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# Atrial Natriuretic Factor Regulates the Calcium Current in Frog Isolated Cardiac Cells

Marie-Pierre Gisbert and Rodolphe Fischmeister

The effect of external application of synthetic atrial natriuretic factor (ANF) on calcium current ( $I_{Ca}$ ) was studied in single cells isolated from frog ventricle using the whole-cell patch-clamp technique. Rat atriopeptin III (APIII) and 3-28 ANF rat (rANF) had negligible effects on basal  $I_{Ca}$  at concentrations up to 200 nM. However, when  $I_{Ca}$  was increased by isoprenaline, both peptides had significant inhibitory effects. rANF (3 nM) decreased isoprenaline-elevated  $I_{Ca}$  by an average of 33% after 3–5 minutes. APIII was slightly less effective than rANF. The effects of rANF and APIII were dose-dependent in a complex manner: one stimulatory and two different inhibitory effects were observed, one being responsible for an irreversible rundown of  $I_{Ca}$ . The effects of ANF were not blocked by atropine and desensitization of the cells to isoprenaline did not play a significant role in the response to ANF. When  $I_{Ca}$  was elevated by intracellular perfusion with cyclic adenosine 3',5'-monophosphate, added to the patch electrode solution or using a perfused pipette, rANF or APIII had less inhibitory effect, and no rundown of  $I_{Ca}$  was observed. It is proposed that adenylate cyclase may be one of several mechanisms by which ANF regulates  $I_{Ca}$ . (*Circulation Research* 1988;62:660–667)

The atrial muscle of heart produces a family of polypeptide hormones, termed atrial natriuretic factors (ANFs), that participate in the regulation of salt and water balance and blood pressure.<sup>1,2</sup> These peptides have been identified and characterized (see reviews in Cantin and Genest<sup>3</sup> and Ballermann and Brenner<sup>4</sup>) and synthetic ANFs have become available. Although the mechanism of action of ANF at the cellular level remains unclear, evidence derived from both in vitro and in vivo studies show that, in most target tissues, ANF action is generally associated with elevation in cyclic guanosine 3',5'-monophosphate (cGMP) levels and that cGMP is the likely second messenger for ANF action.<sup>4,5</sup> For instance, ANF is a potent relaxant of a large variety of vascular and nonvascular smooth muscles that have been contracted by different agonists.<sup>6,7</sup> The relaxing effect of ANF is associated with an elevation in intracellular cGMP levels due to activation of a particulate guanylate cyclase.<sup>7,8</sup> Other cellular mechanisms that may be involved in the action of ANF, whether or not related to cGMP accumulation, include inhibition of adenylate cyclase (in arteries and kidney<sup>9,10</sup>) and inhibition of agonist- but not depolarization-stimulated calcium influx in vascular smooth muscle.<sup>11</sup>

Very little is known about the effect of ANF on heart. Hypotensive doses of atrial extracts were shown to transiently reduce cardiac output and depress the heart rate in anesthetized rats.<sup>12</sup> A similar effect on cardiac output was found with synthetic ANF<sup>13</sup> but with no modification in the heart rate, suggesting a direct inhibitory action of ANF on cardiac contractility. However, in these studies, the effects of the hormone

on cardiac output were contaminated by a simultaneous reduction in total peripheral resistance. Synthetic ANF has also been shown to promote myocardial relaxation in cat and rat papillary muscle<sup>14</sup> and to reduce the activity of adenylate cyclase in crude homogenates of rat cardiocytes,<sup>15</sup> suggesting a participation of ANF in cardiac function. However, other studies have concluded that there are no direct effects of ANF on cardiac contractility or beat rate.<sup>16–18</sup>

Because of the predominant role of calcium ions in the regulation of cardiovascular function, the effects of synthetic ANF on calcium current, which was recorded under voltage-clamp conditions from single isolated cells of frog ventricle, were investigated.<sup>19</sup> Since frog heart, like mammalian atrium, secretes granules<sup>20</sup> that contain a peptide immunologically and biochemically related to mammalian ANF<sup>21</sup> and frog ANF has been shown to induce diuresis and natriuresis in mammals,<sup>22</sup> frog ventricular cells are an appropriate preparation in which to study the effect of ANF. Also, we have recently shown that cGMP regulates the calcium current of these cells,<sup>23,24</sup> so it is possible to make a direct comparison between the actions of cGMP and ANF. To our knowledge, this is the first study of the effect of ANF at the single cell level and the first report of a voltage-clamp study of a response to the polypeptide. A preliminary report has appeared elsewhere.<sup>25</sup>

## Materials and Methods

The methods used for cell dissociation, whole-cell patch-clamp recording, superfusion and internal perfusion of the cells, and data analysis have been extensively described in previous papers<sup>19,23,24</sup> and were used with no major modification in the present study.

Briefly, for routine monitoring of  $I_{Ca}$ , the frog (*Rana esculenta*) ventricular cell was depolarized every 8 seconds from  $-80$  mV holding potential to  $0$  mV for 200 msec. To accurately measure  $I_{Ca}$  with no contamination of other ionic currents, the cells were bathed in

From the Laboratoire de Physiologie Cellulaire Cardiaque, INSERM U-241, Université de Paris-Sud, Orsay, France.

Supported in part by the Fondation pour la Recherche Médicale. Address for correspondence and reprints: Dr. R. Fischmeister, Laboratoire de Physiologie Cellulaire Cardiaque, INSERM U-241, Université de Paris-Sud, Bâtiment 443, F-91405 Orsay, France.

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potassium-free, 20 mM Cs-Ringer's solution containing 88.4 mM NaCl, 20 mM CsCl, 22.9 mM NaHCO<sub>3</sub>, 0.6 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 1.8 mM MgCl<sub>2</sub>, 5 mM *d*-glucose, 5 mM sodium pyruvate, and 0.3 μM tetrodotoxin (Sankyo, Japan). The standard internal solution in the patch electrode (1–3 MΩ resistance) contained CsCl 120 mM, K<sub>2</sub>EGTA 5 mM, MgCl<sub>2</sub> 4 mM, Na<sub>2</sub>CP 5 mM, Na<sub>2</sub>ATP 3 mM, and Na<sub>2</sub>GTP 0.4 mM, adjusted to pH 7.1 with KOH (1N). Solutions were applied to the exterior of the cell by placing the cell at the opening of 250-μm inner diameter capillary tubing (flow rate of 10 μl/min).<sup>19</sup> Solutions were applied to the interior of the cell by the patch electrode and could be modified by a system that permitted perfusion of the patch electrode.<sup>23,24</sup> Under these conditions, I<sub>Ca</sub> was measured on-line as the difference between peak inward current and the current at the end of the 200-msec pulse<sup>19</sup> that was determined from the current trace digitized at 5 kHz (12-bit A/D converter) by a Compaq 286 Desk-Pro computer using programs written in Pascal language. For each cell, membrane capacitance (C<sub>m</sub>) was recorded to give an estimate of the total cell membrane area. When required, kinetics of I<sub>Ca</sub> were analyzed using the computer program EXCALC<sup>26</sup> based on the powerful method described by Yeramian and Claverie.<sup>27</sup> All external solutions were gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> (pH 7.4), and experiments were done at room temperature (19.5–22.5° C).

### Drugs

(±)-Isoprenaline, acetylcholine, atropine, 1-methyl-3-isobutyl xanthine, cyclic adenosine 3',5'-monophosphate (cAMP), and 8-bromo cAMP were obtained from Sigma Chemical, St. Louis, Missouri. Two different synthetic atrial natriuretic factors were used: atriopeptin III (APIII; 5-28 ANF rat, Sanofi, France) and ANF 8-33 (rANF, 3-28 ANF rat, Merck Sharp & Dohme, Teterboro, New Jersey). Quantities (50 μl) of 5-μM ANF stock solutions, made in 0.1 M acetic acid, were thawed from -27° C before each experiment and kept in ice prior to final dilution at 1–200 nM into 5 ml extracellular perfusion solution. Control solutions contained identical amounts of acetic acid as ANF-containing solutions, and pH was readjusted to 7.4 with NaOH (1N) when required.

### Results

L-Type calcium currents<sup>28,29</sup> (I<sub>Ca</sub>) were elicited in frog ventricular cells by voltage-clamp depolarizations from -80 to 0 mV. Figure 1 shows the effect of 100 nM APIII on I<sub>Ca</sub>, which was recorded every 8 seconds during a 200-msec depolarization to 0 mV. APIII had a weak effect on control I<sub>Ca</sub>: in five cells exposed to 100 and 200 nM APIII or rANF, I<sub>Ca</sub> was 95.4 ± 7.8% and 92.7 ± 1.8% of control I<sub>Ca</sub> (mean ± SEM), respectively. But when I<sub>Ca</sub> was enhanced by superfusing the cell with 0.3 μM of the β-adrenergic agonist isoprenaline (ISO), APIII had more pronounced effects. I<sub>Ca</sub> was first reduced by ≈25% after 1 minute of exposure to APIII; after 3 minutes of continuous exposure, this inhibition declined (≈10%). APIII also initiated a

rundown of I<sub>Ca</sub> that was not reversed when APIII was washed out of the cell, although ISO was still present. In fact, the washout of APIII itself induced a further rapid decrease in I<sub>Ca</sub>. A second exposure to APIII stimulated I<sub>Ca</sub> without the transient inhibition that was seen when APIII was first applied. From this experiment, it would appear that, at high concentrations, ANF had a complex pattern of actions on I<sub>Ca</sub> that had been elevated by β-adrenergic agonists: first, a transient inhibitory effect; then, a sustained and irreversible inhibitory action that was responsible for the rundown of I<sub>Ca</sub>; and finally, a small reversible stimulatory effect on I<sub>Ca</sub>.

In an attempt to clarify the action of ANF, lower and more physiological doses were used to separate these multiple effects of the hormone. In Figure 2, 3 nM APIII strongly reduced ISO-enhanced I<sub>Ca</sub> and induced a rundown process. When the concentration of APIII was increased to 10 nM, a transient plateau in this decline was observed that was followed by a further acceleration in the rate of rundown of I<sub>Ca</sub>, suggesting competition between two opposite effects. This competition was confirmed when the concentration of APIII was increased to 30 nM: there was a clear transient increase in I<sub>Ca</sub> and a reduction in the rate of the rundown process. Further increasing the concentration to 100 nM had no additional effect on I<sub>Ca</sub>. When

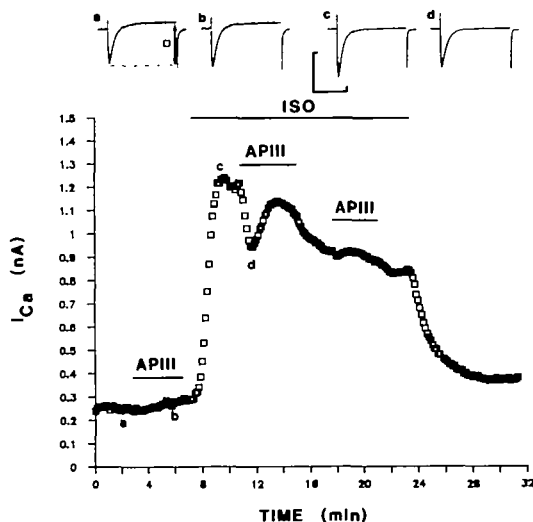


FIGURE 1. Effects of rat atriopeptin III (APIII) on calcium current (I<sub>Ca</sub>). I<sub>Ca</sub> was recorded from an isolated ventricular cell using a patch pipette as described in "Materials and Methods." The four current traces (top) were recorded at times indicated by corresponding letters on bottom graph: a, control; b, 100 nM APIII; c, 0.3 μM isoprenaline; d, isoprenaline + APIII. Current traces were recorded on and replayed from magnetic tape at 3/4 ips and digitized at 5 kHz. Calibration bars: horizontal, 150 msec; vertical, 250 pA (a and b) and 1,000 pA (c and d). Each square (bottom) represents the net calcium current measured by a 200-msec duration depolarizing pulse to 0 mV, every 8 seconds, from -80 mV holding potential as indicated on left individual current trace. Cell was exposed to control cesium-Ringer's solution, except for periods indicated when it was perfused with APIII (100 nM), isoprenaline (ISO, 0.3 μM), or both. (Cell No. 86112703; C<sub>m</sub> = 74 pF.)

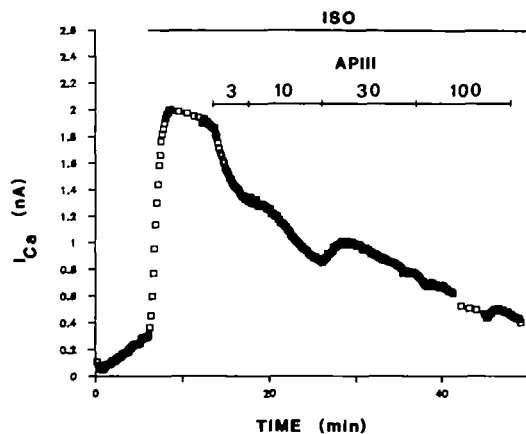


FIGURE 2. Effects of cumulative doses of rat atriopeptin III (APIII) on calcium current ( $I_{Ca}$ ). After  $I_{Ca}$  was elevated by  $0.3 \mu\text{M}$  isoprenaline (ISO), the cell was exposed to ISO plus increasing doses of APIII (3 nM, 10 nM, 30 nM, and 100 nM) during the periods indicated. Finally, the cell was exposed again to ISO alone. (Cell No. 86112002;  $C_m = 75 \text{ pF}$ .)

APIII was washed from the cell,  $I_{Ca}$  did not show any tendency to recover. The dose-dependent effects of rANF are shown in Figure 3. While 5 minutes' successive exposures to 10 and 30 nM rANF depressed ISO-elevated  $I_{Ca}$  by 17% and 60%, respectively, 100- and 300-nM concentrations were less effective. Again, the effects were not reversed when rANF was washed away from the cell.

Desensitization to isoproterenol has been shown to occur relatively quickly in cultured myocytes of chick ventricle.<sup>30,31</sup> A possible contribution of this process to the inhibitory effect of ANF on  $I_{Ca}$  elevated by ISO has been checked. Figure 4 shows an experiment where 3 nM rANF was applied to a cell after more than 40 minutes of continuous exposure to  $2 \mu\text{M}$  ISO. While  $I_{Ca}$  did not decrease during the long exposure to ISO, ANF induced a slow and irreversible inhibition of  $I_{Ca}$  after an initial transient stimulatory period. Thus, electrophysiological desensitization to ISO was slower in our experiments than inotropical desensitization in chick heart<sup>31</sup> and is unlikely to significantly contribute to the response to ANF. The effects of ANF on  $I_{Ca}$  were not voltage-dependent because the current-voltage relation of  $I_{Ca}$  was not affected by the polypeptide (data not shown). However, the time constants of inactivation of  $I_{Ca}$  were slightly reduced by the hormone (Figure 4).

The observation that low doses of ANF only inhibited  $I_{Ca}$  that had been elevated by  $\beta$ -adrenergic stimulation raises questions about the mechanism(s) of action of the peptide.  $\beta$ -Adrenergic agonists stimulate the activity of adenylate cyclase and increase the level of intracellular cAMP. The increase in cAMP is responsible for the  $\beta$ -adrenergic stimulation of  $I_{Ca}$  by increasing the degree of phosphorylation of the calcium channels because a similar action is seen when cAMP is perfused into the cell.<sup>19</sup> In an attempt to clarify whether the action of ANF occurred before or after cAMP synthesis, the effects of ANF on calcium current

that was elevated by direct application of intracellular cAMP were studied. Figure 5 shows a typical experiment where the patch electrode contained  $5 \mu\text{M}$  cAMP in addition to the regular intracellular solution. Immediately after rupture of the patch membrane (time 0),  $I_{Ca}$  began to increase as cAMP permeated the cell and stabilized in about 5 minutes. The application of 3 nM rANF then had a biphasic effect: an initial transient stimulation ( $\approx +7\%$ ) of  $I_{Ca}$  was followed by a sustained inhibition ( $\approx -12\%$ ). In each cell perfused with cAMP, ANF never induced a rundown of  $I_{Ca}$  as had generally been seen when ANF was applied to ISO-elevated  $I_{Ca}$ . The effects of rANF on ISO- and cAMP-elevated  $I_{Ca}$  were compared in the same cell in one experiment (Figure 6). This cell was particularly suitable for this type of comparison because the strong inhibitory effect of 10 nM rANF during first exposure on  $I_{Ca}$  elevated by ISO ( $\approx 60\%$  inhibition after 5 minutes' exposure) was mostly reversible (92%). This provided an opportunity to reexamine the effect of rANF on control  $I_{Ca}$  after all ISO was washed out of the cell and  $I_{Ca}$  returned to control levels and, finally, on  $I_{Ca}$  elevated by perfusion of the patch electrode with  $5 \mu\text{M}$  cAMP. rANF had no significant effect on control  $I_{Ca}$ . Intracellular perfusion with  $5 \mu\text{M}$  cAMP elevated  $I_{Ca}$  less than external application of  $0.3 \mu\text{M}$  ISO, although both manipulations have been shown previously to have similar effects on the amplitude of  $I_{Ca}$ .<sup>19,23,24</sup> This may suggest that rANF had exerted a sustained irreversible inhibitory action on  $I_{Ca}$  after first application. Exposure of the cell to 10 nM rANF under these conditions had a much weaker effect on cAMP-elevated  $I_{Ca}$  than previous application of the peptide on ISO-elevated  $I_{Ca}$ . Unfortunately, the seal between the patch electrode and the cell was suddenly lost during the development of the ANF response, which, thus, was recorded only during 4.5 minutes. However, after a

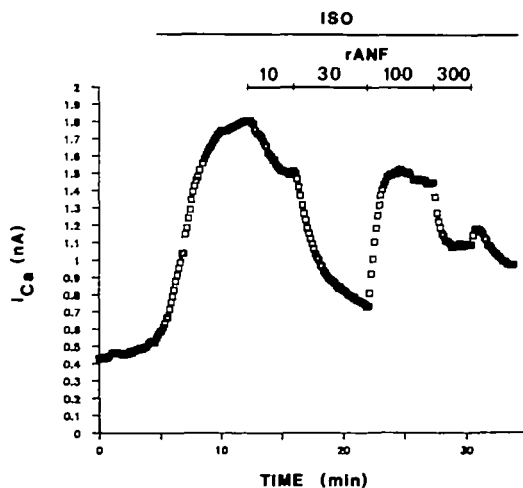


FIGURE 3. Effects of cumulative doses of 3-28 ANF rat (rANF) on calcium current ( $I_{Ca}$ ). After  $I_{Ca}$  was elevated by  $0.3 \mu\text{M}$  isoprenaline (ISO), the cell was exposed to ISO plus increasing doses of rANF (10 nM, 30 nM, 100 nM, and 300 nM) during the periods indicated. Finally, the cell was exposed again to ISO alone. (Cell No. 86120501;  $C_m = 100 \text{ pF}$ .)

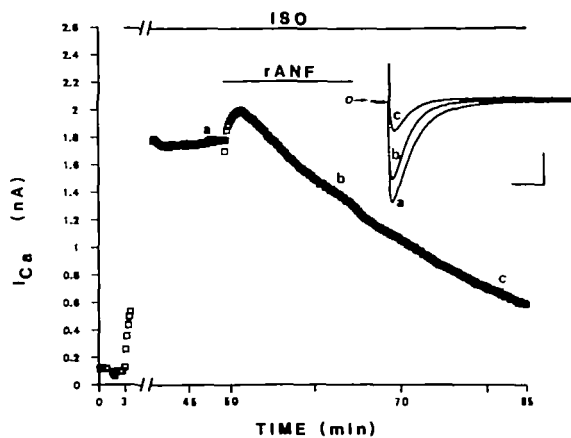


FIGURE 4. Effect of 3-28 ANF rat (rANF) on calcium current ( $I_{Ca}$ ) following a long exposure to isoprenaline (ISO). After the cell had been exposed during 45 minutes to  $2 \mu\text{M}$  ISO and no desensitization of  $I_{Ca}$  to ISO was observed, the cell was exposed to 3 nM rANF during the period indicated. Three current traces were recorded at times indicated by corresponding letters on the graph: a, ISO; b, ISO + 3 nM rANF; c, return to ISO alone. Current traces were recorded on and replayed from VCR tape (16 bits, bandwidth DC to 20 kHz) and digitized at 10 kHz. Calibration bars: horizontal 20 msec, vertical 600 pA; zero current level is indicated by an arrow. Kinetic analysis were performed with EXCALC<sup>26</sup> on six current traces in each three conditions. Two inactivation time constants were detected: a,  $42.5 \pm 1.5$  and  $10.2 \pm 0.2$  msec; b,  $37.9 \pm 2.3$  and  $9.0 \pm 0.2$  msec; c,  $32.8 \pm 3.2$  and  $8.3 \pm 0.2$  msec (mean  $\pm$  SEM,  $n=6$ ). (Cell No. 87072301;  $C_m=58$  pF.)

similar time, rANF had decreased ISO-elevated  $I_{Ca}$  by 60%, while cAMP-elevated  $I_{Ca}$  had been reduced by only 6%.

The strong inhibitory effect of ANF on  $I_{Ca}$  elevated by externally applied ISO as compared with the effect of the peptide on  $I_{Ca}$  enhanced by internally perfused cAMP resembles the differential action of acetylcholine (ACh) on calcium current seen in the same cells.<sup>19</sup> For this reason, a possible participation of muscarinic ACh-receptors in the regulation of  $I_{Ca}$  by ANF was investigated. Figure 7 shows an experiment where the cell was exposed simultaneously to ISO ( $2 \mu\text{M}$ ), ACh ( $1 \mu\text{M}$ ), and atropine ( $2 \mu\text{M}$ ). A concentration of  $1 \mu\text{M}$  ACh was sufficient to completely inhibit the stimulatory action of  $2 \mu\text{M}$  ISO.<sup>19</sup> In the presence of  $2 \mu\text{M}$  atropine, muscarinic ACh-receptors were blocked because ACh did not prevent the stimulatory action of ISO on  $I_{Ca}$ . The addition of 3 nM rANF to the solution induced an inhibitory response similar to those seen when ANF was added to ISO alone (Figure 7). Thus, ANF did not seem to have muscarinic agonist activity in our preparation (see also Ackermann et al<sup>12</sup>).

Table 1 tentatively summarizes the results of the experiments conducted with ANF in the presence of maximal doses of externally applied ISO ( $0.3 \mu\text{M}$ ) or internally applied cAMP ( $5 \mu\text{M}$ ). As shown, the effects of ANF not only varied with the dose of the peptide used but also from cell to cell. Moreover, it should be mentioned that the response to ANF seemed to be

strongly affected by the dissociation procedure because the cells obtained from certain dissociations did not show any response to ANF (rANF) either on control or after  $\beta$ -adrenergic stimulation (see "Discussion"). For this reason, the summary values in Table 1, which includes either all experiments or only those experiments showing ANF responses (for rANF) that were  $>5\%$ , should be regarded as indicative. ISO ( $0.3 \mu\text{M}$ ) increased  $I_{Ca}$  by an average of  $639.5 \pm 62.6\%$  ( $n=35$ ). While 1 nM rANF had negligible effects, 3 and 10 nM rANF significantly reduced  $I_{Ca}$  in the presence of ISO. Low doses of APIII seemed to be somewhat less effective than equivalent concentrations of rANF. At 3- and 10-nM concentrations, the effects of rANF on  $I_{Ca}$  elevated by the  $\beta$ -adrenergic agonist ISO were larger than those observed on  $I_{Ca}$  directly elevated by intracellular perfusion with cAMP.

## Discussion

Although ANF has been shown to antagonize both nonreceptor-induced (e.g., potassium depolarization) and receptor-induced (e.g., angiotensin II, norepinephrine, and histamine) contractions in vascular smooth muscle,<sup>18,32</sup> the effects are more pronounced on the latter.<sup>11,33</sup> Calcium channels may participate in this differential action of the hormone. Indeed, both  $^{45}\text{Ca}$  influx and contraction of isolated rabbit aorta were inhibited by rat atriopeptin II (APII) when prestimulated by norepinephrine but were much less affected by the peptide when prestimulated by high potassium concentration: this suggests a different action of ANF compounds on voltage-operated and receptor-operated calcium channels.<sup>11</sup> However, in rabbit and guinea pig renal arteries<sup>33</sup> and in rat juxtaglomerular cells,<sup>34</sup>  $\alpha$ -human ANF had no effect on calcium influx. Rather than involving sarcolemmal calcium channels, the relaxing effect of ANF on vascular smooth muscle has often been attributed to a reduction in cytosolic free calcium concentration<sup>35</sup> caused by an inhibition of

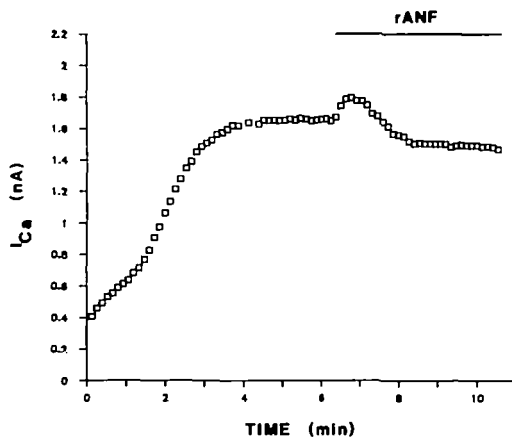


FIGURE 5. Effect of 3-28 ANF rat (rANF) on cyclic AMP (cAMP)-elevated calcium current ( $I_{Ca}$ ). At time zero, perfusion of the cell with intracellular medium containing  $5 \mu\text{M}$  cAMP was begun and continued throughout the experiment. The cell was exposed to 3 nM rANF during the period indicated. (Cell No. 87021001;  $C_m=59$  pF.)

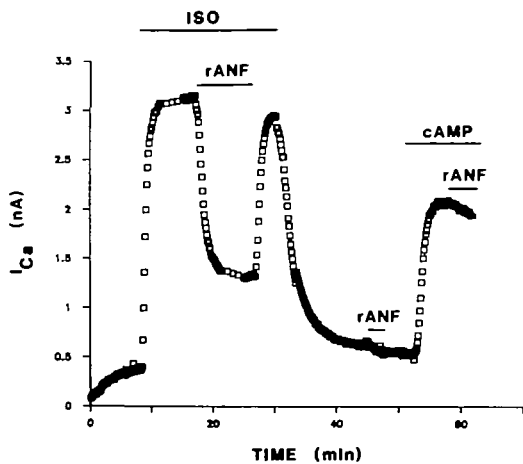


FIGURE 6. Effects of 3-28 ANF rat (rANF) on calcium current ( $I_{Ca}$ ). At time zero, perfusion of the cell with control intracellular medium was begun and continued until the solution in the patch electrode was changed to an intracellular solution containing  $5 \mu\text{M}$  cyclic AMP during the period indicated (cAMP). The cell was exposed externally to  $0.3 \mu\text{M}$  isoprenaline (ISO) and/or  $10 \text{ nM}$  rANF during the periods indicated. (Cell No. 87031104;  $C_m = 63 \text{ pF}$ .)

calcium release from internal stores,<sup>14</sup> following a cGMP-mediated reduction of phosphatidylinositol hydrolysis.<sup>33,36</sup>

Besides demonstrating for the first time a direct action of ANF on calcium influx in heart, our results also suggest that ANF acts preferentially on agonist-stimulated calcium influx. In frog ventricular cells, APIII and rANF were much less effective on control  $I_{Ca}$  than on isoprenaline-elevated  $I_{Ca}$ . In that respect, the effects of ANF on  $I_{Ca}$  resemble those of acetylcholine<sup>19</sup> or internal cGMP.<sup>23,24</sup> In cardiac cells, the stimulatory effect of  $\beta$ -adrenergic agonists on  $I_{Ca}$  involves an increased activity of the adenylate cyclase. Anand-Srivastava and Cantin<sup>15</sup> have demonstrated that adenylate cyclase activity was reduced by ANF in rat cardiocytes. Again, the inhibitory effect of the peptide was more pronounced when the activity of the enzyme had been enhanced by various agonists, including isoproterenol, forskolin, prostaglandins, and others.<sup>15</sup> Interestingly, that the inhibition of adenylate cyclase by ANF was similar when its activity was enhanced by  $\beta$ -adrenergic agonists or forskolin indicates that ANF is unlikely to exert  $\beta$ -adrenergic antagonist activity.<sup>15</sup> In our experiments, adenylate cyclase would clearly seem to be involved in the action of ANF because ANF was more effective when  $I_{Ca}$  was enhanced indirectly by  $\beta$ -adrenergic stimulation than when  $I_{Ca}$  was stimulated directly by intracellular cAMP. A similar observation was obtained in the effects of acetylcholine on  $I_{Ca}$ .<sup>19</sup> However, the ability of ANF (or acetylcholine) to reduce cAMP levels by mechanisms other than inhibition of adenylate cyclase (e.g., cGMP-stimulated cAMP phosphodiesterase) may be less when a massive amount of cAMP is provided by intracellular perfusion than when cAMP is provided locally by the adenylate cyclase.

The accurate analysis of the effect of ANF is limited by the rapid degradation of the peptide when bound to its receptor. Using radiolabeled ANF ( $^{125}\text{I}$ -ANF) in cultured Leydig tumor cells<sup>37</sup> or in isolated mesenteric artery,<sup>38</sup> it was shown that after the binding of ANF to the cell surface receptor, the ligand-receptor complex was rapidly degraded (95% within 3 minutes at  $37^\circ\text{C}$ ) either by a membrane-bound enzyme or after internalization of the complex.<sup>37,39</sup> This could be partly responsible for the transient effects of ANF on  $I_{Ca}$  seen in some of our experiments (Figure 1) and makes it even more difficult to understand the complex multiphasic effects of the hormone on  $I_{Ca}$ . Degradation of ANF in physiological solutions (room temperature) should be relatively small: although some of our cells did not show any response to ANF, other cells exposed up to 2 hours later to the same ANF solutions gave clear effects of the peptide.

Whether the absence of ANF response is an intrinsic property of some cardiac cells or is due to damage of the cells or downregulation of the receptors during the dissociation procedure remains unknown. However, one possible explanation is that cardiac cells secrete a significant amount of ANF during the dissociation procedure and/or in the cell suspension, which would downregulate the receptors and prevent a subsequent response to the hormone. Preliminary radioimmunoassay measurements of ANF levels, using radiolabeled [ $^{125}\text{I}$ ]ANF-(Ser<sup>99</sup>-Tyr<sup>126</sup>) and anti-(rat ANF) antibody,<sup>40</sup> were performed in the recirculating enzymatic dissociation medium (20 ml) collected at the end (45 minutes) of seven cell dissociations (see Fischmeister and Hartzell<sup>19</sup> for composition). ANF content varied from 3 pg/ml to 22 ng/ml, although the cell dissociations were performed under controlled identical conditions. Since purified or synthesized frog ANF is not yet available, it was necessary to assume a complete cross-immunoreactivity of frog ANF with rat ANF, so the measurements of ANF levels were most likely

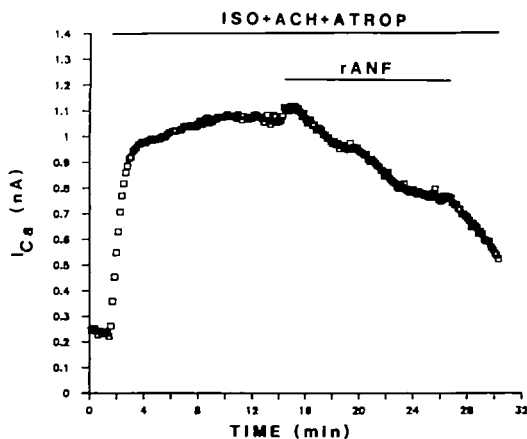


FIGURE 7. Effects of 3-28 ANF rat (rANF) on calcium current ( $I_{Ca}$ ) elevated by isoprenaline (ISO) in the presence of acetylcholine and atropine. After 12 minutes exposure of the cell to ISO ( $2 \mu\text{M}$ ) + acetylcholine (ACH,  $1 \mu\text{M}$ ) + atropine (ATROP,  $2 \mu\text{M}$ ),  $3 \text{ nM}$  rANF was added to solution during period indicated. (Cell No. 87072206,  $C_m = 102 \text{ pF}$ .)

**TABLE 1. Effect of Rat Atriopeptin III and 3-28 ANF Rat on Calcium Current**

| ANF (nM) |       |                | ISO/control (%)    | ISO + ANF/control (%) | cAMP/control (%)   | cAMP + ANF/<br>control (%) |
|----------|-------|----------------|--------------------|-----------------------|--------------------|----------------------------|
| 1        | rANF  | All            | 638.2 ± 64.6 (12)  | 614.3 ± 63.4 (12)     | 659.0 ± 71.8 (5)   | 598.8 ± 152.6 (5)          |
|          |       | Variations >5% | 563.8 ± 102.6 (3)  | 458.8 ± 26.6 (3)      | 623.8 ± 80.6 (4)   | 542.3 ± 179.9 (4)          |
| 3        | APIII |                | 659.5 ± 107.1 (4)  | 563.3 ± 96.0 (4)†     |                    |                            |
| 3        | rANF  | All            | 674.6 ± 124.1 (11) | 455.1 ± 57.2 (11)†    | 593.0 ± 65.9 (15)  | 514.8 ± 77.3 (15)*         |
|          |       | Variations >5% | 789.6 ± 209.2 (6)  | 391.3 ± 79.6 (6)      | 526.2 ± 57.0 (10)  | 410.9 ± 71.2 (10)          |
| 10       | APIII |                | 642.7 ± 141.5 (3)  | 470.1 ± 114.3 (3)     |                    |                            |
| 10       | rANF  | All            | 710.6 ± 156.2 (12) | 527.5 ± 127.4 (12)*   | 920.6 ± 245.0 (11) | 749.1 ± 200.1 (11)†        |
|          |       | Variations >5% | 672.2 ± 201.7 (7)  | 399.9 ± 85.8 (7)      | 991.5 ± 394.4 (6)  | 681.4 ± 274.8 (6)          |
| 30       | APIII |                | 642.7 ± 141.5 (3)  | 486.9 ± 87.4 (3)      |                    |                            |
| 30       | rANF  |                | 510.1 ± 150.5 (3)  | 300.6 ± 42.9 (3)      |                    |                            |
| 100      | APIII |                | 678.0 ± 96.5 (3)   | 491.0 ± 55.9 (3)      |                    |                            |
| 100      | rANF  |                | 628.2 ± 80.9 (3)   | 447.2 ± 67.0 (3)      |                    |                            |

ANF, atrial natriuretic factor; ISO, isoprenaline; cAMP, cyclic adenosine 3',5'-monophosphate; rANF, 3-28 ANF rat; APIII, rat atriopeptin III;  $I_{Ca}$ , calcium current.

$I_{Ca}$  was elevated by either 0.3  $\mu$ M external ISO or 5 or 20  $\mu$ M internal cAMP. For each experiment, currents were expressed with respect to control  $I_{Ca}$  (i.e., prior to ISO or cAMP application). Values are expressed as mean  $\pm$  SEM ( $n$ ).  $I_{Ca}$  values in ANF were measured at the end of ANF application (i.e., after 3–6 minutes) and do not reflect additional transitory effects of the peptide. When  $I_{Ca}$  was elevated by ISO, exposure of the cell of ANF generally induced a rundown of  $I_{Ca}$ ; thus, the values of  $I_{Ca}$  measured at the end of the exposure to ANF are necessarily overestimated. For experiments using rANF, certain cells showed no response to the peptide at any concentration tested. These experiments were either included (all) or discarded (variations >5%) in the summary data; in the latter, the data included only those experiments where ANF responses were >5% of  $I_{Ca}$  elevated by either ISO or cAMP. In the former, † indicates a statistically significant difference from the data in the absence of ANF; \* $p$ <0.1, † $p$ <0.05. (For further details, see text.)

underestimated. However, when ANF levels were below 20 pg/ml (four hearts), eight cells (out of 10) responded electrophysiologically to nanomolar concentrations of rANF. In the three other hearts where ANF levels were >100 pg/ml, only one cell (out of four) gave an effect of rANF on  $I_{Ca}$  (Puc at et al, unpublished observations).

Although stimulation of cardiac guanylate cyclase by ANF has not been demonstrated,\* cGMP may play a role in the action of ANF on  $I_{Ca}$ . In four cells, the action of 10 nM rANF on cAMP-elevated  $I_{Ca}$  was investigated in the presence or in the absence of 100  $\mu$ M external 1-methyl-3-isobutyl xanthine (IBMX). IBMX, known as a nonspecific cyclic nucleotide phosphodiesterase inhibitor, was shown recently to antagonize the inhibitory action of internal cGMP on cAMP-elevated  $I_{Ca}$ .<sup>23,24</sup> In all four cells, the inhibitory effects of rANF on  $I_{Ca}$  were smaller or suppressed by IBMX. In two other experiments, the hydrolysis-resistant 8-bromo derivative of cAMP (8-BrcAMP) was used instead of the native form. Although  $I_{Ca}$  was enhanced as much by 5  $\mu$ M 8-BrcAMP and 5  $\mu$ M cAMP (see also Fischmeister and Hartzell<sup>24</sup>), the effects of subsequent application of 3 nM rANF were negligible. Although more experiments are required to clarify the molecular mechanisms of action, the inhibitory effect of the peptide on  $I_{Ca}$  that had been stimulated by intracellular perfusion with cAMP resembled, though it was much smaller, the inhibitory effect of cGMP that has been seen under similar conditions since IBMX and 8-BrcAMP seemed to

reduce both negative responses.<sup>23,24</sup> This could suggest that a cGMP-stimulated cyclic nucleotide phosphodiesterase<sup>41</sup> participates in the negative action of ANF on  $I_{Ca}$ . Finally, cardiac cells also possess a cGMP-inhibited cyclic nucleotide phosphodiesterase<sup>42</sup> that could be a possible mechanism by which ANF, if it is shown to stimulate cardiac guanylate cyclase, exerts its stimulatory action on  $I_{Ca}$ ; as was seen in several of our experiments. In that respect, it is interesting that ANF has been shown to have vasoconstricting effects in rat postglomerular arterioles<sup>43</sup> and in guinea pig coronary artery<sup>16</sup> (but see Rapoport et al<sup>18</sup>) in contrast to the more commonly observed vasorelaxing effects.

Besides being regulated by ANF, cardiac calcium channels may also play a role in controlling ANF secretion from atrial cells because the calcium agonist Bay K 8644 has been shown to stimulate ANF secretion from perfused rat hearts.<sup>44,45</sup> However, these effects of the calcium agonist could be due to an enhanced atrial contraction frequency that by itself evokes ANF secretion.<sup>46</sup> More interestingly, forskolin<sup>45</sup> and isoproterenol<sup>47</sup> have also been shown to enhance ANF secretion from rat and rabbit heart, respectively. This could be due not only to increased heart beat rate but also to the stimulation of cAMP-dependent protein kinase, which has been shown to phosphorylate ANF-related compounds *in vitro*.<sup>48</sup> Thus, if activators of adenylate cyclase stimulate ANF synthesis and secretion and ANF inhibits adenylate cyclase activity, then ANF may be a feedback regulator of its own secretion. Besides, through its effects on cardiac  $I_{Ca}$  (as reported here), ANF would tend to reduce the positive chronotropic and inotropic effects of sympathetic neuro-mediators and, thus, would act in a qualitatively similar

\*Note added in proof: Cramb et al (*Biochem Biophys Res Commun* 1987;148:962–970) have recently shown a stimulation of cGMP production by ANF in isolated rabbit ventricular myocytes.

way to parasympathetic effectors such as acetylcholine, although through different receptors. This could imply an interaction between acetylcholine and ANF on calcium channels in heart. We are currently investigating this possibility.

In conclusion, our findings demonstrate an inhibitory action of nanomolar concentrations of ANF on cardiac calcium current, which suggests that ANF may exert a negative inotropic effect that could be an additional mechanism by which ANF regulates blood pressure.

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