Calcium, Its Role in Isoproterenol-Stimulated Atrial Natriuretic Peptide Secretion by Superfused Rat Atria

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The β-adrenergic agonist isoproterenol stimulates immunoreactive atrial natriuretic peptide (IR-ANP) secretion by superfused rat atria in vitro. β-Adrenergic agonists alter the cellular handling of calcium, which culminates in a rise in the systolic calcium concentration. This is achieved by increasing calcium influx through voltage-dependent calcium channels and by increasing the storage pool of calcium in the sarcoplasmic reticulum (SR). We therefore asked the question whether isoproterenol-stimulated IR-ANP secretion was dependent on the protein kinase A–induced rise in systolic calcium or was due to a direct effect of protein kinase A activation. Isolated rat left atria paced at 3 Hz were superfused in vitro. IR-ANP secretion was determined by radioimmunoassay of timed collections of the superfusate. Superfusion with 0.1 μM isoproterenol or 0.5 mM dibutyryl cyclic AMP increased IR-ANP secretion twofold. Stimulated IR-ANP secretion was lowered to near baseline by lowering the buffer calcium concentration from 1.8 to 0.2 mM or by adding to the superfusate 10 μM nitrendipine (a calcium-channel blocker) or 1 μM ryanodine (an inhibitor of SR calcium release). Superfusion of nonbeating, electrically quiescent left atria with 0.1 μM isoproterenol failed to stimulate IR-ANP secretion. We conclude: 1) Isoproterenol-stimulated IR-ANP secretion is dependent on calcium influx through voltage-dependent calcium channels and on the release of calcium from the SR. 2) Enhanced calcium influx alone is not adequate to maintain isoproterenol-stimulated IR-ANP secretion. 3) The SR appears to be the primary source of calcium for isoproterenol-stimulated IR-ANP secretion. 4) The stimulatory effect of isoproterenol on IR-ANP secretion is dependent on electrical membrane activity. 5) Thus, protein kinase A activation does not appear to have a direct effect on IR-ANP secretion. Its effect appears to be mediated by protein kinase A–directed changes in the handling of calcium by atrial cardiocytes. These results suggest that calcium-channel blockers may lower plasma ANP levels in man when sympathetic tone is high. Lowering plasma ANP may be responsible, in part, for fluid retention in patients treated with calcium-channel blockers. (Circulation Research 1989;65:600–606)
tion. We studied the dependency of cAMP stimuli on extracellular calcium, calcium influx through nitrendipine-inhibitable calcium channels, and on ryanodine-inhibitable calcium release from the SR. The results of this study suggest that cAMP-stimulated ANP secretion is a calcium-dependent event.

**Materials and Methods**

**Materials**

Isoproterenol, propranolol, atropine, and dibutyryl cAMP were from Sigma Chemical (St. Louis, Missouri). Nitrendipine and Bay K 8644 were gifts from Alexander Scriabine, Miles Institute of Preclinical Pharmacology (New Haven, Connecticut). Ryanodine was from Progressive Agri-Systems (Wind Gap, Pennsylvania), and goat antibody to rabbit globulin was supplied by Peninsula Laboratories (Belmont, California), and goat antibody to rabbit globulin was supplied by Calbiochem (San Diego, California). Phentolamine was a gift from CIBA-Geigy (Suffern, New York).

**Atrial Superfusion Studies**

Experiments were performed as previously described. Briefly, rat left atria were suspended between two hooks, one of which was an electrode. The second hook was attached to an isometric force transducer. Resting tension was initially set at 1.25 g without further adjustment. Atria were superfused at 1.2 ml/min with Medium 199 (potassium 4.0 mM) at 37°C bubbled with 95% O2-5% CO2. Atria were paced at 3 Hz. Studies involving isoproterenol were performed at 0.1% trifluoroacetic acid (TFA) in water to prevent precipitation. Nitrendipine, Bay K 8644, and ryanodine were dissolved in ethanol, final concentration was maintained constant throughout the study.

**ANP Radioimmunoassay**

ANP was quantitated by radioimmunoassay (RIA) as previously described. Bound and free hormones were separated by the double antibody precipitation technique. Rat α-ANP was iodinated by the chloramine T method. [125I]IodoANP was purified by high-pressure liquid chromatography on a Vydac 4.6 mm x 25 cm column using a linear gradient of 0.1% trifluoroacetic acid (TFA) in water to acetonitrile:water:TFA (60:40:0.1) over 60 minutes at a flow rate of 1 ml/min. [125I]IodoANP eluted between 42–45 minutes.

**Data Presentation and Analysis**

All calculations were performed separately on each atrium. Some of the data are expressed as a percent of baseline with the baseline defined as the mean of seven measurements obtained immediately before superfusion with a test agent. The remainder of the data are expressed as a percent of the net increase in ANP secretion. In these studies, the average of seven baseline samples was first subtracted from all of the ANP measurements. The average measurement of samples collected immediately before the introduction of 0.2 mM calcium, nitrendipine, or ryanodine was assigned a value of 100%. The number of samples averaged was four or two for isoproterenol and dibutyryl cAMP experiments, respectively. These mean values were divided into all of the measurements from which baseline was subtracted. These calculations rendered results expressed as a percent of the net increase in ANP secretion. The results were statistically analyzed by paired t test or by one-way analysis of variance. All results are expressed as mean±SEM.

**Results**

We first examined the effect of lowering the extracellular calcium concentration on isoproterenol-stimulated immunoreactive ANP (IR-ANP) secretion. In preliminary experiments the effects of lowering the superfusate calcium concentration on basal IR-ANP secretion was studied. Lowering the superfusate calcium from 1.8 mM to no added calcium resulted in a slow but dramatic rise in IR-ANP secretion by paced atria (Figure 1A). This rise was reversed by superfusion with Medium 199 containing 1.8 mM calcium. However, continued superfusion in the absence of calcium resulted in a further rise in IR-ANP secretion. Secretion by atria continuously superfused with 1.8 mM calcium fell slightly over the same period of time (Figure 1B). Superfusion with 0.2 mM calcium, the calcium concentration chosen for these experiments, also resulted in a delayed rise in IR-ANP secretion but to a lesser degree (data not shown). However, IR-ANP secretion remained stable during the 30 minutes of superfusion with 0.2 mM calcium used in these studies (Figure 2).

Figure 3 illustrates the effect of lowering the extracellular calcium concentration on IR-ANP secretion stimulated by 0.1 μM isoproterenol. Isoproterenol increased IR-ANP secretion by 2.5±0.2-fold during superfusion with 1.8 mM calcium. Lowering the calcium concentration to 0.2 mM resulted in a fall in IR-ANP secretion to baseline. Developed tension fell by 84% (Table 1). Isoproterenol-stimulated IR-ANP secretion remained elevated by atria continuously superfused with 1.8 mM calcium (Figure 3). Lowering the superfusate calcium concentration from 1.8 to 0.2 mM under basal conditions for 30 minutes did not significantly affect IR-ANP secretion (Figure 2) even though developed tension fell by 90% (Table 2).

To ensure that the manipulations used in these experiments did not interfere with isoproterenol-stimulated cAMP production, atria were stimulated with dibutyryl cAMP. Superfusion with 0.5 mM dibutyryl cAMP increased IR-ANP secretion by 2.3±0.3-fold. Lowering the superfusate calcium concentration from 1.8 to 0.2 mM again resulted in a fall in IR-ANP secretion to near baseline (Figure
FIGURE 1. Plots showing effect of lowering the superfusate calcium concentration on immunoreactive atrial natriuretic peptide (IR-ANP; ANP-IR on figure) secretion. Panel A: Rat left atria paced at 2 Hz were initially superfused with 1.8 mM calcium. At 15 minutes (first arrow), atria were superfused with Medium 199 containing no calcium. At 135 minutes (second arrow), atria were again superfused with 1.8 mM calcium (•; n=3) or continued with no added calcium (○; n=2). Results are expressed as a percent of the net increase in IR-ANP secretion with secretion at 135 minutes defined as 100%. Basal IR-ANP secretion was 269±58 pg/ml and rose to 920±114 pg/ml at 135 minutes. Panel B: Basal IR-ANP secretion by atria (n=3) paced at 2 Hz continuously superfused with 1.8 mM calcium. Results are expressed as a percent of the mean of seven measurements taken between 0-15 minutes. IR-ANP secretion between 0-15 minutes was 242±43 pg/ml.

4). Developed tension fell by 89% (Table 1). IR-ANP secretion remained elevated with continued superfusion of 1.8 mM calcium (Figure 4). Thus, IR-ANP secretion, stimulated by increases in intracellular cAMP, is dependent on the extracellular calcium concentration. This dependence suggests that calcium influx is necessary to maintain cAMP-dependent stimuli of IR-ANP secretion.

β-Adrenergic agonists enhance calcium influx by voltage-dependent calcium channels. For this reason, we examined the effect of 10 μM nitrendipine on 0.1 μM isoproterenol and 0.5 mM dibutyryl cAMP-stimulated IR-ANP secretion. Isoproterenol and dibutyryl cAMP increased IR-ANP secretion by 2.2±0.2- and 2.0±0.1-fold, respectively, in these experiments. The addition of nitrendipine to the superfusate lowered isoproterenol and dibutyryl cAMP-stimulated IR-ANP secretion to near baseline (Figure 5). The addition of nitrendipine to the
superfusate lowered developed tension by 63% and 27% with isoproterenol and dibutyryl cAMP stimulation, respectively (Table 1). When nonstimulated atria were superfused with 10 μM nitrendipine, IR-ANP secretion fell slightly but significantly (p<0.03) when compared with control (Figure 2). Nitrendipine lowered developed tension by 56% (Table 2). These results suggest that enhanced calcium influx by nitrendipine-inhibitable calcium channels appears to play a significant role in stimulated secretion by cAMP-dependent agonists of IR-ANP release.

To further examine the role of enhanced calcium influx via voltage-dependent calcium channels on IR-ANP secretion, the effect of Bay K 8644, a calcium-channel activator,7 was examined. Bay K 8644 was superfused with 1 μM propranolol, 10 μM phentolamine, and 10 μM atropine to inhibit effects of the potential release of norepinephrine or acetylcholine from endogenous nerve fibers. Superfusion of paced left atria with 1 μM Bay K 8644 increased IR-ANP secretion by 1.9±0.1-fold (Figure 6). The pattern of the IR-ANP secretory response was different from that of isoproterenol (Figure 3). Superfusion with Bay K 8644 increased developed tension by 3.1±0.2-fold. Resting tension was 0.24±0.07 g and did not change with the addition of Bay K 8644. Superfusion of Bay K 8644 with 50 μM nitrendipine negated stimulated IR-ANP secretion (Figure 6), suggesting that stimulation by Bay K 8644 was mediated by enhanced calcium influx via voltage-dependent calcium channels. In a second control study, nonbeating left atria were superfused with dibutyryl cAMP 0.5 mM Calcium 0.2 mM.

![Figure 3](image1)

**FIGURE 3.** Plot showing effect of lowering the superfusate calcium concentration on isoproterenol-stimulated immunoreactive atrial natriuretic peptide (IR-ANP; ANP-IR on figure) secretion. Rat left atria paced at 3 Hz were continuously superfused with 0.1 μM isoproterenol from 15 to 105 minutes. At 60 minutes, the atria were superfused with Medium 199 containing 0.2 mM calcium (·; n=8), or superfusion was continued with 1.8 mM calcium (○; n=4). Results are expressed as a percent of the net increase in IR-ANP secretion. Basal IR-ANP secretion was 388±37 pg/ml and rose 929±83 pg/ml at 60 minutes with isoproterenol.

**FIGURE 4.** Plot showing effect of lowering the superfusate calcium concentration on dibutyryl cyclic AMP (cAMP)-stimulated immunoreactive atrial natriuretic peptide (IR-ANP; ANP-IR on figure) secretion. Left atria paced at 3 Hz were continuously superfused with 0.5 mM dibutyryl cAMP from 15 to 105 minutes. At 60 minutes, atria were superfused with Medium 199 containing 0.2 mM calcium (·; n=5), or superfusion was continued with 1.8 mM calcium (○; n=4). Results are expressed as in Figure 3. Basal IR-ANP secretion was 427±56 pg/ml and rose to 883±100 pg/ml at 60 minutes with dibutyryl cAMP.

### Table 1. Measurements of Developed and Resting Tension With Stimulation

<table>
<thead>
<tr>
<th>Agent</th>
<th>Developed tension (g)</th>
<th>Resting tension (g)</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>0.14±0.02</td>
<td></td>
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<tr>
<td>0.1 μM isoproterenol</td>
<td>0.44±0.05*</td>
<td>0.37±0.03</td>
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<tr>
<td>+0.2 mM calcium</td>
<td>0.07±0.02†</td>
<td>0.37±0.03</td>
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<tr>
<td>None</td>
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<tr>
<td>0.5 mM dibutyryl cAMP</td>
<td>0.35±0.04*</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>+0.2 mM calcium</td>
<td>0.04±0.01†</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>None</td>
<td>0.09±0.02</td>
<td></td>
</tr>
<tr>
<td>0.1 μM isoproterenol</td>
<td>0.30±0.06*</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>+10 μM nitrendipine</td>
<td>0.11±0.02†</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>None</td>
<td>0.09±0.02</td>
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<tr>
<td>0.5 mM dibutyryl cAMP</td>
<td>0.37±0.04*</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>+10 μM nitrendipine</td>
<td>0.27±0.04†</td>
<td>0.33±0.01</td>
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<tr>
<td>None</td>
<td>0.09±0.01</td>
<td></td>
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<tr>
<td>0.1 μM isoproterenol</td>
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<tr>
<td>+1 μM ryanodine</td>
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<tr>
<td>0.5 mM dibutyryl cAMP</td>
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<td>0.27±0.02</td>
</tr>
<tr>
<td>+1 μM ryanodine</td>
<td>0.02±0.01†</td>
<td>0.30±0.02†</td>
</tr>
</tbody>
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Values are mean±SEM.

* p<0.005 relative to baseline.
† p<0.005 relative to stimulated alone.
‡ p<0.02 relatively to stimulated alone.
FIGURE 5. Plot showing effect of nitrendipine on isoproterenol and dibutyryl cyclic AMP (cAMP)-stimulated immunoreactive atrial natriuretic peptide (IR-ANP; ANP-IR on figure) secretion. Left atria paced at 3 Hz were continuously superfused with 0.1 μM isoproterenol (●; n=5) or 0.5 mM dibutyryl cAMP (○; n=8) from −45 minutes to the end of the experiment. At time 0, 10 μM nitrendipine was added to the superfusate. Results are expressed as a percent of the net increase in IR-ANP secretion before the addition of nitrendipine. Basal and stimulated IR-ANP secretion were 521±153 and 1,158±322 pg/ml for isoproterenol and 489±28 and 971 pg/ml for dibutyryl cAMP.

with Bay K 8644. In electrically quiescent atria, calcium influx through voltage-dependent calcium channels should be only minimally affected on exposure to Bay K 8644. Likewise, IR-ANP secretion should not increase if the effect of Bay K 8644 on IR-ANP secretion is mediated by enhanced voltage-dependent calcium-channel calcium influx. Bay K 8644 failed to stimulate IR-ANP secretion by nonbeating left atria (Figure 6) lending further support to the specificity of its effect. These studies suggest that enhanced calcium influx via voltage-dependent calcium channels is able to stimulate IR-ANP secretion, which is one of the known effects that isoproterenol has on atrial cardiocytes.

We next examined the effect of ryanodine, an inhibitor of SR calcium release, on IR-ANP secretion stimulated by isoproterenol or dibutyryl cAMP. Atria were superfused with 0.1 μM isoproterenol or 0.5 mM dibutyryl cAMP, which resulted in a 2.9±0.4- or 2.2±0.2-fold increase in IR-ANP secretion, respectively. The addition of 1 μM ryanodine to the superfusate resulted in a fall in stimulated IR-ANP secretion to baseline (Figure 7). Ryanodine lowered developed tension by 95% and 94% with isoproterenol and dibutyryl cAMP stimulation, respectively (Table 1). Superfusion with 1 μM ryanodine alone, without prior stimulation, did not significantly influence IR-ANP secretion (Figure 2) in spite of a fall in developed tension to undetectable levels (Table 2). These results suggest that the release of calcium from an expanded calcium storage pool is necessary to maintain cAMP-dependent stimuli of IR-ANP secretion.

The above results are consistent with a calcium-dependent mechanism of isoproterenol-stimulated ANP secretion. Further proof of this thesis is offered by data presented in Figure 8. If isoproterenol-stimulated IR-ANP secretion is indeed calcium-dependent, isoproterenol would not be expected to increase IR-ANP secretion by nonbeating left atria since its ability to increase cytosolic calcium is dependent on electrical membrane activity. When nonbeating atria were superfused with 0.1 μM isoproterenol, IR-ANP secretion not only failed to rise but actually fell significantly relative to control atria superfused with 0.1 μM isoproterenol and 1 μM propranolol (Figure 8). Thus, isoproterenol-stimulated IR-ANP secretion appears not to be due to a direct effect of activated protein kinase A but

| Table 2. Measurements of Developed and Resting Tension Without Stimulation |
|-----------------------------|-----------------------------|
| Agent                        | Developed tension (g)       | Resting tension (g) |
| None                         | 0.10±0.01                   | 0.34±0.02           |
| +0.2 mM calcium              | 0.01±0*                     | 0.34±0.02           |
| None                         | 0.09±0.01                   | 0.38±0.03           |
| +10 μM nitrendipine          | 0.05±0.01*                  | 0.38±0.03           |
| None                         | 0.09±0.01                   | 0.30±0.03           |
| +1 μM ryanodine              | 0±0*                        | 0.30±0.03           |

Values are mean±SEM.

*p<0.005 relative to baseline.
Calcium and Isoproterenol-Stimulated ANP Secretion

Discussion

This study illustrates the multiple calcium-dependent points of isoproterenol-stimulated IR-ANP secretion. Extracellular calcium is necessary to maintain isoproterenol-stimulated IR-ANP secretion. The dependency on extracellular calcium appears to primarily reflect the need for calcium influx through voltage-dependent calcium channels as illustrated by nitrendipine inhibition of stimulated secretion. Isoproterenol-stimulated IR-ANP secretion is also largely dependent on calcium release from the SR. It appears that most of the cytosolic calcium necessary for isoproterenol-stimulated secretion is derived from the SR since ryanodine is able to totally inhibit stimulated secretion. Thus, calcium influx alone cannot maintain isoproterenol-stimulated IR-ANP secretion. However, nitrendipine lowers isoproterenol-stimulated IR-ANP secretion to baseline. Calcium influx is considered to be necessary for calcium-induced calcium release from the SR. Thus, in the presence of nitrendipine, calcium influx is decreased, and this decrease leads to a reduction in the amount of calcium released from the SR. This results in a fall in developed tension and IR-ANP secretion.

These observations demonstrate that isoproterenol-stimulated IR-ANP secretion is calcium dependent. Simply activating protein kinase A alone is not sufficient to stimulate IR-ANP secretion, but rather it is the effect that protein kinase A has on the handling of cellular calcium that is responsible for influencing secretion. This conclusion is based on observations made with the inhibitors nitrendipine and ryanodine. It is theoretically possible that nitrendipine or ryanodine may block isoproterenol binding, couple with adenylyl cyclase, or directly inhibit adenylyl cyclase activity. These effects would tend to lower isoproterenol-stimulated IR-ANP secretion. However, nitrendipine and ryanodine also block dibutyryl cAMP-stimulated IR-ANP secretion; this blockage suggests that inhibition of IR-ANP secretion occurs distal to the generation of cAMP.

The conclusion that activation of protein kinase A is not sufficient to stimulate IR-ANP secretion is further supported by the observation that isoproterenol fails to stimulate IR-ANP secretion by nonbeating left atria. In fact, IR-ANP secretion fell. The fall in secretion may reflect a reduction in cytosolic calcium by enhanced calcium uptake by the SR upon exposure to isoproterenol. The failure of isoproterenol to stimulate IR-ANP secretion by nonbeating atria may also be a partial explanation for the discrepancy between our reported positive results of the effect of isoproterenol using paced atria and the negative results of others using atria that may not have been beating. It has recently been reported that forskolin lowers ANP secretion in nonbeating cultured atrial cardiocytes; this finding is in agreement with the results of our study. The effect of the calcium-channel activator Bay K 8644 was examined to determine the effect of enhanced calcium-channel calcium influx on IR-ANP secretion since β-adrenergic agonists also increase calcium influx via a similar mechanism. Bay K 8644 increased IR-ANP secretion, suggesting that enhanced calcium influx is able to augment IR-ANP secretion. The specificity of the response to Bay K 8644 is supported by inhibition of Bay K 8644-stimulated IR-ANP secretion by nitrendipine and the failure of Bay K 8644 to stimulate IR-ANP secretion.
by nonbeating atria. The pattern of the IR-ANP secretory response to Bay K 8644 was different from that of isoproterenol. This difference suggests that mechanisms, in addition to an increase in calcium influx, are responsible for isoproterenol-stimulated IR-ANP secretion. One additional mechanism suggested from this study is an increase in calcium uptake by the SR with isoproterenol stimulation.

The unanticipated result was that of a rise in IR-ANP secretion by superfusion with no added calcium in the buffer. The significant delay in the rise of IR-ANP secretion suggests that cardiocyte calcium depletion may be responsible for enhanced IR-ANP secretion. We speculate that calcium depletion may alter the integrity of cellular cytoskeletal proteins and lead to enhanced secretory granule migration to the cell membrane and/or enhanced exocytosis. The duration of calcium depletion in this experiment does not appear to have been cytotoxic, since IR-ANP secretion returned to baseline when calcium was returned to the buffer. The transient rise in IR-ANP secretion upon reintroduction of calcium may reflect the calcium paradox in which a decrease in extracellular calcium produces an increase in intracellular sodium accumulation. When extracellular calcium is subsequently raised, cytosolic calcium increases due to altered Na-Ca exchange. Thus, IR-ANP secretion may increase due to a transient rise in cytosolic calcium. It is doubtful that the results of studies conducted in the absence of extracellular calcium reflect calcium's second messenger role in IR-ANP secretion, but rather an unrelated effect possibly on cytoskeletal integrity or function.

Ryanodine slightly raised resting tension in those atria superfused with isoproterenol or dibutyryl cAMP (Table 1). In contrast, ryanodine did not change resting tension when superfused alone (Table 2). The mean rise in resting tension with the introduction of ryanodine, 0.03 g with both isoproterenol and dibutyryl cAMP, were similar to the fall in resting tension induced by superfusion of isoproterenol and dibutyryl cAMP, 0.02 g with each. The fall in resting tension by isoproterenol is believed to reflect increased calcium uptake by the SR resulting in a fall in the diastolic calcium concentration. Some investigators have found that ryanodine also enhances SR calcium efflux. This would be predicted to raise diastolic calcium concentrations toward normal and elevate resting tension. Thus, the fall in resting tension induced by cAMP may be negated by ryanodine. However, IR-ANP secretion fell in spite of the predicted increase in the diastolic calcium concentration by the addition of ryanodine to isoproterenol or dibutyryl cAMP.

In conclusion, the second messenger cAMP stimulates IR-ANP secretion by increasing systolic calcium. Activation of protein kinase A alone without concomitant changes in calcium fluxes is not sufficient to stimulate IR-ANP secretion. Clinically, these observations suggest that administration of calcium-channel blockers may lower plasma ANP levels in patients with conditions such as congestive heart failure in which sympathetic tone is high. This effect may be responsible, in part, for the sodium-retaining properties of calcium-channel blockers.

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