time course of cell volume reduction caused by $1 \mu\text{M}$ ANF, a maximally effective concentration (Clemo and Baumgarten, 1991a), is illustrated in Fig. 1 B. The ANF-induced shrinkage and washout were more rapid than those for 8-Br-cGMP. The decrease in cell volume with ANF, 0.918 ± 0.006 ($n = 5$), was complete at 2 min, and control volume was restored by 5 min of washout. As with 8-Br-cGMP, the decrease in volume occurred without a significant change in cell length. The slower onset and washout of 8-Br-cGMP were probably due to the time required for it to cross the cell membrane. Nevertheless, the effect of $10 \mu\text{M}$ 8-Br-cGMP on cell volume was slightly greater than the maximum effect of ANF.

An important class of agents that increase cGMP levels in heart is the nitrovasodilators, such as sodium nitroprusside (Diamond, Ten Eick, and Trapani, 1977), which

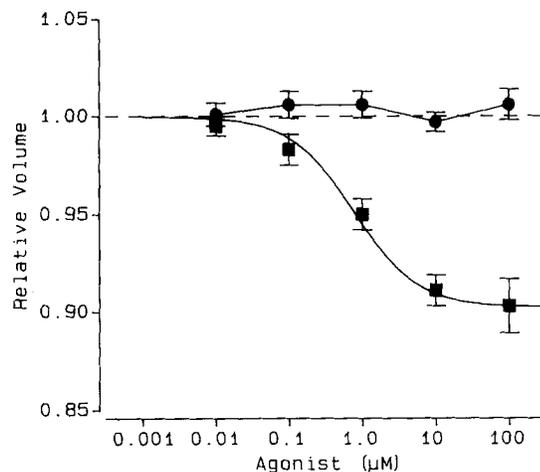


FIGURE 2. Cumulative dose-response relationships for reduction of cell volume by 8-Br-cAMP and 8-Br-cGMP. Cells were exposed to 0.01, 0.1, 1, 10, and $100 \mu\text{M}$ 8-Br-cGMP (squares, $n = 7$) or 8-Br-cAMP (circles, $n = 10$) for 5-min periods. 8-Br-cAMP did not significantly affect cell volume. The response of each cell to 8-Br-cGMP was analyzed separately assuming 1:1 binding. The ED_{50} for 8-Br-cGMP was $0.77 \pm 0.33 \mu\text{M}$, and the smooth dose-response curve was drawn from the average parameters.

activate soluble guanylate cyclase (Murad, Mittal, Arnold, Katsuki, and Kimura, 1978). As shown in Fig. 1 C, exposure to $100 \mu\text{M}$ sodium nitroprusside (SNP) also rapidly and reversibly decreased cell volume. After 20 min in SNP, cell volume was 0.889 ± 0.009 ($n = 5$) relative to that in control. As in the previous protocols, the decrease in cell volume occurred without a significant change in cell length. The decrease in cell volume achieved by elevating cGMP with SNP was indistinguishable from that observed with 8-Br-cGMP. Thus, effects on cardiac cell volume were not peculiar to 8-Br-cGMP.

Volume reduction on application of 8-Br-cGMP was dose dependent, and a cumulative dose-response curve is depicted in Fig. 2. Relative volume was significantly less than control after 5-min exposures to 0.01, 0.1, 1, 10, and $100 \mu\text{M}$ 8-Br-cGMP, but the shrinkages induced by 10 and $100 \mu\text{M}$ 8-Br-cGMP were not significantly different. A dose-response curve was constructed for each cell assuming a single occupancy binding model. Based on this analysis, the ED_{50} was 0.77 ± 0.33

μM ($n = 7$). Because there are probably several steps between the binding of 8-Br-cGMP and reduction of cell volume, the ED_{50} is likely to differ from the K_d for cyclic nucleotide binding.

Whereas 8-Br-cGMP significantly reduced cell volume, cell volume was unaffected by 0.01–100 μM 8-Br-cAMP (Fig. 2). For example, the volume of ventricular cells after a 5-min exposure to 100 μM 8-Br-cAMP was 1.006 ± 0.008 ($n = 10$), a value not different from control. Lengthening 8-Br-cAMP exposure to 10 min was also ineffective. Finally, a 10-min exposure to 50 μM forskolin, an activator of adenylate cyclase, did not alter cell volume ($n = 5$; see also Fig. 7).

Although the simplest explanation for the data in Figs. 1 and 2 is that cell volume is modulated by cGMP and not by cAMP, an alternative mechanism should be considered. A cGMP-dependent phosphodiesterase that is stimulated by cGMP and catalyzes the hydrolysis of cAMP has been reported (Beavo, 1988; Weishaar, Kobylarz-Singer, Keiser, Haleen, Major, Rapundalo, Peterson, and Panek, 1990). Furthermore, cAMP levels in heart tissues of some species are decreased by ANF while cGMP is elevated (Anand-Srivastava and Cantin, 1986; Anand-Srivastava et al., 1989). It is possible, then, that 8-Br-cGMP decreases cAMP levels by increasing its rate of degradation, and that a decline of cAMP levels, rather than elevation of cGMP, is the proximate cause of the fall in cell volume.

To test the idea that a decrease in cAMP leads to reduction of cell volume, cells were pretreated with 100 μM 8-Br-cAMP to “clamp” the cAMP level and then exposed to 8-Br-cGMP. Cell volume did not change during the 5-min pretreatment with 100 μM 8-Br-cAMP. Upon addition of 10 μM 8-Br-cGMP, however, volume decreased to 0.910 ± 0.008 ($n = 5$) in 10 min. This decrease in volume was indistinguishable from that observed without 8-Br-cAMP in the bath, and the results suggested that a fall in cAMP is not required for 8-Br-cGMP to elicit cell shrinkage.

cGMP Blocks $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ Cotransport

We previously reported that inhibiting $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport decreases cardiac cell volume (Clemo and Baumgarten, 1991a; Drewnowska and Baumgarten, 1991). Fig. 3 tests the hypothesis that 8-Br-cGMP reduces cardiac cell volume by blocking the same cotransporter. If the hypothesis is correct, inhibiting $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport with BUM should preclude the action of 8-Br-cGMP. Fig. 3A depicts the effect of pretreating with 10 μM BUM in isotonic Tyrode's solution. BUM decreased myocyte volume to 0.887 ± 0.009 ($n = 5$), and exposure to 10 μM 8-Br-cGMP failed to induce further shrinkage of BUM-treated cells (squares). Volume returned to its control value after 20 min of washout of BUM and 8-Br-cGMP. The crucial issue, the effect of 8-Br-cGMP on BUM-treated cells, is more clearly addressed by calculating cell volume relative to that in BUM (circles). Expressed in this way, cell volume in BUM plus 8-Br-cGMP was 0.996 ± 0.009 , a value indistinguishable from unity.

Although Fig. 3A is consistent with the idea that cGMP inhibits $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport, it might be argued that physical shrinkage caused by BUM, rather than its inhibition of the cotransporter, attenuated the effect of 8-Br-cGMP on cell volume. To rule out this possibility, the effect of BUM pretreatment on the response to 8-Br-cGMP was evaluated in osmotically swollen cells (Fig. 3B). Previously we showed that the volume of rabbit myocytes remains stable in hypotonic solutions rather than

undergoing a regulatory volume decrease (Drewnowska and Baumgarten, 1991). When placed in 0.8T Tyrode's solution, myocytes swelled to 1.168 ± 0.015 ($n = 6$) times their control volume. Although cell volume still decreased upon exposure to $10 \mu\text{M}$ BUM, it remained slightly greater than control, 1.042 ± 0.007 (squares). Nevertheless, $10 \mu\text{M}$ 8-Br-cGMP failed to decrease cell volume after pretreatment with BUM. After addition of 8-Br-cGMP, cell volume was 1.040 ± 0.014 of the control volume. Expressed relative to volume in 0.8T plus BUM, cell volume was

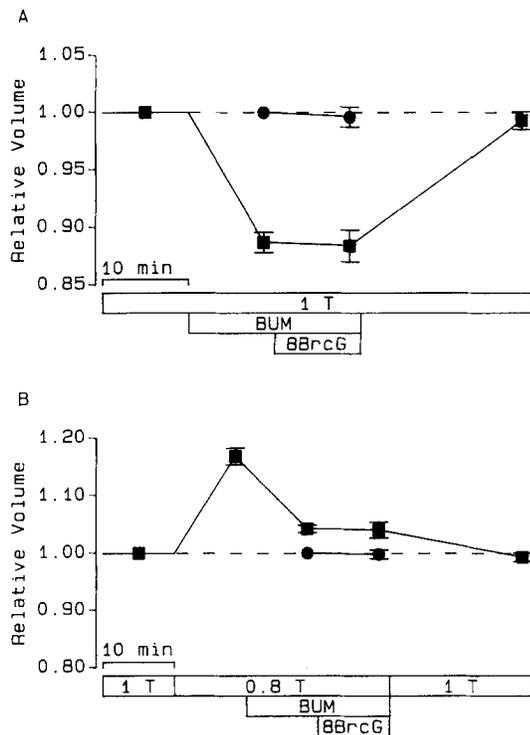


FIGURE 3. Inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport with BUM prevents 8-Br-cGMP-induced cell shrinkage. In this and following figures, squares represent cell volume relative to the control volume in 1T Tyrode's solution and describe volume changes over the duration of the experiment; circles represent selected cell volumes relative to volumes in the preceding solution. (A) In 1T Tyrode's solution, a 10-min exposure to $10 \mu\text{M}$ BUM decreased cell volume to 0.887 ± 0.009 (squares), and addition of $10 \mu\text{M}$ 8-Br-cGMP (8Br-cG) for 10 min failed to alter cell volume. Cell volume in 8-Br-cGMP plus BUM was 0.996 ± 0.009 relative to that in BUM alone (circles). $n = 5$ cells. (B) To eliminate the chance that BUM-induced shrinkage prevented ANF's effect, the study was repeated in

cells preswollen in 0.8T media for 10 min. Although cell volume after a 10-min exposure to BUM in 0.8T solution remained greater than the volume in 1T solution (squares), 8-Br-cGMP still did not significantly alter volume after 10 min. Cell volume in BUM plus 8-Br-cGMP was 0.998 ± 0.008 relative to that in BUM alone (circles). $n = 6$ cells. In both panels volume returned to its initial value after 20 min of washout in 1T.

0.998 ± 0.008 after the challenge with 8-Br-cGMP (circles). These results argue that cell shrinkage itself cannot account for the suppression of the effect of 8-Br-cGMP by BUM.

$\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport requires extracellular Na^+ and Cl^- , and removal of these ions from the bathing solution blocks the cotransport process (Geck, Pietrzyk, Burckhardt, Pfeiffer, and Heinz, 1980; O'Grady et al., 1987). If 8-Br-cGMP shrinks cells by blocking $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport, it should have no effect on cells that are

bathed in either Na^+ - or Cl^- -free media since $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport would have already been inhibited. This prediction is tested in Fig. 4. Cell volume decreased to 0.952 ± 0.005 ($n = 5$) after 10 min in Cl^- -free 1T media (Fig. 4A). Exposure to $10 \mu\text{M}$ 8-Br-cGMP did not decrease cell volume further. Cell volume was 0.947 ± 0.007 of the control volume (squares) or 0.995 ± 0.006 of that in Cl^- -free medium alone (circles). To ensure that shrinkage caused by Cl^- -free medium was not responsible for blocking cGMP's effect, this experiment was repeated in five osmotically swollen cells, as shown in Fig. 4B. When the bathing solution was changed from Cl^- -free 1T to Cl^- -free 0.8T solution, relative cell volume increased over 10 min from 0.943 ± 0.006 to 1.092 ± 0.006 . 8-Br-cGMP did not significantly change cell volume under these conditions either. Relative to cell volume in 0.8T Cl^- -free media, cell volume was 1.003 ± 0.005 after exposure to $10 \mu\text{M}$ 8-Br-cGMP (circles).

The Na^+ dependence of cGMP's effect is demonstrated in Fig. 4C. Removal of Na^+ for 10 min decreased cell volume to 0.950 ± 0.010 ($n = 4$). In the absence of Na^+ , $10 \mu\text{M}$ 8-Br-cGMP did not significantly alter cell volume. Relative to cell volume in Na^+ -free solution, cell volume was 1.007 ± 0.014 after exposure to 8-Br-cGMP (circles). To check whether the shrinkage caused by Na^+ -free solution interfered with the action of cGMP, this experiment was repeated in five osmotically swollen cells (Fig. 4D). Exposure to Na^+ -free 0.8T solution increased volume from 0.954 ± 0.007 to 1.086 ± 0.009 . Again, $10 \mu\text{M}$ 8-Br-cGMP did not significantly change cell volume. After treating cells with 8-Br-cGMP, cell volume was 1.001 ± 0.014 of that in Na^+ -free 0.8T solution (circles). These experiments establish that the effect of 8-Br-cGMP depends on two of the ions required for $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport, extracellular Na^+ and Cl^- .

Is cGMP the Second Messenger for ANF-induced Cardiac Cell Shrinkage?

The experiments presented so far show that 8-Br-cGMP and elevating cGMP with SNP mimic the effect of ANF on cell volume (Fig. 1) and suggest that cGMP, like ANF (Clemo and Baumgarten, 1991a), reduces cell volume by inhibiting $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport. Because ANF increases cytosolic cGMP levels in heart by stimulating guanylate cyclase (Cramb et al., 1987; Anand-Srivastava et al., 1989; Rugg et al., 1989) and ANF modulates $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport in other tissues via cGMP (O'Grady et al., 1985; O'Donnell and Owen, 1986; O'Donnell, 1989), it is appealing to postulate that cGMP is the second messenger for ANF-induced cardiac cell shrinkage.

One test of the idea that cGMP is the second messenger of ANF-induced cardiac cell shrinkage is shown in Fig. 5. As expected for a second messenger system, pretreating cells with 8-Br-cGMP, an analogue of the putative second messenger, attenuated the effect of ANF. Exposure to $10 \mu\text{M}$ 8-Br-cGMP shrank cells in isotonic Tyrode's solution to 0.887 ± 0.012 ($n = 5$) of their control volume, and the addition of $1 \mu\text{M}$ ANF did not further affect cell volume (Fig. 5A; squares). Relative to cell volume in 8-Br-cGMP-containing media, cell volume after addition of ANF was 1.006 ± 0.009 (circles). As before, this experiment was repeated in osmotically swollen cells to demonstrate that shrinkage caused by 8-Br-cGMP did not interfere with ANF's action (Fig. 5B). In cells swollen by exposure to 0.8T solution, $10 \mu\text{M}$ 8-Br-cGMP reduced cell volume from 1.177 ± 0.013 to 1.046 ± 0.008 ($n = 7$). After

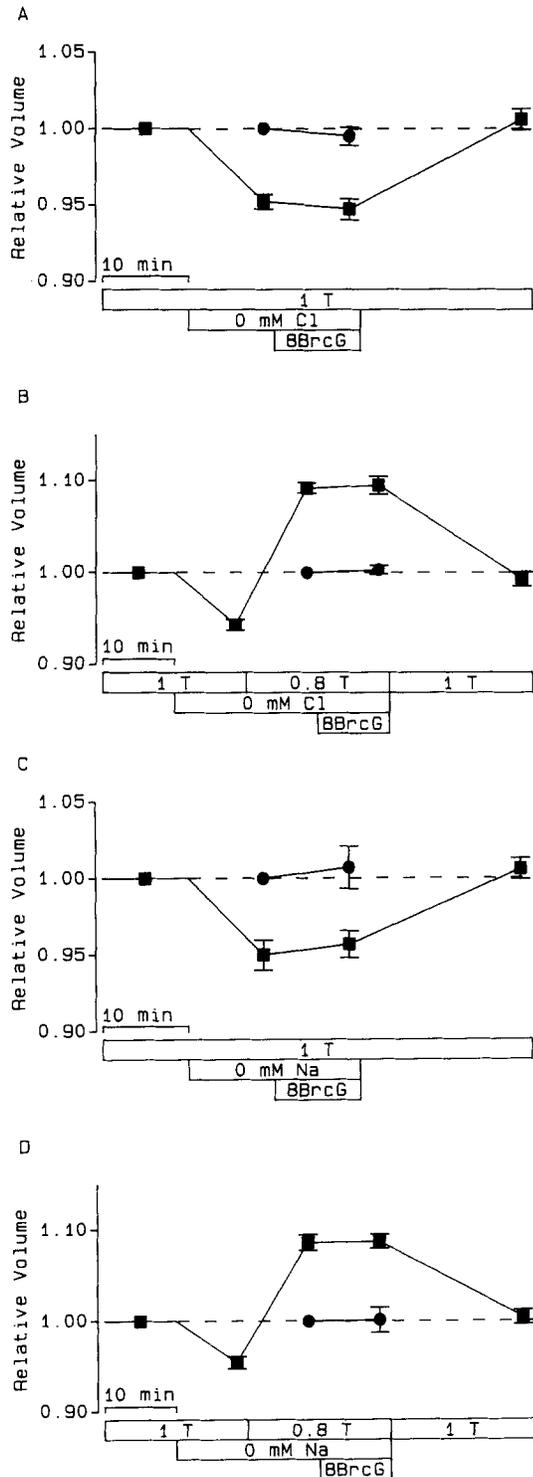


FIGURE 4. Inhibition of Na⁺/K⁺/2Cl⁻ cotransport by replacement of [Cl⁻]_o with methanesulfonate (A and B) or [Na⁺]_o with NMDG (C and D) prevents 8-Br-cGMP-induced cell shrinkage. After 10 min in Cl⁻- or Na⁺-free 1T solutions (A and C), cell volume decreased to 0.952 ± 0.005 ($n = 5$) and 0.950 ± 0.010 ($n = 4$) relative to control, respectively, and adding $10 \mu\text{M}$ 8-Br-cGMP (8Br-cGMP) did not alter volume further (squares). Relative to volume in Cl⁻- or Na⁺-free medium alone, volume after a 10-min exposure to $10 \mu\text{M}$ 8-Br-cGMP was 0.995 ± 0.006 and 1.007 ± 0.014 , respectively (circles). Shrinkage in Cl⁻- or Na⁺-free medium was not responsible for lack of effect of 8-Br-cGMP (B and D). Cells were exposed to Cl⁻- or Na⁺-free 1T medium for 10 min, and then were swollen in Cl⁻-free or Na⁺-free 0.8T medium for 10 min. Cell volume remained greater than control in 0.8T Cl⁻-free and 0.8T Na⁺-free solutions, 1.092 ± 0.006 ($n = 5$) and 1.086 ± 0.009 ($n = 5$), respectively (squares), but 8-Br-cGMP still failed to reduce cell volume. Relative to cell volume in Cl⁻- and Na⁺-free 0.8T media, volume was 1.003 ± 0.005 and 1.001 ± 0.014 , respectively, after a 10-min exposure to $10 \mu\text{M}$ 8-Br-cGMP (circles). In each case, initial volume was restored after 20 min of washout.

addition of 1 μM ANF, cell volume was 1.044 ± 0.004 relative to the control volume or 0.998 ± 0.007 relative to the volume in 8-Br-cGMP 0.8T solution (circles). This small change in cell volume was not significant.

If ANF causes cell shrinkage by increasing intracellular cGMP levels, pretreatment of cells with ANF should attenuate the effect of exogenously applied 8-Br-cGMP just as pretreatment with 8-Br-cGMP blocked the action of ANF. To demonstrate this, the experiments depicted in Fig. 5 were repeated with the order of application of ANF and 8-Br-cGMP reversed. Fig. 6A shows the protocol in isotonic Tyrode's solution. Exposure to 1 μM ANF reduced cell volume to 0.924 ± 0.003 ($n = 5$), and addition

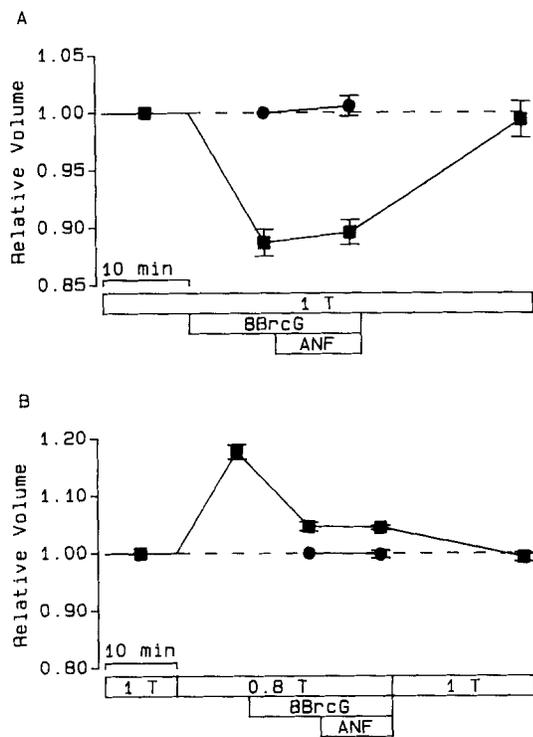


FIGURE 5. 8-Br-cGMP prevents ANF-induced cell shrinkage. (A) A 10-min exposure to 10 μM 8-Br-cGMP (8Br-cG) reduced cell volume to 0.887 ± 0.012 in 1T media (squares). Relative to cell volume in 8-Br-cGMP alone, volume 10 min after addition of 1 μM ANF was 1.006 ± 0.009 (circles). $n = 5$ cells. (B) ANF's failure was not a result of cell shrinkage itself. Cells were first swollen in 0.8T solution for 10 min, and then exposed for 10 min to 8-Br-cGMP in 0.8T solution. Volume in 0.8T solution with 8-Br-cGMP was 1.046 ± 0.008 (squares), a value slightly greater than control. ANF was not effective under these conditions either. Relative to cell volume in 0.8T solution with 8-Br-cGMP, volume after a 10-min exposure to ANF was 0.998 ± 0.007 ($n = 7$; circles). In both cases, initial volume was restored after 20 min of washout.

of 10 μM 8-Br-cGMP caused a further significant reduction of cell volume to 0.896 ± 0.007 or 0.969 ± 0.007 of the volume of ANF-treated cells (circles). Although the 3.1% decrease in cell volume caused by 8-Br-cGMP after pretreatment with ANF was significant, it was significantly smaller than the 10.8% decrease caused by 8-Br-cGMP alone (see Fig. 1). That is to say, ANF markedly attenuated the effect of 8-Br-cGMP but did not block it completely. Similar results were obtained when this protocol was repeated in osmotically swollen cells, as is illustrated in Fig. 6B. Treatment with 1 μM ANF reduced cell volume from 1.181 ± 0.005 to 1.105 ± 0.009 , and addition of 10 μM 8-Br-cGMP reduced volume further to 1.057 ± 0.007 ($n = 5$). Relative to the

volume in 0.8T media containing ANF, cell volume after exposure to 8-Br-cGMP was 0.957 ± 0.010 . Again, this 4.3% reduction of cell volume is much less than that caused by 8-Br-cGMP alone.

As discussed above, 8-Br-cAMP did not effect cell volume and 8-Br-cGMP did not shrink cells by decreasing intracellular cAMP. Fig. 7 illustrates two sets of experiments that rule out the possibility that a component of ANF's effect on cardiac cell volume is mediated by a reduction of cAMP levels. Two methods were used to activate the cAMP system. Fig. 7A shows the effect of pretreatment of cells with 100 μ M

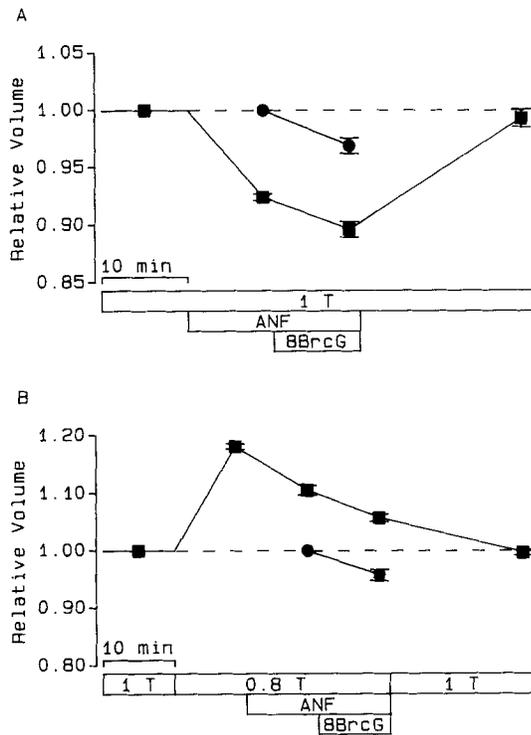


FIGURE 6. ANF attenuates 8-Br-cGMP-induced cell shrinkage. (A) A 10-min exposure to 1 μ M ANF reduced cell volume to 0.924 ± 0.003 in 1T media (squares). Relative to the cell volume in ANF, volume after a 10-min exposure to 10 μ M 8-Br-cGMP (8Br-cG) was 0.969 ± 0.007 ($n = 5$; circles). The decrease in cell volume caused by 8-Br-cGMP was statistically significant. However, the 3.1% reduction in volume after pretreatment with ANF was significantly smaller than the 10.8% decrease in cell volume observed with 8-Br-cGMP alone (cf. Fig. 1). (B) Attenuation of the effect of 8-Br-cGMP by ANF was not a result of cell shrinkage itself. Cells were first swollen in 0.8T solution for 10 min, and then exposed to ANF in 0.8T solution for 10 min. Volume in 0.8T solution with

ANF was 1.105 ± 0.009 (squares), a value slightly greater than control. The response to 8-Br-cGMP was reduced under these conditions as well. Relative to the volume in 0.8T solution with ANF, cell volume after a 10-min exposure to 8-Br-cGMP was 0.957 ± 0.010 ($n = 5$; circles). In both cases, initial volume was restored after 20 min of washout.

8-Br-cAMP, and Fig. 7B shows pretreatment with 50 μ M forskolin, an activator of adenylate cyclase. As expected from the data illustrated in Fig. 2, neither agent significantly altered cell volume. A more important result is the response to ANF while the cAMP system was activated. After pretreatment with 8-Br-cAMP, 1 μ M ANF reduced cell volume from 0.995 ± 0.007 to 0.923 ± 0.007 ($n = 5$), and after forskolin, from 1.004 ± 0.008 to 0.925 ± 0.008 ($n = 5$). The $\sim 8\%$ decreases in cell volume induced by 1 μ M ANF in these pretreatment protocols were indistinguishable from that observed with 1 μ M ANF alone, 8.2% (Fig. 1; also see Clemo and

Baumgarten, 1991a). The results argue that ANF does not cause cell shrinkage by virtue of decreasing cytoplasmic cAMP levels.

Another prediction of the hypothesis that cGMP mediates the cell volume effect of ANF can be tested. If cGMP is the second messenger for ANF, the effect of ANF should be attenuated by manipulations that decrease cGMP production and should be amplified by manipulations that decrease cGMP breakdown. Fig. 8 illustrates the effect of LY83583, which inhibits guanylate cyclase in heart (Schmidt, Sawyer, Truex, Marshall, and Fleisch, 1985; MacLeod and Diamond, 1986) and should suppress an ANF-induced increase in cGMP. First, the responsiveness of cells to ANF was verified; in these six cells, 1 μ M ANF decreased cell volume by 8% to 0.920 ± 0.004 . Then,

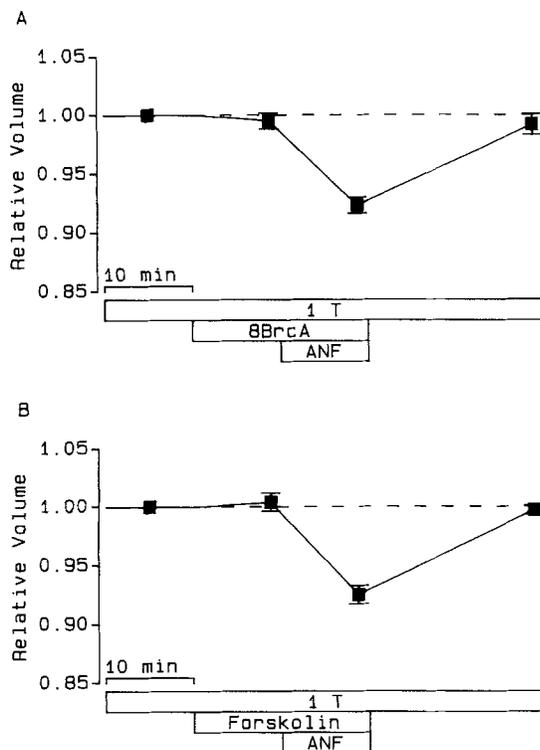


FIGURE 7. Activating the cAMP system with 8-Br-cAMP or forskolin did not attenuate ANF-induced cell shrinkage. A 10-min pretreatment of cells with 100 μ M 8-Br-cAMP (A, 8Br-cA, $n = 5$) or 50 μ M forskolin (B, $n = 5$) did not significantly alter cell volume; the volumes after treatment were 0.995 ± 0.007 and 1.004 ± 0.008 , respectively. Exposure to 1 μ M ANF for 10 min reduced cell volumes to 0.923 ± 0.007 in the presence of 8-Br-cAMP and to 0.925 ± 0.008 in the presence of forskolin. The shrinkage observed under these conditions is not significantly different from that observed with ANF alone, 0.918 ± 0.006 (see Fig. 1). In both cases, initial volume was restored after 20 min of washout.

after washout of ANF, cells were exposed to 10 μ M LY83583. LY83583 caused a small but significant increase in cell volume from 0.999 ± 0.004 to 1.019 ± 0.005 (squares) after 10 min, and similar data were obtained after only 5 min. Finally, ANF was added to the bath. Cell volume significantly decreased when LY83583-treated cells were exposed to 1 μ M ANF, but the change in volume was much smaller than with ANF alone. Relative to volume in LY83583, 1 μ M ANF decreased volume to 0.980 ± 0.004 (circles) in treated cells, a 2% change. Attenuation of the effect of ANF on cell volume by LY83583 is consistent with the idea that cGMP is the second messenger for ANF.

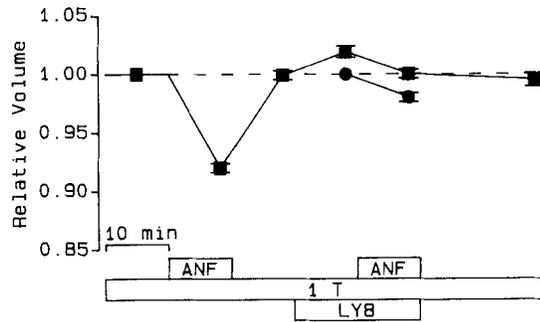


FIGURE 8. LY83583, a guanylate cyclase inhibitor, attenuated the effect of ANF. A 10-min exposure to 1 μM ANF decreased cell volume to 0.920 ± 0.004 of control. After return of cell volume to its initial value with washout of ANF for 10 min, the response to LY83583 (LY8) was studied. Exposure to 10 μM LY83583 for 10 min increased cell volume from

0.999 ± 0.004 to 1.019 ± 0.005 (squares). When 1 μM ANF was added to LY83583-treated cells for 10 min, volume decreased to 0.980 ± 0.004 of that in LY83583 alone (circles). This 2% decrease in cell volume was significantly less than the 8% decrease in cell volume to caused by 1 μM ANF alone. Initial volume was restored after 20 min of washout of both ANF and LY83583. $n = 6$ cells.

According to our working hypothesis, slowing degradation of cGMP should potentiate the effect of a submaximal dose of ANF. To test this idea, we used M&B22948, a cGMP-specific phosphodiesterase (class Ic) inhibitor (Lugnier, Stierle, Beretz, Schoeffter, Lebec, Wermuth, Cazenave, and Stoclet, 1983; Brunkhorst, v der Leyden, Meyer, Nigbur, Schmidt-Schumacher, and Scholz, 1989; Weishaar et al., 1990). Fig. 9 shows the response to 0.01 μM ANF, a dose near threshold for detecting a volume change; the ED_{50} for ANF-induced volume reduction is 0.072 μM (Clemo and Baumgarten, 1991a). This dose of ANF, which was 100-fold lower than that used in the preceding experiments, caused a small decrease of cell volume to 0.977 ± 0.004 ($n = 6$). After washout of ANF, cells were treated with 100 μM M&B22948, which reduced cell volume from 1.005 ± 0.006 to 0.967 ± 0.004

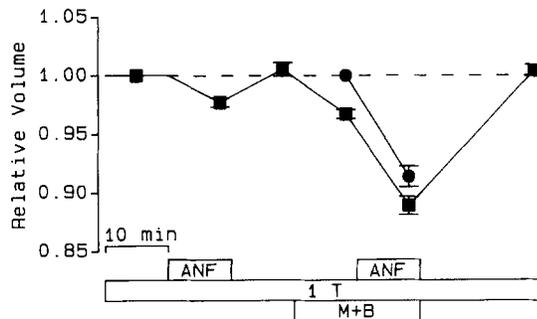


FIGURE 9. M&B22948, a cGMP-specific (class Ic) phosphodiesterase inhibitor, potentiated the effect of a low concentration of ANF. A 10-min exposure to 0.01 μM ANF decreased cell volume to 0.977 ± 0.004 of control. After return of cell volume to its initial value with washout of ANF for 10 min, the response to M&B22948 (M+B) was studied.

Treatment with 100 μM M&B22948 for 10 min decreased cell volume to 0.967 ± 0.004 (squares). When 0.01 μM ANF was added to M&B22948-treated cells for 10 min, volume decreased to 0.914 ± 0.009 relative to cell volume in M&B22948 alone (circles). Initial volume was restored after 20 min of washout of both ANF and M&B22948. The 8.6% decrease in cell volume in 0.01 μM ANF plus 100 μM M&B22948 was greater than the 2.3% decrease of cell volume due to 0.01 μM ANF alone; it was also greater than the sum of the volume decreases induced by ANF alone and M&B22948 alone. $n = 6$ cells.

(squares). The effect of 0.01 μM ANF on M&B22948-treated cells was 3.7-fold greater than that of 0.01 μM ANF alone. Normalized by the volume of M&B22948-treated cells, ANF reduced volume to 0.914 ± 0.009 (circles). Calculated relative to the control volume, ANF plus M&B22948 decreased cell volume to 0.889 ± 0.008 . This decrease was significantly greater than the sum of the decreases caused separately by ANF and M&B22948. Rather than exhibiting additive effects, M&B22948 potentiated the ability of a submaximal dose of ANF to reduce cell volume. Such potentiation is expected if the rate of degradation of the second messenger is slowed.

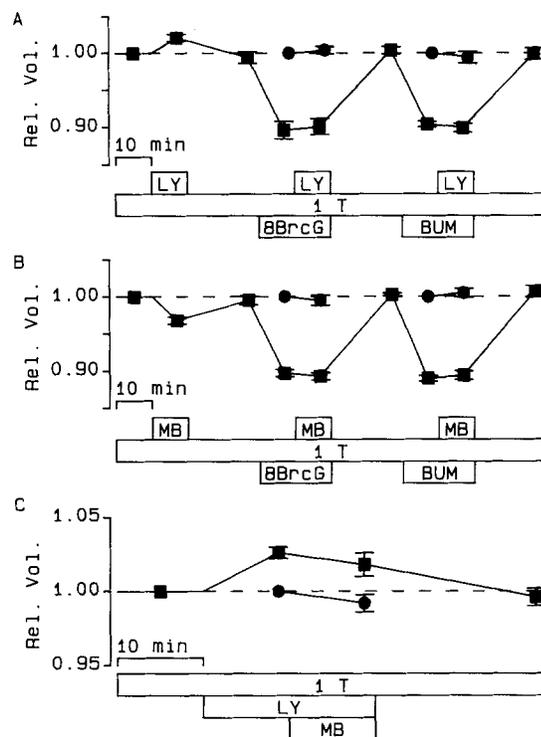


FIGURE 10. Specificity of the effects of LY83583 (LY) and M&B22948 (MB). Pretreatment of cells for 10 min with either 10 μM BUM to inhibit $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport, or 10 μM 8-Br-cGMP (8Br-cG) to activate the cGMP system, abolished the volume increase caused by a 10-min exposure to 10 μM LY83583 (A, $n = 4$) and the volume decrease caused by a 10-min exposure to 100 μM M&B22948 (B, $n = 4$). After each treatment, cell volume returned to its initial value with a 20-min washout. Volumes relative to control (squares); volumes relative to cells treated with just BUM or 8-Br-cGMP (circles). These results are expected if LY83583 and M&B22948 alter cell volume by a cGMP-dependent modulation of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. C confirms that treatment for 10

min with the guanylate cyclase inhibitor LY83583 increased cell volume. In the presence of 10 μM LY83583, a 10-min exposure to 100 μM M&B22948 failed to alter cell volume; volume was 0.992 ± 0.006 ($n = 5$) relative the volume of cells in LY83583 alone (circles). Cell volume returned to its initial value with a 20-min washout.

The Mechanism of Action of LY83583 and M&B22948

Fig. 10 depicts experiments designed to test whether the effects of LY83583 and M&B22948 on cell volume involve cGMP and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Although LY83583 and M&B22948 are inhibitors of cardiac guanylate cyclase (Schmidt et al., 1985; MacLeod and Diamond, 1986) and cardiac cGMP-specific phosphodiesterase (Brunkhorst et al., 1989), and we have suggested that cGMP modulates cell volume

via $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport, it is possible that these agents affect cardiac cell volume by multiple means. Fig. 10A illustrates the interactions between LY83583 and both 8-Br-cGMP and BUM. By itself, 10 μM LY83583 caused a small but significant increase of cell volume to 1.021 ± 0.008 ($n = 4$). LY83583 was ineffective after $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport was inhibited by 10 μM 8-Br-cGMP or 10 μM BUM, however (circles). This is consistent with the idea that LY83583 acts solely by augmenting the production of cGMP and thereby decreasing ion accumulation by $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport. 8-Br-cGMP and BUM should inhibit cotransport, preventing the effect of LY83583.

Fig. 10B depicts similar experiments with M&B22948. Treatment with 10 μM M&B22948 induced a small but significant decrease of cell volume to 0.968 ± 0.006 ($n = 4$). As expected for an agent acting solely by inhibiting cGMP-specific phosphodiesterase, M&B22948 did not alter cell volume after exposure to 10 μM 8-Br-cGMP or 10 μM BUM (circles). Inhibition of cotransport by 8-Br-cGMP or BUM should avert the effect of the accumulation of cGMP on cell volume. Fig. 10C verifies the mechanism of action of M&B22948 in another way. Block of guanylate cyclase by LY83583 is expected to abolish production of cGMP. Consequently, inhibition of the cGMP-specific phosphodiesterase should alter neither the cytoplasmic cGMP level nor cell volume. This expectation was confirmed. Relative to cell volume in 10 μM LY83583, cell volume after exposure to 10 μM M&B22948 was 0.992 ± 0.006 ($n = 5$; circles), a value indistinguishable from unity.

Effects of ANF, LY83583, and M&B22948 on cGMP Levels in Myocytes

Interpretation of the results presented up to this point depends in part on previous descriptions of the effects of ANF (Cramb et al., 1987; Anand-Srivastava et al., 1989; Rugg et al., 1989; McCall and Fried, 1990), LY83583 (Schmidt et al., 1985; MacLeod and Diamond, 1986), and M&B22948 (Lugnier et al., 1983; Brunkhorst et al., 1989; Weishaar et al., 1990) on cGMP levels in heart. Therefore, ^{125}I -cGMP radioimmunoassay was used to confirm that the key elements of previous findings were applicable to isolated ventricular myocytes under the present experimental conditions.

Fig. 11A demonstrates that 1 μM ANF rapidly increased cGMP levels in isolated myocytes and that elevated cGMP levels were well maintained throughout a 20-min exposure to ANF. cGMP was 0.25 ± 0.01 pmol/ 10^6 cells in control myocytes, increased to 0.60 ± 0.01 pmol/ 10^6 cells at 2 min, and reached 0.74 ± 0.04 pmol/ 10^6 cells after 10 min in 1 μM ANF, a threefold increase. Thus, the time course of the rise of cGMP was consistent with that of the fall of cell volume (see Fig. 1B).

The interactions of ANF and both LY83583 and M&B22948 were examined in another set of experiments, illustrated in Fig. 11B, which exactly paralleled the volume measurement studies (see Figs. 8 and 9). As expected from Fig. 11A, exposure to 1 μM ANF for 10 min increased cGMP to 0.72 ± 0.02 pmol/ 10^6 cells, nearly threefold greater than in control cells. The guanylate cyclase inhibitor LY83583 significantly decreased, but did not totally eliminate, the response to 1 μM ANF. After 10 min in 10 μM LY83583, exposure to 1 μM ANF for 10 min in the continued presence of LY83583 increased cGMP to 0.42 ± 0.02 pmol/ 10^6 cells, an increase of only $\sim 50\%$ rather than threefold. By itself, however, LY83583 did not significantly alter cGMP levels.

The response to the cGMP-specific phosphodiesterase inhibitor M&B22948 also was as expected (Fig. 11 B). First, a 10-min exposure to 100 μ M M&B22948 more than doubled the cGMP level. Second, M&B22948 markedly potentiated the ability of a low dose of ANF to elevate cGMP. By itself, treatment with 0.01 μ M ANF for 10 min caused a small but significant increase in cGMP from 0.28 ± 0.02 (10 min control) to 0.35 ± 0.01 pmol/ 10^6 cells. After 10 min of pretreatment with M&B22948, however, exposure to 0.01 μ M ANF in the continued presence of M&B22948 elevated cGMP to 1.36 ± 0.56 pmol/ 10^6 cells, a fourfold higher level.

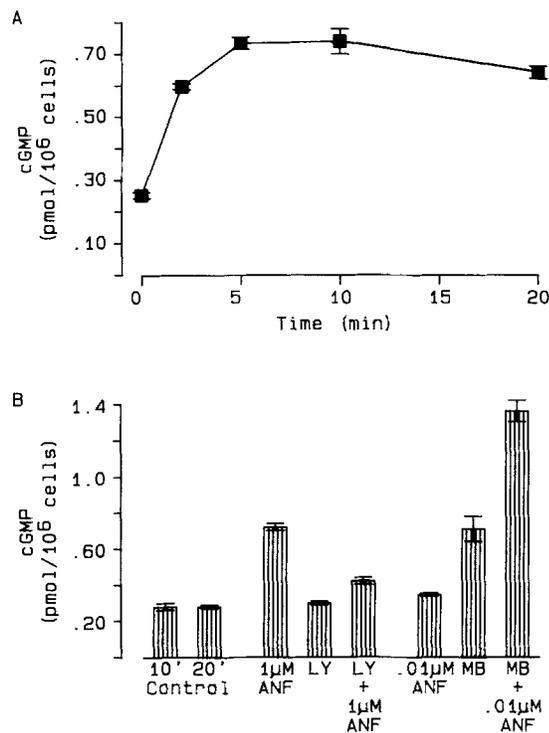


FIGURE 11. 125 I-cGMP radioimmunoassay determinations of cGMP levels in isolated cardiac myocytes. Separate aliquots of cells were used for each time point or condition, and each cGMP assay was done in duplicate, averaged, and corrected for recovery. (A) Exposure to 1 μ M ANF for 20 min induced a rapid and sustained elevation of cGMP. cGMP was already significantly greater than in control cells after a 2-min exposure to ANF, and was 0.64 ± 0.02 pmol/ 10^6 cells, 2.6 times higher than control, after 20 min. $n = 3$ or 2 per time point. (B) Inhibition of guanylate cyclase by 10 μ M LY83583 (LY) significantly decreased ANF-induced accumulation of cGMP, and inhibition of cGMP-specific phosphodiesterase by 100 μ M M&B22948 (MB) significantly increased ANF-induced accumulation of cGMP. Bars represent (left to right) 10- and 20-min untreated controls and the effects of 1 μ M ANF for 10 min, LY for 10 min, LY for 10 min followed by exposure to both 1 μ M ANF and LY for 10 min, 0.01 μ M ANF for 10 min, MB for 10 min, and MB for 10 min followed by exposure to both 0.01 μ M ANF and MB for 10 min. $n = 5$ or 4 per condition.

These studies confirm that under present conditions LY83583 and M&B22948 have the expected effects on ANF-induced cGMP accumulation.

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DISCUSSION

These experiments demonstrate for the first time that cGMP and 8-Br-cGMP, its membrane permeant analogue, modulate ventricular cell volume. Furthermore, they suggest that cGMP is the second messenger in the reduction of cell volume by ANF.

Previously it was shown that ANF activates guanylate cyclase in heart and increases the cytoplasmic concentration of cGMP (Cramb et al., 1987; Anand-Srivastava et al., 1989; Rugg et al., 1989; McCall and Fried, 1990), a finding confirmed in the present study. We propose that the increase in cGMP causes shrinkage of myocytes by inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. The results are summarized diagrammatically in Fig. 12.

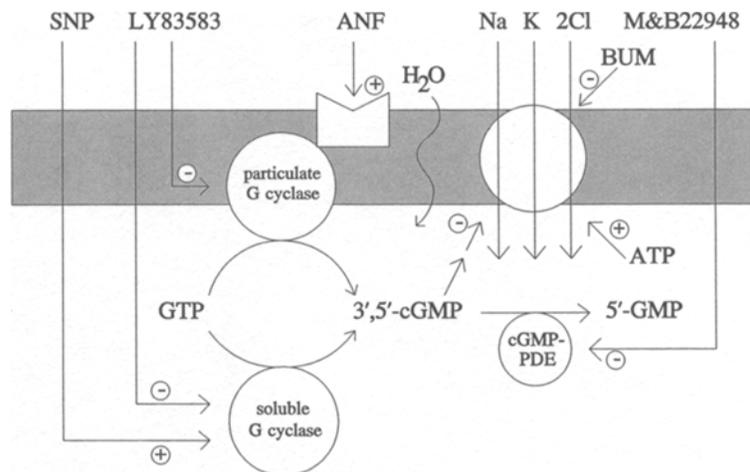


FIGURE 12. Schematic model of the mechanism of action of ANF and cGMP on cell volume. Binding of ANF to a sarcolemmal receptor activates (+) a particulate guanylate cyclase, which may be a portion of the receptor molecule (Willey et al., 1992), leading to the conversion of guanosine triphosphate (*GTP*) to 3',5'-cGMP. SNP activates a soluble guanylate cyclase and also elevates cGMP (Diamond et al., 1977; Murad et al., 1978). By one or more steps, cGMP inhibits (-) $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. LY83583 inhibits guanylate cyclase, resulting in reduced accumulation of cGMP upon stimulation with ANF, and M&B22948 inhibits cGMP-specific phosphodiesterase, resulting in enhanced accumulation of cGMP upon stimulation with ANF. Other phosphodiesterases (not shown) may also contribute to the degradation of cGMP to 5'-GMP. Adenosine triphosphate (*ATP*) is required at a regulatory site. In the steady state, the influx of osmolytes via $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport and obligate influx of water must be balanced exactly by efflux processes (not shown). Therefore, inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport results in the net efflux of osmolytes and water, and cell shrinkage ensues.

Does cGMP Reduce Cell Volume by Inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ Cotransport?

Cell shrinkage represents the net loss of osmolytes and cell water. Either a stimulation of the efflux or an inhibition of the influx of osmotic equivalents by cGMP or 8-Br-cGMP could explain the shrinkage of ventricular myocytes. The most likely possibility is that cGMP and 8-Br-cGMP inhibit ion uptake by $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. This conclusion is based on the finding that BUM, a blocker of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport (Schlatter, Greger, and Weidtko, 1983; Clemo and Baumgarten, 1991a), prevented volume reduction by 8-Br-cGMP. Once $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport is fully inhibited by BUM, agents that act solely by inhibiting the same transporter should be

ineffective. The ED_{50} for reduction of cell volume by 8-Br-cGMP, $\sim 0.8 \mu\text{M}$, is comparable to the 8-Br-cGMP sensitivity of $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport in other tissues (O'Donnell and Owen, 1986; O'Donnell, 1989).

Additional evidence that 8-Br-cGMP brings about cell shrinkage by inhibiting $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport is that removal of either Na^+ or Cl^- from the bathing media blocked its action. The ionic requirements for cell shrinkage by 8-Br-cGMP match those for $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport (Geck et al., 1980; O'Grady et al., 1987). Replacing Na^+ or Cl^- with ions that are not transported should preclude further inhibition of transport by 8-Br-cGMP. The dependence of 8-Br-cGMP's effect on extracellular K^+ was not tested because complete removal of extracellular K^+ results in spontaneous activity of myocytes.

Shrinkage on removal of Na^+ or Cl^- was less than that observed with 8-Br-cGMP, however. Shrinkage was not limited by the leak of Na^+ and Cl^- substitutes into the cell. If they did leak in, volume should overshoot on returning to Na^+ or Cl^- -containing solutions, but no overshoot was detected. The same apparent discrepancy was noted previously with BUM and ANF. By themselves, these agents decrease cell volume by ~ 12 and $\sim 8\%$, but like 8-Br-cGMP, their actions are totally blocked by removing Na^+ or Cl^- with only a $\sim 4\%$ decrease in volume (Clemo and Baumgarten, 1991a). It may be inferred that besides a common effect to block $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport, either BUM, ANF, and 8-Br-cGMP or Na^+ and Cl^- removal must have additional effects on ion transport. There is an intrinsic difference in stopping $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport in these two ways. On removal of Na^+ or Cl^- , intracellular cation or anion cannot be lost alone because of the requirement for macroscopic electroneutrality. If loss of Na^+ is partially compensated for by gain of K^+ or loss of Cl^- by gain of HCO_3^- , the decrease in volume would be less than expected, as has been shown to be the case (Clemo and Baumgarten, 1991a). In contrast, inhibiting $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport directly does not disturb macroscopic electroneutrality and causes the net loss of these ions with a fixed stoichiometry. Taken together, these data suggest but do not prove that the differences in shrinkage may be a consequence of how macroscopic electroneutrality is maintained in each case.

An alternative idea is that 8-Br-cGMP produced cell shrinkage by stimulating an efflux of osmolytes. In some tissues, passive K^+ and Cl^- efflux by separate conductive pathways coupled by macroscopic electroneutrality causes a volume regulatory decrease after osmotic swelling (Hoffman and Simonsen, 1989). cGMP activates a poorly selective cation channel in retinal outer rod segments (Fesenko, Kolesnikov, and Lyubarsky, 1985; Yau and Nakatani, 1985; Furman and Tanaka, 1990), olfactory receptor cilia (Nakamura and Gold, 1987), A6 renal epithelial cells (Marunaka, Ohara, Matsumoto, and Eaton, 1991), and a Cl^- channel in the T_{84} colonic cells (Lin, Nairn, and Guggino, 1991). Although BUM appears specific for $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransport in heart, it has been reported to block certain Cl^- channels in epithelia and nerve (Gallagher, Nakamura, and Shinnick-Gallagher, 1983; Landry, Reitman, Cragoe, and Al Awqati, 1987; Wang and Cooke, 1990). Thus, one might argue that 8-Br-cGMP stimulates ion efflux and cell shrinkage by opening a BUM-sensitive Cl^- channel or a K^+ channel that is linked to a BUM-sensitive Cl^- channel by macroscopic electroneutrality. The results of several experiments run counter to this hypothesis, however. First, if BUM blocked Cl^- and K^+ efflux, it should increase cell

volume. As shown here and previously (Clemo and Baumgarten, 1991a; Drewnowska and Baumgarten, 1991), BUM significantly decreased cell volume. Second, BUM decreases intracellular Cl^- activity (Baumgarten and Duncan, 1987; Liu et al., 1987). This is contrary to expectations for Cl^- channel block because the electrochemical gradient for Cl^- is outward in rabbit myocytes (Désilets and Baumgarten, 1986). Third, BUM-induced cell shrinkage is inhibited by removing Na^+ from the bathing solution (Clemo and Baumgarten, 1991a). The conductive efflux of K^+ and Cl^- should not require extracellular Na^+ .

Although the primary effect of cGMP appears to be $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport inhibition, the resultant modification of ionic gradients (Frelin et al., 1986; Baumgarten and Duncan, 1987; Liu et al., 1987) may affect other transport processes. For example, inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport decreases intracellular Na^+ activity (Liu et al., 1987) and therefore affects the Na^+-K^+ pump, $\text{Na}^+-\text{Ca}^{2+}$ exchange, and Na^+-H^+ exchange. These processes may contribute to regulation of the cell volume directly by performing osmotic work or indirectly by altering intracellular Ca^{2+} and pH. Furthermore, we cannot exclude other direct effects of cGMP on transport processes.

Does 8-Br-cGMP Modulate Cell Volume by Altering cAMP Levels?

Although 8-Br-cGMP is often used to specifically activate cGMP-dependent processes, other pathways may be affected also. For example, 8-Br-cGMP stimulates a cAMP-specific phosphodiesterase (Beavo, 1988; Weishaar et al., 1990), and myocyte shrinkage might ultimately depend on cAMP rather than cGMP-dependent mechanisms. Increasing cAMP stimulates $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in avian erythrocytes and epithelial cells (Palfrey and Rao, 1983) but inhibits the cotransporter in bovine aortic endothelial cells (O'Donnell, 1989), human erythrocytes (Garay and Ciccone, 1982), and vascular smooth muscle (Owen, 1984). In myocytes, however, cell volume was unaffected by up to 100 μM 8-Br-cAMP and by activation of adenylyl cyclase with 50 μM forskolin. Furthermore, the possibility that 8-Br-cGMP acts by decreasing cAMP levels was also excluded. Because $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport is decisive in setting cardiac cell volume (Clemo and Baumgarten, 1991a; Drewnowska and Baumgarten, 1991), one can conclude that cardiac $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport is not modulated by cAMP.

Is cGMP the Second Messenger for ANF-induced Myocyte Shrinkage?

It is well established that ANF receptors are linked to and activate guanylate cyclase and elevate cGMP in a number of tissues (Waldman and Murad, 1987; Tremblay et al., 1988; Wildey et al., 1992) including the heart (Cramb et al., 1987; Anand-Srivastava et al., 1989; Rugg et al., 1989; McCall and Fried, 1990). The increase in cGMP levels is transient in a few cell lines, but is maintained in many others including bovine aortic smooth muscle, canine kidney epithelial (MDCK), rat mammary epithelial, human lung fibroblast, bovine adrenal cortical, rat glioma, and rat pheochromocytoma cells (Fiscus, Roles, Waldman, and Murad, 1987; Leitman, Andresen, Catalano, Waldman, Tuan, and Murad, 1988). In the present study, the ANF-induced increase in cGMP was well maintained in isolated ventricular myocytes for 20 min. This is consistent with the observation that cGMP in ventricular muscle is

elevated after a 30-min exposure to ANF (McCall and Fried, 1990). In contrast, in the presence of the phosphodiesterase inhibitor 3-isobutyl-*l*-methylxanthine (IBMX, 1 mM) and reduced extracellular Ca^{2+} (0.5 mM), ventricular cGMP levels are reported to begin to decrease after exposure to ANF for ~10 min but remain significantly greater than control at 20 min (Cramb et al., 1987).

cGMP appears to be the second messenger for ANF in the sequence of steps leading to a reduction of myocyte cell volume. First, ANF rapidly increased cGMP levels in isolated myocytes. Second, 8-Br-cGMP and elevation of cGMP by SNP mimicked ANF in decreasing cell volume. Third, inhibition of guanylate cyclase and the production of cGMP with the quinolinedione LY83583 (Schmidt et al., 1985; MacLeod and Diamond, 1986) blunted both the ANF-induced elevation of cGMP and ANF-induced reduction of cell volume. LY83583 also attenuates ANF's inhibition of ion fluxes mediated by $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in vascular endothelial cells (O'Donnell, 1989). Fourth, inhibition of cGMP-specific phosphodiesterase and the degradation of cGMP with M&B22948 (Lugnier et al., 1983; Brunkhorst et al., 1989; Weishaar et al., 1990) potentiated the effect of a threshold dose of ANF (0.01 μM) so that myocyte shrinkage was comparable to that induced by a maximally effective dose. Correspondingly, after M&B22948 treatment, a threshold dose of ANF gave rise to a maximally effective increase in cGMP. M&B22948 also potentiates ANF's vasodilatory and natriuretic effects in rats and rabbits (Weishaar et al., 1990) and its stimulation of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport in vascular smooth muscle (O'Donnell and Owen, 1986). Fifth, pretreatment with a maximally effective dose of 8-Br-cGMP eliminated the ability of ANF to decrease cell volume. Such inhibition is required if ANF acts by elevating cGMP levels. Sixth, in the converse experiment, a maximally effective dose of ANF (Clemo and Baumgarten, 1991a) markedly diminished cell shrinkage on exposure to 8-Br-cGMP. ANF did not totally prevent 8-Br-cGMP-induced cell shrinkage, however. Only a partial inhibition of 8-Br-cGMP-induced cell shrinkage is expected if the maximum level of cGMP attained by activating ANF receptors does not saturate the cGMP-dependent step. Consistent with this idea, the maximum decrease of cell volume observed with 8-Br-cGMP, 10.8%, was slightly greater than the 8.2% decrease with ANF (Fig. 1).

Although these experiments satisfy classic criteria for identifying a second messenger, some of the results could also be explained by ANF and cGMP acting independently, in parallel on a common final target, $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport. Such a parallel scheme cannot easily explain why modulating cGMP with LY83583 and M&B22948 should have affected the efficacy of ANF as it did, however. In a parallel scheme, the effects of these agents should have been reversed from what they were.

Does Basal Production of cGMP Modulate Cell Volume?

LY83583 alone increased cell volume by 2–3% and M&B22948 alone decreased cell volume by 3%. These observations may be explained by the basal production of cGMP in the absence of stimulation of guanylate cyclase. Then, inhibition of either guanylate cyclase or cGMP-specific phosphodiesterase is expected to perturb the basal cGMP concentration, modulate $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport, and thereby adjust cell volume. In keeping with this idea, M&B22948 alone significantly elevated cGMP, but LY83583 alone did not significantly effect cGMP. We are uncertain why we were

unable to detect the small decrease in cGMP expected with LY83583. One possibility is that noise introduced by the unavoidable presence of damaged myocytes in the aliquots of cells used for determining cGMP masked a small effect.

Physiological Implications

What are the physiological consequences of cell volume changes on activation of the ANF-cGMP pathway and inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport? We suggested previously that the decrease of cell volume induced by ANF might serve as a local mechanism for feedback inhibition of ANF release (Clemo and Baumgarten, 1991a). In addition, one can postulate that inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport will alter cardiac contractile function. By decreasing the influx of Na^+ , inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport lowers intracellular Na^+ activity (Liu et al., 1987). In turn, enhanced efflux of Ca^{2+} via $\text{Na}^+-\text{Ca}^{2+}$ exchange and a decrease in contractility are expected. Cell volume perturbations also may affect the interaction of the cross-bridges. Furthermore, diastolic properties may be modified. Ventricular muscle mass is an important determinant of diastolic compliance (for review, see Gaasch, Apstein, and Levine, 1985), and by itself reduction of cell volume should increase compliance and improve cardiac filling. Preliminary studies on rabbit heart Langendorff preparations instrumented with a left ventricular balloon (Clemo and Baumgarten, 1991c) support these predictions. Exposure to 10 μM BUM, 10 μM 8-Br-cGMP, or 100 μM SNP decreased isovolumetric systolic pressure over a wide range of left ventricular end diastolic volumes indicating a reduction in contractility (see also Meulemans, Sipido, Sys, and Brutsaert, 1988). In addition, these agents shifted the diastolic pressure-volume curve downward and to the right, indicating an increase in ventricular compliance. Negative inotropic effects of cGMP derivatives were noted previously, and the role of cGMP in the contractile effects of muscarinic agonists has been the subject of extensive debate (Linden and Brooker, 1979; Opie, 1982; Diamond and Chu, 1985). Our suggestion that cGMP decreases contractility in part by inhibiting $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport may be relevant in this controversy.

In summary, the present results suggest that ANF decreases cardiac cell volume via a cGMP-mediated inhibition of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport and that the level of cGMP helps determine the volume a cell attains. cGMP is modulated physiologically by a number of mechanisms (Waldman and Murad, 1987; Tremblay et al., 1988). Consequently, alteration of cardiac cell volume may be far more common than previously thought.

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