Inhibition of AKT in Human Pancreatic, Renal and Colorectal Cancer Cells by Four Cardiac Hormones

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Abstract. *Background: Protein kinase-B (AKT) is a serine/threonine protein kinase that has a key role in cell proliferation and cancer cell invasiveness. Four cardiac peptide hormones, namely vessel dilator, atrial natriuretic peptide (ANP), kaliuretic peptide, and long-acting natriuretic peptide (LANP) have anticancer effects both in vitro and in vivo. Materials and Methods: Four cardiac hormones were examined for their ability to inhibit AKT, measured with a solid-phase enzyme-linked immunosorbent assay (ELISA) in human colorectal, pancreatic, and renal cancer cells. Results: Vessel dilator, kaliuretic peptide, ANP, and LANP maximally reduced the concentration of AKT by 47%, 45%, 52%, and 46% in human colorectal cancer cells ($p<0.0001$), by 60%, 61%, 64%, and 59% in human pancreatic carcinoma cells ($p<0.0001$), and by 31%, 32%, 31%, and 31% in renal adenocarcinoma cells ($p<0.001$). Conclusion: These four cardiac hormones are significant inhibitors of AKT in human cancer cells, as part of their anticancer mechanism(s) of action.*

Protein kinase-B (AKT) is a serine/threonine-protein kinase that has a key role in cell proliferation and is a major factor in many types of cancer (1-6). The name AKT derives from the Ak mouse strain that developed spontaneous thymic lymphomas where the "t" stands for thymoma in addition to Ak of the mouse strain (7). AKT (now also called AKT1) is involved in the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway and is associated with tumor cell invasiveness as well as proliferation (1-6). PI3K activates AKT *via* phosphorylation at Thr^{308} to transmit signals from rat sarcoma-bound guanosine triphosphate (RAS) and growth factors (6). AKT is overexpressed in colorectal cancer cells but not in normal colonic mucosa and hyperplastic polyps (8). Four endogenous cardiac hormones, namely vessel dilator, kaliuretic peptide, atrial natriuretic peptide (ANP), and long-acting natriuretic peptide (LANP), have anticancer effects *in vivo* (9-11) and reduce by 89% to 97% the number of viable human colon adenocarcinoma cells *in vitro* (12). Part of the rationale for investigating the effects of cardiac hormones on AKT is that they are known to inhibit 95% of basal RAS activity (13, 14) and to inhibit the ability of growth factors, such as epidermal growth factor, to stimulate RAS (15), which, in turn, stimulates AKT (6).

The present investigation was designed to ascertain if the four cardiac hormones inhibit AKT in human colon adenocarcinoma cells. To determine if their potential ability to inhibit AKT relates to other types of cancers in addition to colorectal cancer, these four cardiac hormones were also studied for their ability to inhibit AKT in human pancreatic cancer where they were found to eliminate up to 80% of such human tumors *in vivo* growing in mice (10), and in renal cancer cells.

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Materials and Methods

Materials. The four cardiac hormones were obtained from Phoenix Pharmaceuticals, Inc., Belmont, CA, USA.

Human colorectal, pancreatic, and renal cancer cells. Human colorectal cancer cells [American Type Culture Collection (ATCC) number CCL-225], pancreatic carcinoma cells (ATCC number CRL-1469, panc-1) and renal adenocarcinoma cells (CRL-1611) were obtained from the ATCC, Manassas, VA, USA. The ATCC authenticated these cell lines and performed the genotype and phenotype evaluations, including DNA profiles [short tandem repeats (STR)] and cytogenetic analyses.

Culture of human colorectal adenocarcinoma cells. Propagation of the human colorectal adenocarcinoma cells was performed in Roswell Park Memorial Institute (RPMI)-1640 medium with 2 mM glutamine, adjusted with the addition of 1.5 g/l sodium bicarbonate,

4.5 g/l glucose, 10 mM HEPES, 1 mM of 90% sodium pyruvate and 10% fetal bovine serum (FBS) (Sigma Chemical Company, St. Louis, MO, USA), at a temperature of 37˚C with 5% CO_2, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

Culture of human pancreatic carcinoma cells. Propagation of the human pancreatic carcinoma cells was performed in Dulbecco's modified Eagle's plus Ham's F12A 1:1 mixture containing 1.2 g/l of sodium bicarbonate (Sigma Chemical Co.) supplemented with 15 mM of HEPES and FBS 10% with 5% CO_2, at a temperature of 37˚C, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

Culture of human renal adenocarcinoma cells. Propagation of human renal cell adenocarcinomas was performed in Eagle's minimum essential medium supplemented with 2 mM glutamine adjusted with the addition of 1.5 g/l sodium bicarbonate, 1 mM of 90% sodium pyruvate and 10% FBS (Sigma Chemical Co.) with 5% CO_2 at a temperature of 37˚C, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days.

AKT research protocol. The human colorectal adenocarcinoma, pancreatic carcinoma, and renal adenocarcinoma cells were subcultured for 24 h, then approximately 5,000 cells of each line in 50 μl of their respective media were seeded in 96-well plates with 50 μl of media containing 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, and 100 pM concentrations of each of the four cardiac hormones separately (*i.e.* six concentrations of four cardiac hormones measured six times at each concentration; n=6 for each concentration). The standards from R&D Systems were diluted using Reagent Diluent and added to blank wells to serve as reference points of known AKT1 concentrations. In this assay, absorbance was examined at 540 nM wavelength using a 96-well BioTek Gen5, Synergy Mx microplate reader (Winooski, VT, USA) set according to the parameters recommended by the kit manufacturer. There were 32 controls for each cell line in these experiments (n=32) and six experimental determinations for each of the six concentrations of the four cardiac hormones in the three cancer cell lines (n=6).

ELISA for AKT. The DuoSet® IC Intracellular human AKT1 (R&D Systems, Inc., Minneapolis, MN, USA) is a 6-hour solid-phase ELISA which measures V-AKT murine thymoma viral oncogene homolog 1 (AKT1) in cell lysates. In this assay, an immobilized capture antibody specific for AKT1 binds to AKT1 using a standard Streptavidin-conjugated to horseradish-peroxidase. The human total AKT1 Duo Set IC ELISA specifically recognizes AKT1. Multiple factors related to AKT1, including AKT2, AKT3, and related AGC kinase family members such as serum- and glucocorticoid-inducible kinase-1 (SGK1), ribosomal S6 kinase-1 (RSK1), and p70 S6 kinase were assayed by R&D Systems and no cross-reactivity or interference was found to occur with this AKT1 ELISA assay. The AKT1 ELISA was calibrated against a highly-purified *Escherichia coli*-expressed recombinant human AKT1 produced by R&D systems. The standard curve for this assay was computer-calculated using a four-parameter logistic (4-PL) curve-fit.

Statistical analysis. Data are expressed as the means±SEM. Statistical analysis of the data was performed by the Student's t-test for unpaired values. A value of $p<0.05$ was considered the criterion for statistical significance.

Results

AKT concentration is reduced in human colorectal cancer cells by cardiac hormones. Vessel dilator maximally reduced AKT by 47% in human colorectal cancer cells (Figure 1A). There was a 45% decrease of AKT in human colorectal cancer cells by kaliuretic peptide (Figure 1B). ANP caused a maximal decrease of 52% of AKT in human colorectal cancer cells (Figure 1C). The maximal decrease observed for colorectal cancer cells treated with LANP was 46% (Figure 1D). As one observes in Figure 1, in human colorectal cancer cells, each of the cardiac hormones had a similar significant ($p<0.0001$) ability to inhibit AKT over a concentration range of 100 pM to 10 μM.

Inhibition of AKT in human pancreatic carcinoma cells. Vessel dilator caused a maximal decrease of 60% of AKT in human pancreatic cancer cells (Figure 2A). Kaliuretic peptide maximally reduced AKT in human pancreatic cancer cells by 61% (Figure 2B). ANP maximally reduced AKT by 64% in human pancreatic cancer cells (Figure 2C). LANP reduced AKT in human pancreatic cancer cells by 59% (Figure 2D). Each of the cardiac hormones had a similar significant ($p<0.0001$) ability to inhibit AKT in human pancreatic cancer cells over a concentration range of 100 pM to 10 μM (Figure 2).

Inhibition of AKT in human renal adenocarcinoma cells. Vessel dilator maximally reduced AKT in human renal cancer cells by 31% (Figure 3A). Kaliuretic peptide maximally reduced AKT by 32% (Figure 3B). The maximal decrease of AKT observed for renal cancer cells treated with ANP was 31% (Figure 3C). There was a 31% decrease of AKT in human renal cancer cells by LANP (Figure 3D). When comparing Figures 1-3, one can discern that the four cardiac hormones decreased AKT the most, in human pancreatic cancer cells followed by their effects in human colorectal cancer cells and then in renal adenocarcinoma cells.

Discussion

Vessel dilator, kaliuretic peptide, ANP, and LANP decreased the levels of AKT by 41-52% in human colorectal cancer cells, by 54-64% in human pancreatic carcinoma cells, and by 29-32% in human renal cancer cells (Figures 1-3), in the present investigation. This significant decrease of AKT in these cancer cell lines suggests that AKT is a target of the four cardiac hormones in these human cancer types. The present investigation further suggests that a complex

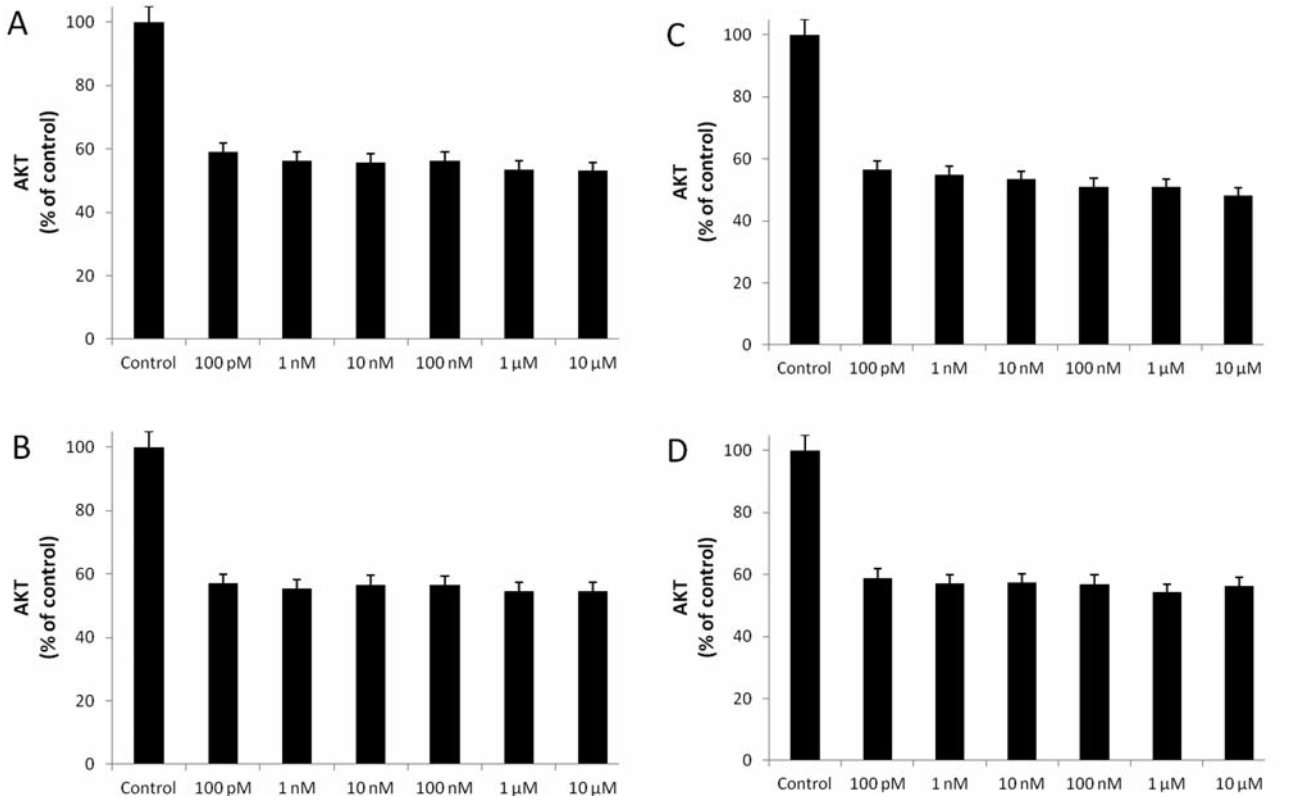

Figure 1. *Vessel dilator (A), kaliuretic peptide (B), atrial natriuretic peptide (C) and long-acting natriuretic peptide (D) maximally reduced protein kinase B (AKT) in pg/ml in human colorectal cancer cells by 47%, 45%, 52%, and 46%, respectively. Data for each concentration of each of the cardiac hormones is illustrated here and each of these decreases was significant at p<0.0001 when evaluated by the Student's t-test for unpaired values. Each bar represents the mean±SEM of 32 determinations for control and six determinations at each concentration for the experimental groups.*

interplay of AKT, rat sarcoma-bound guanosine triphosphate (RAS) and vascular endothelial growth factor (VEGF) in causing cancer and maintenance of cancer cell growth (1-6, 16), is inhibited by these four cardiac hormones. There is cross-talk in the activation of AKT and its inhibition by the cardiac hormones, which is summarized as follows: RAS activates AKT (6). Growth factors such as epidermal growth factor also activate RAS with a resultant downstream activation of AKT (6). The effects of VEGF on cancer growth and metastasis (16-24) are mediated by binding to the VEGFR2 receptor which, in turn, activates the PI3K-AKT pathway (21, 22). Thus, RAS, growth factors which stimulate RAS, and VEGF activate AKT (6, 21, 22). Furthermore, VEGF itself has growth-enhancing effects *via* stimulating RAS (23, 24). Oncogenic KRAS, in turn, up-regulates the activity of VEGF, and both RAS and VEGF contribute to the pathobiology of colonic cancer (25). Each of the four cardiac hormones inhibit RAS up to 95% (13, 14) and they also strongly reduce the expression of VEGF and its VEGFR2 (*i.e.* up to 92% inhibition) (26), which both enhance AKT. The present investigation also demonstrates that the cardiac hormones directly inhibit AKT.

In addition to the above relationships upstream of AKT, it has recently been shown that the cross-talk between AKT and downstream Int (integration-1 gene in breast cancer) and wg (wingless) (WNT) signaling is influenced by ANP (27). AKT (28), RAS (29) and VEGF (29) pathways all stimulate WNT. KRAS and WNT pathways can cooperate to regulate the *VEGF* gene (16). Thus, there is a complicated interrelationship between AKT, RAS, VEGF and WNT. The four cardiac hormones studied here also inhibit WNT to a similar extent in the same cancer cells of the present investigation (unpublished observation). Thus, each of the above pathways are targeted by the four cardiac hormones, which helps to explain their dramatic effects on eliminating up to 86% of human carcinomas growing in mice (9) and the fact that once eliminated, these human carcinomas never recur in the primary site in the lifespan of the mice (9-11). If a metastatic lesion occurs, the cardiac hormones can also eliminate the metastatic lesion when utilized in a sequential manner (9). The ability of

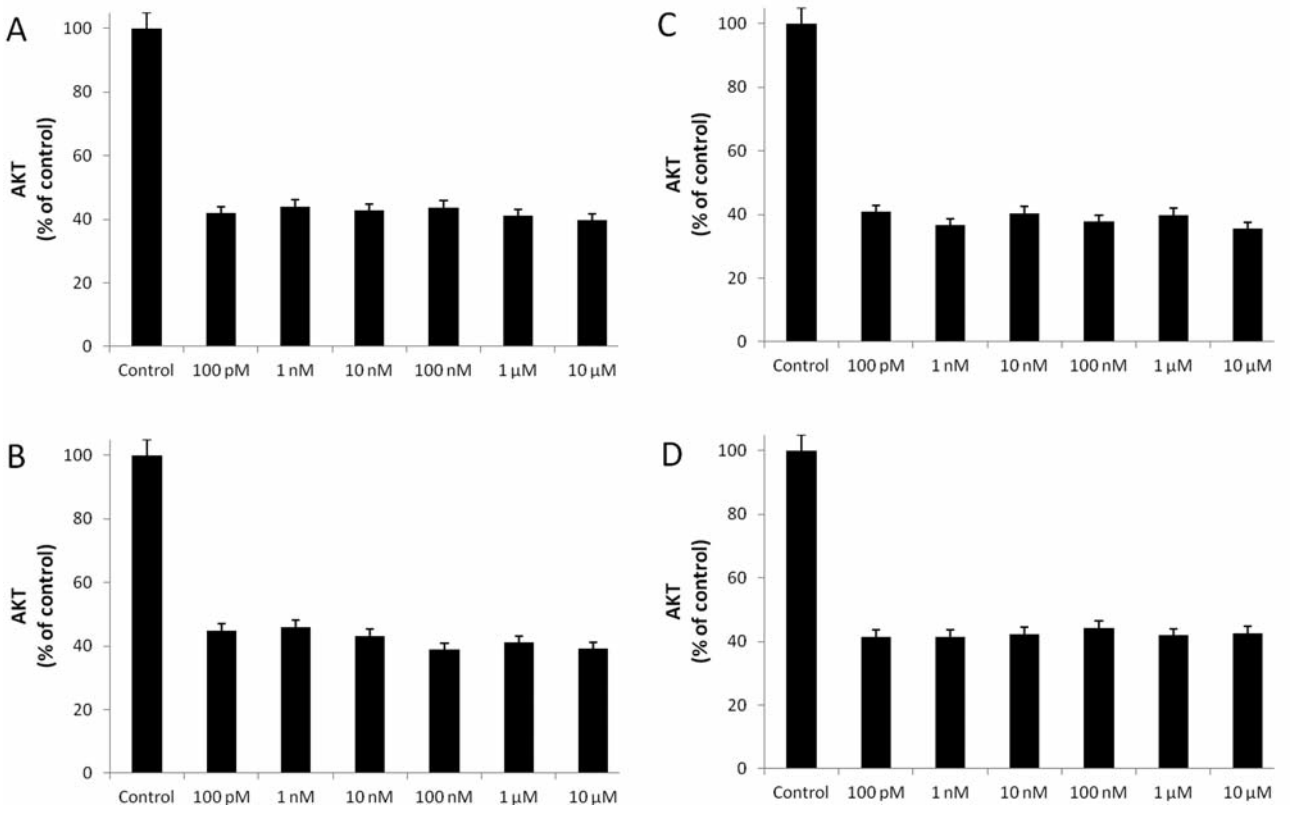

Figure 2. *AKT in pg/ml in human pancreatic cancer cells was maximally decreased by 60%, 61%, 64%, and 59%, by vessel dilator (A), kaliuretic peptide (B), atrial natriuretic peptide (C), and long-acting natriuretic peptide (D), respectively. Data for each concentration of each of the cardiac hormones is illustrated here and each of these decreases in AKT in pancreatic cancer cells was significant at p<0.0001 when evaluated by the Student's t-test for unpaired values. Each bar represents the mean±SEM of 32 determinations for the control and six determinations at each concentration of the cardiac hormone treated groups.*

the four cardiac hormones to eliminate different cancer types we would hypothesize is due to their ability to significantly inhibit AKT directly, as demonstrated in the present investigation, as well as indirectly, *via* inhibiting RAS (13, 14) and VEGF (26) which activate AKT (21, 22).

There was a difference in the ability of the cardiac hormones to inhibit AKT depending on the type of cancer. The inhibition of AKT was greater in human pancreatic cancer than in human colorectal cancer. AKT was inhibited more in the colorectal cancer cells than in human renal cell adenocarcinoma cells. AKT was inhibited two-fold more in human pancreatic cancer cells than in renal adenocarcinoma cells ($p<0.01$). The exact reason for this difference in the degree of inhibition of AKT in the different cancer types is unknown but may be due, in part, to the fact that AKT has been shown to be highly-expressed in pancreatic cancer (30), while its expression in colorectal cancer, on the other hand, is low (31), and renal cancer expresses AKT weakly (32). In the present investigation, the control cells exhibited the highest concentration of AKT in pancreatic >colorectal> renal. Thus, the high expression of AKT in human pancreatic cancer cells compared to colorectal and renal cancer cells could be a reason why AKT in pancreatic cancer cells is reduced more by the cardiac hormones. It is important to note that patients whose tumors have a high expression of AKT have significantly lower overall survival *versus* those with tumors with low expression of AKT (30). We conclude that the four cardiac hormones inhibit multiple steps in the production and growth of carcinomas that cross-talk with each other in that they inhibit AKT, RAS, WNT, and VEGF pathways.

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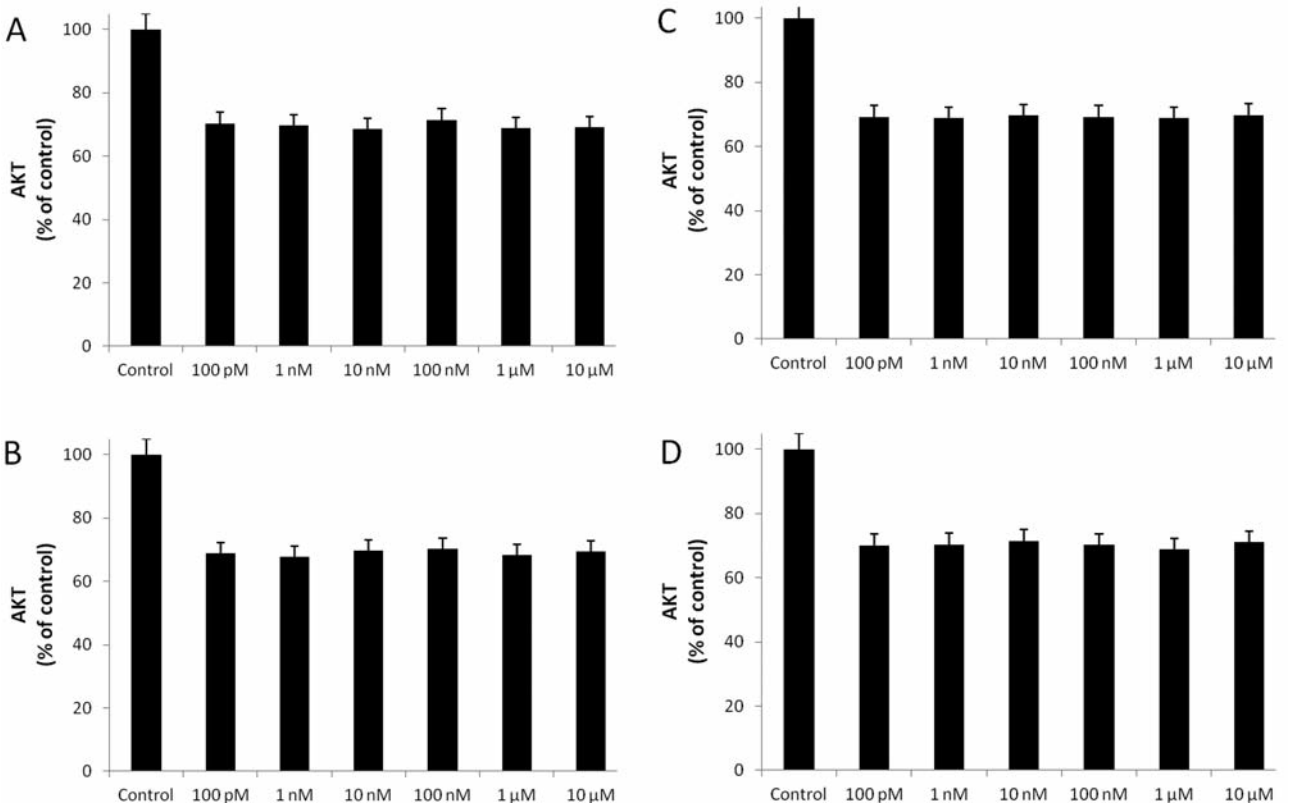

Figure 3. *AKT in pg/ml in human renal adenocarcinoma cells was maximally decreased by 31%, 32%, 31%, and 31%, by vessel dilator (A), kaliuretic peptide (B), atrial natriuretic peptide (C), and long-acting natriuretic peptide (D), respectively. Data for each concentration of each of the cardiac hormones is illustrated here and each of these decreases in AKT in human renal adenocarcinoma cells was significant at p<0.001, when evaluated by the Student's t-test for unpaired values. Each bar represents the mean±SEM of 32 determinations for the control and six determinations at each concentration of the experimental groups.*

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