Long-term treatment with atrial natriuretic peptide inhibits ATP production and insulin secretion in rat pancreatic islets

Hui You and Suzanne G. Laychock

Department of Pharmacology and Toxicology, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York

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You H, Laychock SG. Long-term treatment with atrial natriuretic peptide inhibits ATP production and insulin secretion in rat pancreatic islets. Am J Physiol Endocrinol Metab 300: E435–E444, 2011. First published October 19, 2010; doi:10.1152/ajpendo.00398.2010.—Atrial natriuretic peptide (ANP) levels correlate with hyperglycemia in diabetes mellitus, but ANP effects on pancreatic islet α-cell insulin secretion are controversial. ANP was investigated for short- and long-term effects on insulin secretion and mechanisms regulating secretion in isolated rat pancreatic islets. A 3-h incubation with ANP did not affect basal or glucose-stimulated islet insulin secretion. However, 7-day culture of islets with 5.5 mM glucose and ANP (1 nM - 1 μM) markedly inhibited subsequent glucose (11 mM)-stimulated insulin secretion; total islet insulin content was not affected. Following ANP removal for 24 h, the islet insulin-secretory response to glucose was restored. The insulin-secretory response to other insulin secretagogues, including α-ketoisocaproic acid, forskolin, potassium chloride, and ionomycin were also markedly inhibited by chronic exposure to ANP. However, the combination of potassium chloride and α-ketoisocaproic acid was sufficient to overcome the inhibitory effects of ANP on insulin secretion. The glucose-stimulated increases in islet ATP levels and the ATP/ADP ratio were completely inhibited in ANP 7-day-treated islets vs. control; removal of ANP for 24 h partially restored the glucose response. ANP did not affect islet glycolysis. ANP significantly increased levels of islet ATP levels and the ATP/ADP ratio were completely inhibitory effects of ANP on insulin secretion. The glucose-stimulated intracellular phase 0 [Ca2+] changes that regulate insulin secretion in rat islet β-cells (25) and to inhibit insulin secretion in rats and mice (1) and glucagon secretion in isolated rat pancreatic islets (52). There are also reports of a lack of effect of ANP on insulin secretion (51) or of a stimulatory effect (39, 50). The aforementioned in vitro studies were short term (hours) and did not fully elucidate the cellular mechanisms of ANP action on insulin secretion. In guanylyl cyclase A receptor knockout mice, insulin secretion in response to glucose stimulation was enhanced (39). In long-term in vitro studies (up to 7 days) ANP has been reported to promote rat islet β-cell Ser-Thr kinase Akt/PKB, FoxO1a and cyclin D2 signaling, and cell growth by a process mediated by phosphatidylinositol 3'-kinase (PI3K) activation (59). Akt has been reported to be important in insulin exocytotic events but not for more proximal events in β-cell glucose-stimulated insulin secretion (4). In contrast, activation of PI3K has been reported to adversely affect glucose-stimulated insulin secretion (60). Thus, putative mediators of ANP and cGMP signaling may have pleiotropic effects on β-cell regulatory processes.

ANP has also been reported to be a lipolytic agent in adipocytes and to act through the phosphorylation and activation of hormone-sensitive lipase (HSL) (42). cGMP/PKG also activates HSL (19) and has been reported to reduce intracellular Ca2+ levels in cells by inhibiting Ca2+ influx through voltage-dependent Ca2+ channels and activation of Ca2+ ATPases and the Na+/Ca2+ exchanger promoting Ca2+ efflux and sequestration of intracellular Ca2+, among other actions (6). The present study was undertaken to test the hypothesis that chronic exposure to ANP affects insulin secretion. The results demonstrate that long-term, but not acute, ANP treatment inhibited glucose-stimulated metabolism and insulin secretion in isolated rat pancreatic islets.

MATERIALS AND METHODS

Materials. ANP (1–28, rat, disulfide bridge: 7–23) was from AnaSpec (San Jose, CA). 8-Bromo-cGMP (8-Br-cGMP), 8-Br-cAMP, α-ketoisocaproic acid (KIC), forskolin, phosphoenolpyruvate, sodium molybate, and GMP were from Sigma Chemical (St. Louis, MO). Ionomycin was from Calbiochem (La Jolla, CA). NPR-A antiserum was from Abcam (Cambridge, MA). NPR-C (the peptide clearance receptor) lacks intrinsic guanylyl cyclase enzyme activity, and, while found in many different tissues (35), in islets this protein is expressed only in pancreatic α-cells (5). Interestingly, there is a link between ANP and diabetes mellitus. Acute hyperglycemia in type 1 diabetes mellitus is associated with the elevation of plasma ANP levels (31). Cardiac ANP mRNA is also augmented in streptozotocin-induced diabetic rats compared with normal rats (30). However, the effect of ANP on insulin secretion is controversial. ANP was reported to inhibit glucose-stimulated intracellular phase 0 [Ca2+] changes that regulate insulin secretion in rat islet β-cells (25) and to inhibit insulin secretion in rats and mice (1) and glucagon secretion in isolated rat pancreatic islets (52). There are also reports of a lack of effect of ANP on insulin secretion (51) or of a stimulatory effect (39, 50). The aforementioned in vitro studies were short term (hours) and did not fully elucidate the cellular mechanisms of ANP action on insulin secretion. In guanylyl cyclase A receptor knockout mice, insulin secretion in response to glucose stimulation was enhanced (39). In long-term in vitro studies (up to 7 days) ANP has been reported to promote rat islet β-cell Ser-Thr kinase Akt/PKB, FoxO1a and cyclin D2 signaling, and cell growth by a process mediated by phosphatidylinositol 3'-kinase (PI3K) activation (59). Akt has been reported to be important in insulin exocytotic events but not for more proximal events in β-cell glucose-stimulated insulin secretion (4). In contrast, activation of PI3K has been reported to adversely affect glucose-stimulated insulin secretion (60). Thus, putative mediators of ANP and cGMP signaling may have pleiotropic effects on β-cell regulatory processes.

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The present study was undertaken to test the hypothesis that chronic exposure to ANP affects insulin secretion. The results demonstrate that long-term, but not acute, ANP treatment inhibited glucose-stimulated metabolism and insulin secretion in isolated rat pancreatic islets.
ENLITEN ATP Assay System Bioluminescence Detection kit was from Promega (Madison, WI). Pyruvate kinase and ADP were from Calzyme Laboratories (San Luis Obispo, CA). ATP sulfurylase was from New England Biolabs (Boston, MA). D-[5-3H]glucose was from American Radiolabeled Chemicals (St. Louis, MO).

Isolation of rat islets and culture of cells and tissue. Pancreatic islets were isolated from pancreata of adult male Sprague-Dawley rats by use of collagenase, as described previously (57). All animal procedures were approved by the SUNY Buffalo Institutional Animal Care and Use Committee. Freshly isolated (fresh) islets were either used immediately or cultured in CMRL-1066 for up to 7 days in the presence or absence of various stimuli, as described in the text. CMRL-1066 medium contained 5.5 mM glucose, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. Culture conditions were 5% CO2–95% air and 37°C.

Immunoblotting. Islet homogenates were prepared for immunoblotting essentially as described previously (24). Equal amounts of protein per sample (40 μg) for each experiment were separated on 8% SDS-PAGE gels and transferred to PVDF (0.45 μm) membranes. Immunoblotting was carried out essentially as described previously (24), with primary antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody. β-Actin was also immunoblotted for each sample to normalize the data. Chemiluminescence was digitized using a Fuji luminoimager. The density of each band was determined by densitometric scanning using Molecular Analyst software.

cGMP enzyme-linked immunoassay. Cultured islets (25 islets/sample) were washed twice with fresh medium. 3-Isobutyl-1-methylxanthine (IBMX) (20 μM) was added to each sample. After 20 min of incubation with IBMX, samples were treated with ANP for an additional 10 min. The same ANP concentration was present during the incubation with IBMX as was present during the pretreatment. Ice-cold HCl (final concentration 0.1 M) was added to stop the reaction. Samples were frozen and thawed three times, sonicated, and centrifuged at 500 rpm for 10 min. Supernatants were assayed for cGMP by enzyme-linked immunoassay with acetylation.

Insulin secretion of islets. Islet insulin secretion was determined essentially as described previously (57). Islets were cultured with 5.5 mM glucose and 10% FBS in the presence or absence of ANP as indicated for 7 days. The islets (10 islets/sample) were washed and resuspended in Krebs-Ringer bicarbonate-HEPES (KRHB) buffer, pH 7.4, containing 5.5 mM glucose and 0.1% insulin-free BSA and incubated at 37°C under an atmosphere of 5% CO2–95% O2 in a shaking water bath. After 30 min, the KRHB buffer was replaced with fresh buffer, and an aliquot of buffer was removed to determine the time 0 insulin level. After an additional 60-min or 3-h incubation in the presence or absence of stimuli indicated in the text, an aliquot of buffer was removed for determination of insulin release. Insulin release values are minus time 0 insulin values. Insulin was extracted from the islets in 70% ethanol containing 1 M HCl. Total insulin values include extracted plus secreted insulin. Insulin levels were quantitated by RIA using rat insulin as the standard.

ATP and ADP content of islets. The ATP content of islets was measured using a modified method of Fadda et al. (11). Cultured isolated islets (10 islets/sample) were washed twice with KRHB buffer containing 2.8 mM glucose. TCA (final concentration 2.4%) was added to each sample. Samples were kept on ice for 30 min and then sonicated for 20 s. TCA was removed by three extractions of samples with water-saturated ether. Islet ADP levels were quantitated in the same samples as those used for ATP after conversions of ATP to AMP and ADP to ATP, as described previously (41). The net samples were assayed for ATP content using the ENLITEN ATP Assay System Bioluminescence Detection kit and a Berthold lumimeter. Briefly, background light emission B (mV), islet supernatant (10 μl) light emission S (mV), and ATP standard (2 μM) I (mV) light emission were measured. Samples were assayed in duplicate. ATP concentration of the 10-μl samples was calculated by the formula: ATP (pmol) = (S–B)/(I–S) × 2 pM, with correction for dilution of the original sample. The recovery of ATP standards after TCA extraction was ~92%.

Glucose utilization. Islet (50 islets/sample) utilization of D-[5-3H]glucose was determined essentially as described previously, by quantitating the conversion of D-[5-3H]glucose (10 mM) to H2O (21). All sample values were corrected for recovery using H2O as a standard and subtraction of blank values with D-[5-3H]glucose (50 μM) added to each sample. All islets washed in phosphate-buffered saline were used for protein determination, as described above. Data are expressed as nanomoles glucose utilized per microgram of protein during a 60-min incubation at 37°C under an atmosphere of O2–CO2 (95:5).

RNA isolation and real-time PCR analysis. Total RNA was extracted from islets (15 islets/sample). RNA isolation and real-time PCR were carried out essentially as described previously (59). Equal amounts of cDNA sample were used. Amplification conditions, after the initial denaturation period of 5 min at 95°C, were annealing at 61°C for UCP2, 65°C for PPARα, 65°C for PPARγ, 63°C for granulin, with extension for 45 s at 72°C and a final extension for 7 min. Typically, 40 cycles were sufficient for achieving exponential amplification of 18S rRNA and the other five primers. The sequences of primer pairs for rat UCP2 were sense 5'-GGTCG-GAGATAACGAGAC-3', antisense 5'-GAGAGTCGTCTGCTCAT-GAG-3' (180 bp); rat PPARα sense 5'-TTGACTGTCAAGCT-CAGG-3', antisense 5'-AAGCTCGAAGCTCTCC-3' (227 bp); rat PPARγ sense 5'-CTGCGCAAGTCCAGCCAT-3', antisense 5'-GAAGAGGCTGTGAAGTGG-3' (218 bp); rat PPARγ sense 5'-TGGAGTCTGATGGTGTAAG-3', antisense 5'-CACAACGGCTTC-CTCTTCTG-3' (215 bp); rat granulin sense 5'-AGGCCTACG-TGGATATGG-3', antisense 5'-AAGTCTGGGCACCTCTGAC-3' (258 bp).

Temperature gradient PCR was run to determine efficiency. A melt curve was plotted to distinguish the nonspecific amplification products. An optimal amplification PCR cycle number was chosen for each gene. The 2–ΔΔCt method was applied to analyzes the relative changes in gene expression.

Statistical analysis. Values are means ± SE. Significant differences between treatment groups were determined by Student’s t-test (paired, two-tailed) or one-way analysis of variance with post hoc analysis using a Student-Newman-Keuls multiple comparison test. Western blotting results were analyzed by two-tailed, one-sample t-test. Most statistical analysis software was used. Values of P < 0.05 were accepted as significant.

RESULTS

Glucose-stimulated insulin secretion from islets after short-term ANP treatment. Freshly isolated rat pancreatic islets were incubated with either 5.5 mM glucose (basal) or 11 mM glucose (stimulated) in the presence or absence of ANP for 3 h. Acute treatment with ANP (100 nM and 1 μM) did not significantly affect the insulin-secretory response to 11 mM glucose (a half-maximal glucose-stimulatory concentration) after 3 h of incubation (90 ± 30 and 104 ± 37% of control, respectively, P > 0.05); basal insulin secretion was also unaffected by the presence of ANP at these concentrations (108 ± 5 and 86 ± 4% of control, respectively, P > 0.05). Even when islets were preincubated with ANP at basal glucose levels for 1 h followed by a 1-h glucose (11 mM) stimulation, there was no significant difference in insulin secretion between control and ANP-pretreated islets (100 nM ANP: 99 ± 5%, 1 μM ANP: 95 ± 7% of control, P > 0.05).
Insulin content and insulin secretion in islets after long-term 7-day ANP pretreatment. Islets were cultured for up to 7 days with ANP, and time-dependent changes in the insulin-secretory response were observed. Although glucose (11 mM) stimulated a 10-fold increase in insulin secretion consistently during the 7-day culture period, ANP-treated islets showed a progressive decline in the glucose-stimulated response. After 1 day, insulin secretion from ANP-treated islets was not significantly different from control; however, after 3 days there was a 25% decrease in the glucose-stimulated insulin-secretory response (Fig. 1A). A further decrease in glucose-stimulated insulin secretion was observed after 5 days of ANP treatment, and after 7 days a marked suppression of glucose-stimulated insulin secretion to only 9% of control levels was observed (Fig. 1A).

To determine whether the changes in insulin secretion reflected changes in insulin content, the islet total insulin levels were quantitated. Although the secretion of insulin was inhibited in ANP-pretreated islets, the islet total insulin content was not significantly changed (122 ± 8% of control) after 7 days with ANP (Fig. 1B).

Concentration-dependent ANP-induced changes in insulin secretion were also determined. Basal (5.5 mM glucose) insulin secretion from islets cultured with ANP at 1 nM to 1 μM for 7 days was not significantly changed compared with control (Fig. 2A). In contrast, the 7-day culture of islets with ANP resulted in a concentration-dependent loss of the insulin-secretory response to glucose (11 mM) stimulation (1 nM ANP: 50 ± 15%, 100 nM ANP: 42 ± 7%, and 1 μM ANP: 10 ± 3% of glucose-stimulated control values; Fig. 2A).

To determine whether ANP affected the viability of the islets and whether the inhibitory response was reversible, islets were cultured for 7 days at 5.5 mM glucose in the absence (−) or presence (+) of ANP (1 μM), as indicated. Insulin secretion under basal 5.5 mM glucose conditions (−) or in response to 11 mM glucose (Glu) stimulation (+) for 60 min was determined on days indicated. B: insulin was extracted from 7-day cultured islets in control (C; 5.5 mM glucose) and ANP (1 μM)-pretreated groups following 60-min incubation with 5.5 mM glucose for insulin secretion, and total insulin content including the insulin secreted was determined for each group. Values are means ± SE for A (n = 3) and B (n = 7) independent determinations. *P < 0.05 vs. control islets for each day-group, as determined by one-way ANOVA and multiple comparison test.

Insulin content and insulin secretion in islets after long-term 7-day ANP pretreatment. Islets were cultured for up to 7 days with ANP, and time-dependent changes in the insulin-secretory response were observed. Although glucose (11 mM) stimulated a 10-fold increase in insulin secretion consistently during the 7-day culture period, ANP-treated islets showed a progressive decline in the glucose-stimulated response. After 1 day, insulin secretion from ANP-treated islets was not significantly different from control; however, after 3 days there was a 25% decrease in the glucose-stimulated insulin-secretory response (Fig. 1A). A further decrease in glucose-stimulated insulin secretion was observed after 5 days of ANP treatment, and after 7 days a marked suppression of glucose-stimulated insulin secretion to only 9% of control levels was observed (Fig. 1A).

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washed and cultured in the absence of ANP for 24 h. The removal of ANP restored the islet insulin-secretory response to 11 mM glucose to approximately control levels (1 nM ANP: 97 ± 2%, 100 nM ANP: 89 ± 22%, 1 μM ANP: 102 ± 27% of control; Fig. 2B).

KIC and forskolin effects on insulin secretion from ANP-pretreated islets. KIC, a potent insulin secretagogue, is the product of leucine transamination and has glucose-like effects on islet Ca2+ regulation and insulin release. KIC (11 mM) stimulation evoked a fourfold higher level of insulin secretion in 7-day control islets compared with 11 mM glucose-stimulated islets (Table 1). A 7-day culture of islets with 1 mM, 100 nM, and 1 μM ANP significantly inhibited the KIC insulin-secretory response in a concentration-dependent manner (Table 1). Forskolin, a direct activator of adenyl cyclase, increases intracellular cAMP biosynthesis, activates PKA, and potentiates glucose-stimulated insulin secretion in islets (27). In 7-day cultured islets, a 1-h incubation with forskolin dramatically potentiated 11 mM glucose-induced insulin secretion (165 ± 59%; Table 1). However, in ANP-pretreated islets, forskolin-induced secretory increases were also inhibited in a concentration-dependent manner by the peptide, although the levels of insulin secretion remained above levels released in response to glucose alone (Table 1). Similar results were obtained when islets were stimulated with the phosphodiesterase-resistant cAMP analog 8-Br-cAMP. Insulin secretion from islets during a 60-min incubation in the presence of 8-Br-cAMP (5 mM) together with 11 mM glucose was inhibited in ANP (1 μM)-pretreated islets to only 26.1 ± 15% of glucose stimulation alone (P < 0.05).

ANP 7-day pretreatment effects on KCl- and ionomycin-stimulated insulin secretion. A nonnutritive insulin secretagogue that augments intracellular Ca2+ levels was investigated for effects on ANP-pretreated islets. KCl induces β-cell depolarization, the subsequent opening of L-type voltage-dependent Ca2+ channels, and insulin secretion (15). Ionomycin, a Ca2+ ionophore, raises intracellular [Ca2+] levels and stimulates insulin secretion (56). KCl (35 mM) and ionomycin (2 μM) each stimulated insulin secretion to a similar level that was 10-fold higher than basal levels in control islets (Table 1). Although 1 nM ANP did not have a significant effect on KCl-stimulated insulin secretion, 100 nM and 1 μM ANP inhibited KCl-induced secretion by as much as 50% (Table 1). The insulin-secretory response to ionomycin was significantly inhibited by 1 nM, 100 nM, and 1 μM ANP islet pretreatment (Table 1). These data suggested that an increase in intracellular [Ca2+] was not sufficient to overcome the inhibitory effects of ANP on insulin secretion.

KIC and KCl costimulated insulin secretion from islets with long-term ANP pretreatment. To determine whether a combined stimulus that augmented both ATP production and Ca2+ would affect ANP-pretreated islets, KCl and KIC were added together to the islets. Even though the insulin-secretory response to KIC was not augmented by the presence of KCl, the combination of KCl with KIC almost fully reversed the inhibitory effect of ANP on insulin secretion at all concentrations of ANP tested (1 nM: 94 ± 8%, 100 nM: 90 ± 11%, 1 μM: 95 ± 11% of control; Table 1). These results suggested that an augmented Ca2+ level in β-cells could protect the KIC-induced secretory response from ANP-induced inhibitory effects and that both metabolism and Ca2+ mobilization pathways are involved in the ANP inhibitory process.

Effects of 7-day ANP on ATP production and ATP/ADP ratio. In response to glucose stimulation, metabolism and mitochondrial energy production increase in pancreatic β-cells, causing an elevation of ATP and an increase in the ATP/ADP ratio. The increase in ATP leads to closure of ATP-sensitive K+ (KATP) channels and depolarization of the β-cell membrane that opens voltage-dependent Ca2+ channels and stimulates insulin secretion (3). ATP levels under basal glucose (5.5 mM) conditions were markedly reduced in 7-day ANP-treated islets at each concentration studied (1 nM: 53 ± 9%, 100 nM: 28 ± 5%, 1 μM: 23 ± 4% of control; Table 2). The removal of ANP after 7-day treatment partially recovered ATP production in washed ANP-pretreated islets with significant changes observed at 100 nM ATP (51 ± 5%) and 1 μM ANP (38 ± 7% of control untreated islets) (Table 2). Following incubation with 11 mM glucose for 25 min, ATP levels increased more than 3.9-fold in control islets (Table 2). In contrast, ATP levels of ANP-treated islets were significantly lower than glucose-stimulated control values, reaching only 6% of control (Table 2). Moreover, after the ANP was washed away, the glucose-stimulated ATP production significantly increased at each concentration of ANP compared with islets in the continued presence of ANP but did not fully recover to levels in glucose-stimulated ANP-untreated control samples (Table 2).

Since the ratio of ATP to ADP is important for regulating insulin secretion, the ratio of these nucleotides in islets treated

<p>| Table 1. Effects of ANP on insulin secretion by nutrient and nonnutrient secretagogues |</p>
<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Glucose, 5.5 mM (Basal)</th>
<th>Glucose, 11 mM</th>
<th>KIC, 11 mM</th>
<th>FSK, 10 μM + Glucose 11 mM</th>
<th>KCl, 35 mM</th>
<th>Iono, 2 μM</th>
<th>KIC, 11 mM + KCl 35 mM</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>0.4 ± 0.1</td>
<td>12.6 ± 2.5</td>
<td>43.9 ± 7.4</td>
<td>71.1 ± 13.7</td>
<td>3.9 ± 0.4</td>
<td>5.1 ± 0.1</td>
<td>46.0 ± 6.0</td>
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<tr>
<td>ANP, 1 nM</td>
<td>0.2 ± 0.04</td>
<td>6.7 ± 2.1*</td>
<td>31.3 ± 1.4*</td>
<td>45.7 ± 3.4*</td>
<td>3.7 ± 0.2</td>
<td>3.9 ± 0.5*</td>
<td>43.2 ± 3.8</td>
</tr>
<tr>
<td>ANP, 100 nM</td>
<td>0.1 ± 0.01</td>
<td>5.7 ± 1.0*</td>
<td>15.0 ± 2.0*</td>
<td>22.7 ± 6.7*</td>
<td>2.2 ± 0.8</td>
<td>2.6 ± 0.2*</td>
<td>41.1 ± 5.1</td>
</tr>
<tr>
<td>ANP, 1 μM</td>
<td>0.3 ± 0.06</td>
<td>2.7 ± 0.6*</td>
<td>11.6 ± 2.1*</td>
<td>12.0 ± 3.3*</td>
<td>1.8 ± 0.6*</td>
<td>1.9 ± 0.1*</td>
<td>43.8 ± 4.9</td>
</tr>
</tbody>
</table>

Values are means ± SE; no. of independent determinations shown in parentheses. Islets were cultured for 7 days in the absence (Control) or presence of atrial natriuretic peptide (ANP), as indicated. KIC, α-ketoisocaproic acid; FSK, forskolin; KCl, potassium chloride; Iono, ionomycin. Insulin secretion was determined during 60-min incubation with stimuli, as indicated. *P < 0.05 vs. control in the same stimulated group, as determined by one-way ANOVA and multiple comparison test.

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Table 2. Effects of ANP on ATP levels and ATP/ADP ratio

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<tbody>
<tr>
<td>Control</td>
<td>113 ± 12</td>
<td>443 ± 71*</td>
<td>74 ± 5*</td>
<td>98 ± 11§§</td>
<td>2.7 ± 0.1</td>
<td>7.9 ± 0.7*</td>
<td>0.6 ± 0.1*</td>
<td>1.0 ± 0.1§</td>
</tr>
<tr>
<td>ANP, 1 nM</td>
<td>60 ± 10</td>
<td>66 ± 68</td>
<td>58 ± 6*†</td>
<td>63 ± 8§§</td>
<td>0.5 ± 0.1*</td>
<td>0.7 ± 0.1§</td>
<td>0.7 ± 0.1*</td>
<td>1.2 ± 0.1§</td>
</tr>
<tr>
<td>ANP, 100 nM</td>
<td>32 ± 4*</td>
<td>43 ± 48</td>
<td>43 ± 8*§</td>
<td>54 ± 6§§</td>
<td>0.3 ± 0.1*</td>
<td>0.6 ± 0.1§</td>
<td>0.5 ± 0.1*</td>
<td>0.9 ± 0.1§</td>
</tr>
<tr>
<td>ANP, 1 µM</td>
<td>26 ± 4*</td>
<td>28 ± 1§</td>
<td>43 ± 8*§</td>
<td>54 ± 6§§</td>
<td>0.3 ± 0.1*</td>
<td>0.6 ± 0.1§</td>
<td>0.5 ± 0.1*</td>
<td>0.9 ± 0.1§</td>
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Values are means ± SE; n = 3-7. Islets were cultured for 7 days at 5.5 mM glucose in the absence (Control) or presence of ANP as indicated in the left-hand column. In some experiments, ANP was washed (Wash) from the islets and incubation was continued for 24 h, as indicated. ATP and ADP levels were determined during 25-min incubation with glucose 5.5 mM (5.5 G) or 11 mM (11 G), as indicated. *P < 0.05 vs. control, †P < 0.01 vs. 5.5 G, ‡P < 0.02 vs. 11 G, §P < 0.05 vs. glucose-stimulated control, as determined by one-way ANOVA and multiple comparison test.

with ANP was determined. After 7-day treatment with ANP concentrations ranging from 1 nM to 1 µM, the ATP/ADP ratios at 5.5 mM glucose were significantly reduced to ~10–20% of control values and reflected the marked inhibition of ATP levels in these islets. Removal of the ANP for 24 h did not restore ATP/ADP ratios to control levels, although the small increases observed were significantly higher than in the unwashed groups (Table 2). Glucose (11 mM) stimulation increased the ATP/ADP ratio almost threefold; however, pretreatment with 1 nM to 1 µM ANP resulted in significant reductions in the ratios to levels found in ANP-treated islets stimulated with only 5.5 mM glucose (Table 2). In washed islets, the removal of ANP allowed a small but significant increase in glucose (11 mM)-stimulated ATP/ADP ratios at each concentration of ANP studied. However, these levels remained at ~20% of control values in the absence of ANP. Thus, ANP pretreatment of islets reduced the total ATP levels and the ATP/ADP ratios.

Since costimulation with KIC and KCl overcame the inhibitory effect of ANP on insulin secretion, the effects of these agents on islet ATP production were investigated. Incubation with KIC alone elevated ATP levels threefold above control islets (Fig. 3). Pretreatment of islets with ANP for 7 days reduced KIC-stimulated ATP levels to below control levels (Fig. 3). Even though the levels of ATP in the ANP-treated islets were low, stimulation by KIC significantly elevated the ATP levels (1 nM ANP: 140 ± 4%, 100 nM ANP: 147 ± 18%, 1 µM ANP: 190 ± 4% of paired ANP only treated values; Fig. 3). KCl did not have any significant effects on ATP levels of either control or ANP-treated islets (Fig. 3). A combination of KIC and KCl did not increase ATP levels of ANP-treated islets above the levels observed with KIC alone (Fig. 3).

To determine whether ANP affected glucose uptake and metabolism through glycolysis, the effects of ANP on glycolysis were determined. Glucose utilization in islets treated with ANP (1 µM; 0.41 ± 0.03 nmol glucose utilized/µg protein) for 7 days was not different (P > 0.05) compared with control islets cultured in the absence of ANP (0.43 ± 0.04 nmol glucose utilized/µg protein).

**ANP evoked effects on mediators of metabolic activity.** HSL, activatable by cGMP-dependent protein kinase (19), regulates cellular lipid homeostasis and catalyzes the hydrolysis of stored triglyceride (55). ANP (100 nM and 1 µM) evoked marked increases in activated phospho-HSL levels in islets after only 30 min of stimulation (Fig. 4, A and B).

UCP2 is a mitochondrial carrier protein that mediates proton leak across the inner mitochondrial membrane (18), which in islets decreases the yield of ATP from glucose (7). The expression of UCP2 mRNA in islets was sensitive to ANP stimulation as low as 1 nM, but not 0.1 nM (Fig. 5A). Moreover, 7-day treatment with the analog 8-Br-cGMP elicited a marked increase in UCP2 mRNA expression (Fig. 5A). Culture of islets with 11 mM glucose for 7 days also elicited an increase in UCP2 mRNA levels that was similar to the levels evoked by ANP (Fig. 5A). When ANP was washed from islets for 24 h, the UCP2 mRNA levels fell by ~50% but remained significantly higher than control levels (Fig. 5B).

Since PPARs have been reported to mediate UCP2 gene transcription (54), the ANP effects on islet PPAR expression were investigated. ANP concentrations from 1 nM to 1 µM stimulated concentration-dependent increases in islet PPARα during 8 days of culture (Fig. 6A). Washout of ANP completely reversed the effects of 1 nM and 100 nM ANP and reduced the effect of 1 µM ANP by ~50% (Fig. 6A). Culture alone did not affect basal PPARα mRNA expression. ANP at 100 nM and 1 µM, but not 1 nM, elevated PPARα mRNA levels, and washout of the ANP for 24 h significantly reduced the level at the highest ANP concentration tested (Fig. 6B). Following culture for 8 days, even basal levels of PPARα mRNA were elevated compared with fresh islets (Fig. 6B). In contrast, 8-day ANP at 1 nM (163 ± 37% of control) and 1 µM (132 ±
33% of control) did not significantly affect PPARγ mRNA expression levels (data not shown).

**NPR-A expression and cGMP levels in long-term ANP-treated islets.** NPR-A belongs to the transmembrane guanylyl cyclase family that mediates the effects of natriuretic peptides via the second messenger cGMP. In a previous study, we reported that NPR-A was the predominant guanylyl cyclase-coupled receptor expressed in rat islet cells (59). The protein levels of NPR-A in freshly isolated rat islets and 7-day cultured control islets were similar (Fig. 7A). However, NPR-A expression was reduced by 40–90% of control islet levels following 7-day treatment with 1 nM ANP (Fig. 7, A and B). The freshly isolated islets contained a very low level of intracellular cGMP (Fig. 8). Basal levels of cGMP in 7-day cultured islets was similar to those of fresh islets, and 7-day control islets responded to acute ANP stimulation with an increase in cGMP levels two- to threefold higher than basal levels (Fig. 8). Even though NPR-A levels showed a 40% downregulation in ANP-treated islets, the cGMP levels generated in response to ANP in ANP-treated islets were similar to control islet levels (Fig. 8).

**DISCUSSION**

ANP levels in diabetes correlate with hyperglycemia (30, 31), and whereas ANP levels in normoglycemic rats have been reported to be 158 ± 15 pg/ml, in diabetes these levels almost double (33). Previous studies reported that short-term exposure to ANP (1 nM) inhibited glucagon secretion from isolated islets (52) but failed to significantly affect insulin secretion from insulinoma cells in the presence or absence of a nutrient secretagogue (51). In a recent study, 1 nM ANP potentiated mouse islet insulin secretion in response to a low concentration of glucose (39), whereas glucose-stimulated insulin secretion was inhibited in the mouse and rat following intravenous injection of ANP (1). In the present study, ANP provided at time 0 or for a 60-min pretreatment failed to affect insulin secretion from freshly isolated rat islets in the presence or absence of glucose stimulation. In contrast, 7-day treatment with ANP at a concentration as low as 1 nM reduced insulin secretion by one-half, and higher concentrations of ANP profoundly inhibited the glucose-stimulated secretory response. These results demonstrate for the first time that long-term ANP treatment of isolated rat islets did not affect basal insulin secretion but did inhibit secretagogue-stimulated insulin secre-
In NPR-A knockout mice, there was increased glucose-stimulated islet insulin secretion (39), supporting the conclusion that ANP receptors exert an inhibitory effect on insulin secretion. Since islet total insulin storage levels after 7-day ANP treatment were normal, insulin storage does not account for reduced secretion. Moreover, since the glucose-stimulated insulin secretion was restored following washout of the ANP, islet viability was not compromised by long-term ANP exposure, and the ANP inhibitory effect was reversible.

In contrast to guanylyl cyclase-linked NPR-A type receptors, NPR-C clearance receptors in islet cells (5) have the potential to not only remove ANP but also to inhibit adenylyl cyclase signaling and enhance phosphatidylinositol turnover in cells (32) in the absence of changes in cGMP. The specific actions of NPR-C in islets are undetermined. However, since ANP inhibited the secretory response to exogenously supplied 8-Br-cAMP, and 8-Br-cGMP mimicked effects of ANP, it appears that ANP is acting via mechanisms that involve NPR-A and cGMP.

The mechanisms regulating insulin secretion include glucose transport into the β-cell, metabolism through glycolysis, pyruvate decarboxylation, and the citric acid cycle. Increased ATP levels and the ATP/ADP ratio lead to closure of K<sub>ATP</sub> channels (46), membrane depolarization, the opening of voltage-gated Ca<sup>2+</sup> channels, Ca<sup>2+</sup> influx, and a rise in intracellular [Ca<sup>2+</sup>] that initiates exocytosis of insulin granules (2, 36, 45). The similar glycolytic activities between control and ANP-treated islets makes it unlikely that ANP effects on glucose transport or glycolysis account for the changes in islet ATP production or insulin secretion. KIC, metabolized in the mitochondria to either acetyl-CoA or α-ketoglutarate, which enter the citric acid cycle to generate ATP (12, 26) and trigger insulin release (29), was used as a tool to segregate the effects of ANP on mitochondrial metabolism. ANP inhibited similarly the magnitude of both glucose- and KIC-induced islet insulin secretion, suggesting a similar locus of regulation that may involve mito-chondrial metabolism or a distal path common to glucose and KIC stimulation. Even though we have previously reported that ANP can activate the PI3K-Akt pathway in islets (59), PI3K does not affect KIC-induced secretion in mouse islets (60).

ATP and the ATP/ADP ratio play an important role in regulated insulin secretion (9). Glucose stimulation of control islets increased ATP levels almost fourfold compared with basal levels, in agreement with another report (46). However, 7-day exposure of islets to ANP inhibited basal ATP levels in a concentration-dependent manner, and in glucose-stimulated islets were either freshly isolated (fresh) or cultured for 7 days in the absence (control, C) or presence of ANP. Then, ANP was washed (+) from some batches of islets and the culture continued for 24 h. Islet mRNA was isolated and PPAR<sub>δ</sub> (A) and PPARα (B) mRNA levels were quantitated by real-time PCR using 18S rRNA as an internal control. Values are means ± SE for 3–9 independent determinations. *P < 0.05 vs. C, as determined by two-tailed one-sample t-test; #P < 0.03 vs. 1 μM ANP wash (−), as determined by unpaired t-test.

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islets ATP levels failed to reach basal levels and were in fact as low as one-half of basal values even at 1 nM ANP. The reduced ATP levels are consistent with the loss of glucose-stimulated insulin secretion. Following a 24-h ANP washout, the basal and glucose-stimulated islet ATP levels were significantly elevated compared with similarly treated islets in the continuous presence of ANP, but ATP was still below control levels. The ATP/ADP ratios reflected the changes observed in the ATP levels, and although washout of ANP for 24 h partially restored ATP/ADP values, there was not a complete restoration. Even though the ATP/ADP ratio was significantly lower in ANP-treated islets under basal and glucose-stimulated conditions, glucose still evoked a 75–100% increase in the ATP/ADP ratio in unwashed or washed islets. This is in line with the ∼80% increase in the ATP/ADP ratio previously reported for glucose-stimulated rat islets (14). However, “the absolute concentrations of ATP and ADP” regulate $K_{\text{ATP}}$ channel activity (46) and may be sufficient to explain the ANP inhibitory process. Why the glucose-induced insulin-secretory response but not the ATP level is restored 24 h after removal of ANP is not known. Perhaps the adenine nucleotide levels in the vicinity of the plasma membrane are sufficient to evoke closure of $K_{\text{ATP}}$ channels in ANP recovered islets (46) and stimulate insulin secretion. Cytoplasmic or microdomain ATP may also be important (14).

To determine other possible mediators in the response to ANP, islets were incubated with metabolizable insulin secretagogues (glucose and KIC) or nonmetabolizable secretagogues capable of either potentiating forskolin and 8-Br-cAMP or inducing (KCl and ionomycin) insulin secretion. Forskolin stimulates adenyl cyclase activity and cAMP production, which activates PKA and affects $K_{\text{ATP}}$ channels (43), Na$^+$ transport (49), and the PKC-Ca$^{2+}$/calmodulin-signaling pathway (37) and also potentiates glucose-stimulated insulin release (8, 38). However, cAMP has little or no effect on insulin secretion when the glucose concentration is low (∼2.8mM) (16, 17, 28, 58). Since pretreatment with ANP almost completely abolished forskolin- and 8-Br-cAMP-potentiated 11 mM glucose-stimulated insulin secretion, this suggests that ANP is inhibiting the secretory pathway distal to cAMP generation.

Since increased intracellular Ca$^{2+}$ levels trigger the exocytosis of insulin granules, the direct or indirect elevation of intracellular [Ca$^{2+}$] by ionomycin and KCl, respectively, stimulated insulin secretion. However, these stimulatory effects were significantly reduced by islet ANP pretreatment. Since ionomycin affects bypass cation channel activation, it appears likely that ANP partially inhibits Ca$^{2+}$ responses in β-cells. cGMP/PKG can reduce intracellular Ca$^{2+}$ levels in cells by several mechanisms (6), although ANP closed mouse islet $K_{\text{ATP}}$ channels and increased glucose-stimulated Ca$^{2+}$ signaling in the short term (39). Interestingly, islet costimulation with both KIC and KCl was sufficient to completely overcome the inhibitory effect of ANP on insulin secretion. It is concluded that, since a combination of elevated oxidative metabolism and Ca$^{2+}$ mobilization overcame the inhibitory effects of ANP, these pathways are targets of ANP inhibition.

KIC initiated a significant increase in control islet ATP production, which was abolished with ANP pretreatment. KCl did not affect ATP levels. The costimulation with both KIC and KCl did not fully restore ATP levels in ANP-treated islets, even though the insulin-secretory response was fully recovered. However, there was a significant increase in ATP levels with KIC stimulation in ANP-pretreated islets vs. paired unstimulated ANP-treated islets. This increase likely had a positive impact on insulin secretion and accounts for the ability of KIC to moderately stimulate insulin secretion even in the presence of ANP. Thus, the data suggest that, since insulin secretion is restored by ANP washout or KIC/KCl combination stimulation, both ATP levels and Ca$^{2+}$ levels are important contributors to ANP-suppressed insulin secretion.

The mechanisms mediating ANP effects on mitochondrial activity appear to involve the activation of HSL and PPARs and UCP2 regulation. ANP and cGMP-dependent protein kinase activate HSL in several tissues (19, 42). HSL is expressed as a doublet in rat islets (55), and ANP induced a rapid activation of islet HSL, which would produce free fatty acids due to the hydrolysis and depletion of triacylglycerol. If β-cells are depleted of triacylglycerol stores, glucose-stimulated insulin secretion is negatively impacted (20). Moreover, increased intracellular fatty acid levels might induce lipotoxicity and negatively impact β-cell function (62). Increased fatty acid levels can also activate the family of nuclear receptor PPARs (40). Pancreatic β-cells express all subtypes of PPAR (54), and ANP exposure led to increased islet PPARα and PPARγ gene expression. Fatty acids can stimulate PPARα and associated target genes in β-cells (54, 63), and the increased expression of PPARα suppressed insulin secretion in insulinoma cells and led to uncoupled respiration (47), suggesting that increased expression of PPARs in rat islets also has a negative impact on secretory responsiveness and ATP generation. A mechanism that can account for this alteration in ATP generation is the PPAR-mediated induction of UCP2, one of a family of mito-
ichondrial transporters that perturb the mitochondrial proton gradient and allow protons to pass through the inner mitochondrial membrane without producing ATP. PPARα and PPARγ are major determinants in control of UCP2 gene expression (54), although fatty acid-dependent UCP2 regulation can also be PPAR independent. UCP2 is a negative mediator of insulin secretion and is increased in islets of animal models of type 2 diabetes (61). ANP increased islet UCP2 expression to levels similar to that induced by long-term glucose stimulation, in agreement with a published report (34). Moreover, 8-Br-cGMP markedly increased UCP2 gene expression, providing evidence that cGMP mediated the ANP response. Following ANP washout, islet UCP2 gene expression fell by ~50%, but the remaining UCP2 expression could explain why ATP generation does not return to normal. The evidence taken together builds a case for HSL, PPARs, and UCP2 setting the stage for impaired ATP production and impaired insulin secretion in response to ANP.

Another explanation for why insulin secretion declined during islet long-term exposure to ANP was that a possible stimulatory effect of ANP (50) and cGMP on insulin secretion (22, 23) was lost due to desensitization of NPRs that can be downregulated (6, 10). Islet NPR-A protein levels were reduced 40% after 7-day exposure to ANP. A recent study reported that knockout of NPR-A correlated with a loss of K_{ATP} channel expression and activity in mouse islets (39). Whether a partial downregulation of NPR-A in rat islets could have a similar effect is not known. However, the 7-day ANP-treated islet cGMP levels were similar to those of ANP-stimulated control islets. Since ANP can stimulate Akt (59) and Akt can activate nitric oxide synthase (10), it is possible that ANP regulates cGMP in islet cells through both NPR-A and nitric oxide activation of soluble guanylyl cyclase. The data suggest that ANP-induced elevation of cGMP levels mediates the intracellular signaling in islets with 7-day ANP treatment. cGMP may also affect the exocytotic elements of secretion. However, islet expression of the granulin gene, a key protein characterized as regulating the exocytosis of insulin-secretory granules by linking vesicle transport proteins (48), was not significantly affected by 7-day ANP exposure (H. You, unpublished observations).

In summary, this is the first report that chronic exposure to nanomolar levels of ANP dramatically reduces insulin secretion and the ATP levels and ATP/ADP ratio in β-cells and that HSL, PPARs, and UCP2 mediate the response. Also, ANP does not permanently impair islet glucose metabolism, allowing metabolic and secretory responses to be at least partially restored soon after removal of the peptide. The conclusion is that the inhibitory effects of ANP on insulin secretion involve mitochondrial metabolism, ATP production, and modulation of Ca^{2+} responses.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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