Vessel Dilator and Kaliuretic Peptide Inhibit Ras in Human Prostate Cancer Cells

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Abstract. *Background: Vessel dilator and kaliuretic peptide have anticancer effects in human prostate adenocarcinomas. Materials and Methods: The effects of vessel dilator, kaliuretic peptide and cyclic GMP on Ras were examined in human prostate adenocarcinoma cells. Results: Vessel dilator and kaliuretic peptide decreased the activation of Ras –GTP over a concentration range of 0.01 μM to 1 μM. Vessel dilator and kaliuretic peptide (each 1 μM) inhibited the phosphorylation of Ras by 95% (p<0.0001) and 90% (p<0.0001), respectively. At 0.01 μM of kaliuretic peptide, the maximal inhibition was 95% . The inhibition of Ras lasted for 48 to 72 hours secondary to both peptides. Their ability to inhibit Ras was inhibited by cyclic GMP antibody and cyclic GMP itself inhibited Ras phosphorylation (89% ; p=0.0015). Conclusion: Vessel dilator and kaliuretic peptide both inhibit Ras partially mediated via cyclic GMP as part of their anticancer mechanism(s) of action.*

The Ras-mitogen-activated protein kinase (MAPK)/ extracellular signal-related kinase (ERK) (MEK)-ERK cascade, hereafter referred to as the Ras-MAPK pathway, is a prototypical signal transduction pathway that is aberrantly activated in many neoplasms including prostate and breast cancers (1,2). This pathway's activation is associated with a poor prognosis (2). Structural alteration in the upstream GTPase Ras occur in 25% to 30% of human cancers which allows them the ability to relay mitogen signals in a ligand-independent manner, thereby obviating the need for ligand activation of growth factor receptors that occurs in normal cells (3, 4). In addition, this pathway contributes to enhanced survival of tumor cells while also facilitating their metastatic spread to distant organs (3). The integral role of the Ras- MEK-ERK pathway in mediating multiple hallmarks of cancer has suggested that the different kinases in this pathway maybe targets for the treatment of cancer (3-6). Attempts to target Ras by perturbing its interaction with either SOS or GRB2 have not yielded viable drug development candidates largely because of the inherent difficulties of disrupting protein-protein interactions with drug-like molecules (3). Several drug discovery programs have also been devoted to finding inhibitors of farnesyltransferase as a means to prevent the membrane localization of Ras (3). Despite the successful identification of several chemical leads that effectively inhibited this prenylation enzyme, tumor cells, however, have proved generally to be impervious to the action of this class of inhibitors (3).

The present investigation was designed to determine if two endogenous cardiac peptide hormones, *i.e.* vessel dilator and kaliuretic peptide, which eliminate up to 80% of human pancreatic cancers in athymic mice (7) and two-thirds of human breast cancers in athymic mice (8), affect Ras. These two cardiac hormones are known to inhibit the phosphorylation (*i.e.* activation) of downstream kinases MEK 1/2 (9) by 98% ($p<0.0001$) and ERK 1/2 by 97% ($p<0.0001$) (10). These cardiac hormones have never been investigated for their effects on Ras. When both vessel dilator and kaliuretic peptide were demonstrated to inhibit Ras in dose-response and time-sequenced experiments, it was then investigated whether their effects were specifically mediated by cyclic GMP, the intracellular mediator of many of these peptide hormones' effects (11, 12).

Materials and Methods

Human prostate adenocarcinoma cells. A cell line (ATCC number HTB-81; DU 145) of human prostate adenocarcinoma cells was purchased from American Type Culture Association (ATCC), Manassas, VA, USA. This prostate cancer cell line was derived in 1978 by KR Stone *et al.* (13) from a 69 year old man. These homogenous cells when injected into athymic mice form moderately

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differentiated prostate adenocarcinomas within 21 days (13).

Culture of the prostate adenocarcinoma cells. Propagation of these cells was in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2 mmol/L-glutamine adjusted with addition of 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1 mmol/L sodium pyruvate, and heat-inactivated 10% fetal bovine serum (Sigma Chemical Company, St. Louis, MO, USA) with penicillin, streptomycin and fungizone at a temperature of 37˚C, with 5% CO_2 as recommended by the ATCC.

Ras activity assay protocol. The activation of Ras was evaluated using a Ras activation assay kit (Upstate Cell Signaling Solutions, Temecula, CA, USA) according to the manufacturer's protocol. The human prostate cancers were incubated for 5,15, 30 and 45 minutes, as well as 1, 2, 3, 4, 5 and 24 hours, respectively, in dose-response curves with 0.01 μmol/L to 1 μmol/L of kaliuretic peptide or vessel dilator. After the respective time periods prostate cancer cells were placed on ice, washed with cold prosphate-buffered saline (PBS), and lysed in magnesium lysis wash buffer [MLB] (25 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1% Igepal CA-630, 10% glycerol, 25 mmol/L NaF, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 μg/mL leupeptin and 10 μg/mL aprotinin). Cell lysates were centrifuged at 14,000 rpm for 5 min at 4˚C. Protein concentrations in cell lysate supernatants were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Fresh lysates were utilized in these experiments because GTP-Ras (*i.e.* activated form of Ras) is quickly hydrolyzed to GDP-Ras.

GTPγS/GDP loading for positive and negative controls. The prostate cancer cell extract was aliquoted (0.5 mL) to two microfuge tubes to which 10 mM of EDTA was added. For the positive control 100 μmo/L of GTPγS was added while the negative control had 1 mmol/L of GDP added (both were from Upstate Cell Signaling Solutions). These tubes were incubated for 30 minutes at 30˚C with agitation. Loading was stopped by placing the tubes on ice and adding 60 mmol/L of $MgCl_2$.

Ras pull-down assay. To each 0.5 mL of human prostate cancer cell extract, 10 μg of Ras Assay Buffer Reagent (Upstate Cell Signaling Solutions) consisting of glutathione S-transferase fusion-protein, corresponding to the human Ras Binding Domain residues 1-149 of Raf-1 expressed in *E. coli*, and provided bound to glutathione-agarose, was added. This reagent specifically binds to and precipitates Ras-GTP from cell lysates. These reaction mixtures plus vessel dilator or kaliuretic peptide or controls without either of the cardiac hormones were incubated for 45 minutes at 4˚C with gentle rocking followed by centrifugation at 14,000× *g*, 4˚C for 10 sec to pellet the agarose beads. The beads were washed three times with MLB and then resuspended in 40 μL of 2× Laemmli reducing sample buffer with 2 μL of 1 M dithiothreitol and boiled for 5 minutes. These beads were then pelleted by centrifugation (14,000× *g*, 4˚C) and collected by microcentrifuge pulse.

Immunoblotting analysis: Western blots. Twenty microlitres of each protein sample were separated by 10% SDS-polyacrylamide gels (120 volts for 90 min) and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences Corporation, Piscataway, NJ, USA) for 90 min at 110 volts in transfer buffer. The membranes were blocked with 3% non-fat dry milk for 2 hours with gentle rocking and incubated overnight with anti-Ras, clone Ras 10 containing 0.05 ng/L of purified Ig G2aκ (Upstate) at 4˚C with gentle rocking. These membranes were washed three times (6 mins each time) with PBS. The membranes were then immediately incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) at a dilution of 1:1000 for Ras for 1 hour at room temperature. The membranes were washed again two times and then examined by the chemiluminescent method.

Cyclic GMP effects on ras. Cyclic GMP is one of the known mediators of the biological effects of these peptide hormones (11, 12). For the mechanism of action of these peptide hormones' ability to inhibit Ras in prostate adenocarcinoma cells, 1 μM of 8-bromoguanosine 3',5'-cyclic monophosphate (*i.e.*, 8-bromo-cyclic GMP, Sigma), the cell-permeable analog of cyclic GMP, was utilized.

Do these peptide hormones' ability to inhibit ras in prostate adenocarcinoma cells specifically involve cyclic GMP? To determine if the inhibition of Ras in prostate adenocarcinoma cells was cyclic GMP specific, these peptide hormones (1 μmol/L each) and the specific cyclic GMP antibody in 1:80 dilution (Sigma) were incubated together for 30 min followed by the above research protocol with Immunoblotting.

Statistical analysis. Data are expressed as means±SEM and evaluated using analysis of variance (ANOVA) with repeated measures design for within-group comparisons. A $p<0.05$ was considered the criteria to be statistically significant.

Results

Dose-response and time sequence studies. Vessel dilator inhibited the activation of Ras by a maximum of 95% ($p<0.00001$) which occurred at 45 minutes at its 1 μmol/L concentration (Figure 1A). Within five minutes there was a 62% decrease in the phosphorylation of Ras with the activation of Ras being inhibited by 90% at 15 and 30 minutes with 1 μmo/L of vessel dilator (Figure 1A; $p<0.0001$). At one hour there was still an 83% decrease in the activation of Ras ($p<0.001$) secondary to 1 μmol/L of vessel dilator. When the effects of 1 μmol/L of vessel dilator were investigated for 72 hours to determine when vessel dilator no longer inhibited the phosphorylation of Ras, it was found that vessel dilator (1 μmol/L) could inhibit the activation of Ras for 24 hours ($p<0.0001$) and then the effects began to wane at 48 and 72 hours (Figure 2). Reducing the concentration of vessel dilator 10-fold in dose-response studies to 0.1 μmol/L resulted in an 86% decrease ($p<0.001$) in the activation of Ras at 15 minutes and a 78% decrease ($p<0.001$) at 30 minutes (Figure 1B). At one hour there was still a 63% decrease in the phosphorylation of Ras secondary to 0.1 μmol/L of vessel dilator (Figure 1B). A 100-fold reduction in vessel dilator in these dose-response experiments to 0.01 μmol/L revealed that this concentration of vessel dilator caused a maximal decrease (67% , $p<0.001$) at 30 minutes (Figure 1C). After 30 minutes vessel dilator's effects began to wane with a 23% decrease at 45 minutes ($p<0.05$) and only a 14% decrease at 60 minutes (NS) (Figure 1C).

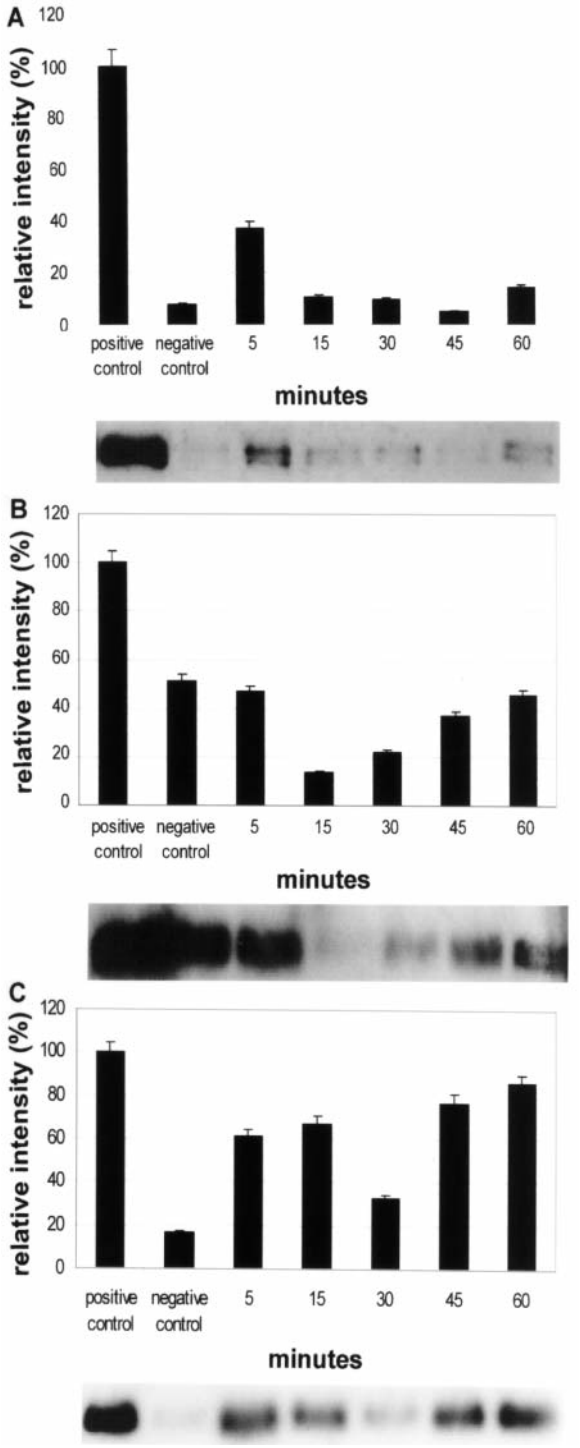

Figure 1. *Dose response of vessel dilator on the activation of Ras in human prostate cancer cells at A) 1 μmol/L, B) 0.1 μmol/L and C) 0.01 μmol/L in time-sequenced experiments at 5, 15, 30, 45 and 60 minutes. There was a significant (p<0.0001) inhibition of the activiation (i.e. phosphorylation) of Ras at each time point when evaluated by analysis of variance (ANOVA). Ras-GPT (i.e. active Ras) is at 21 kD. The relative intensity in these bar graphs is a comparison of three Western blots against the positive control (untreated Ras-GPT) with one typical Western blot illustrated. The illustrated negative controls in these graphs is Ras-GDP at 21 kDa.*

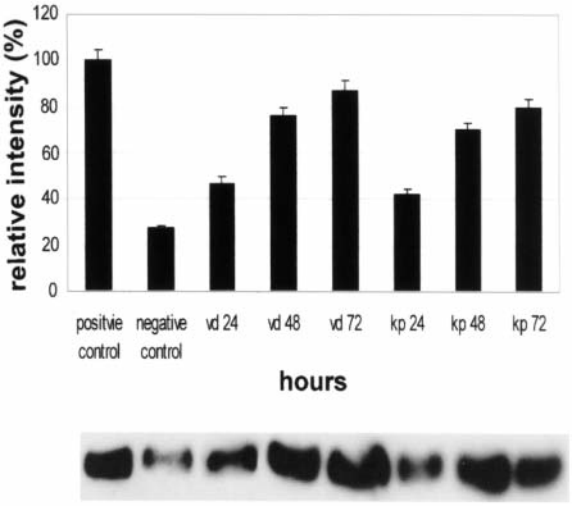

Figure 2. *Time-sequenced experiments to determine when vessel dilator's and kaliuretic peptide's inhibitory effects on Ras are no longer significant. In these experiments their effects on Ras were examined for up to 72 hours. Vessel dilator and kaliuretic peptide's effects on Ras were no longer significant after 72 hours and began to wane by 48 hours when evaluated by ANOVA. Ras-GPT is at 21 kD. The relative intensity in this bar graph is a comparison of the Western blots, with the positive control (untreated) Ras-GTP intensity being 100%. The negative control illustrated in these graphs is Ras-GDP at 21 kD.*

Kaliuretic peptide (1 μmol/L) caused a significant (64%; p=0.003) decrease in Ras activation in 30 minutes with a maximal 90% (p<0.0001) decrease of Ras in human prostate adenocarcinoma cells at 60 minutes (Figure 3A). At this concentration of kaliuretic peptide there was a 77% (p<0.001) decrease in the phosphorylation of Ras at 45 minutes (Figure 3A). When the concentration of kaliuretic peptide was reduced 10-fold to 0.1 μml/L there was a 91% (p<0.0001) decrease in the phosphorylation of Ras within five minutes with a sustained decrease in the phosphorylation by 81% (p<0.001) at 60 minutes (Figure 3B). When the concentration of kaliuretic peptide was reduced to 0.01 μmol/L, the inhibition began as early as five minutes (95% decrease, p<0.0001) where it was maximal (Figure 3C). There was still a very significant (p<0.001) 75% decrease after one hour of exposure to 0.01 μmol/L of kaliuretic peptide (Figure 3C). At 24 hours there was a significant (p<0.01) 58% inhibition of Ras secondary to kaliuretic peptide (Figure 2). This inhibition waned between 48 and 72 hours (Figure 2).

Cyclic GMP antibody specifically blocks vessel dilator and kaliuretic peptide's inhibition of ras kinase. Cyclic GMP (1 μmol/L) inhibited the phosphorylation of Ras by 89% (p<0.001) (Figure 4). When cyclic GMP antibody was added to kaliuretic peptide the inhibition of the activation of Ras

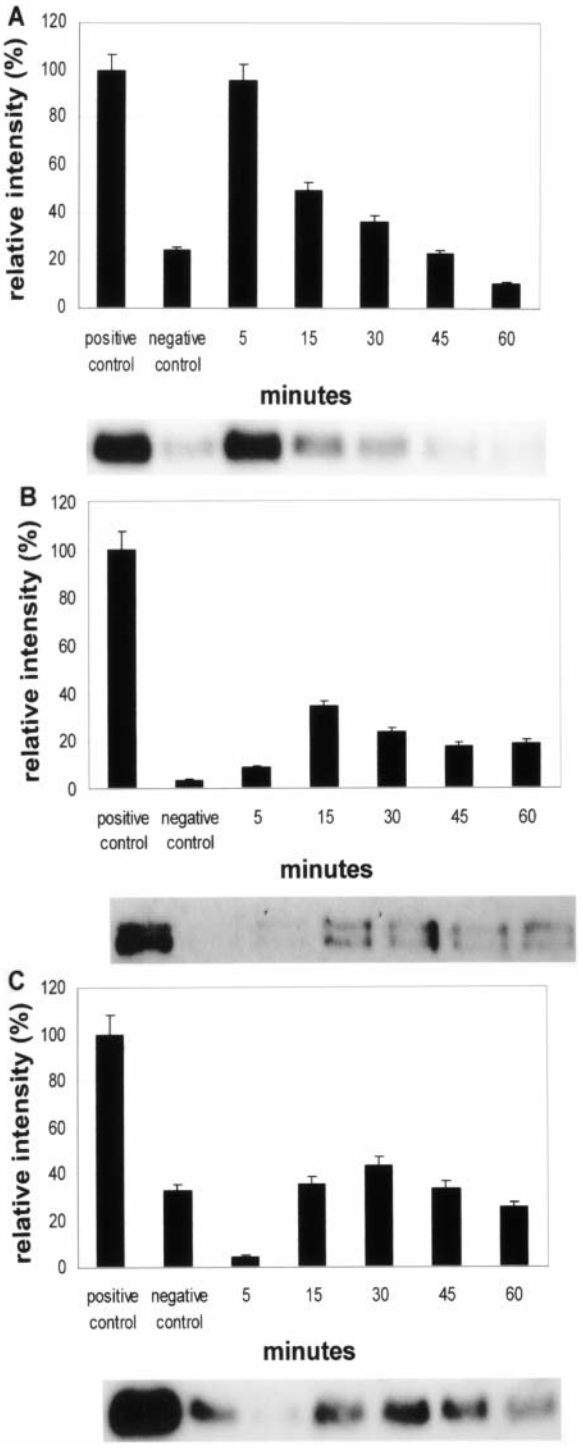

Figure 3. *Dose response of kaliuretic peptide on the activation of Ras in human prostate adenocarcinoma cells at A) 1 μmol/L, B) 0.1 μmol/L and C) 0.01 μmol/L in time sequenced experiments at 5, 15, 30, 45 and 60 minutes. There was a significant inhibition of the phosphorylation (i.e. activation) of Ras at each time point when evaluated by analysis of variance (ANOVA). Ras-GTP (positive control) is at 21 kD. The illustrated negative controls in these graphs is Ras-GDP. The relative intensity in this bar graph is a comparison of three Western blots, with the control blot intensity being 100%.*

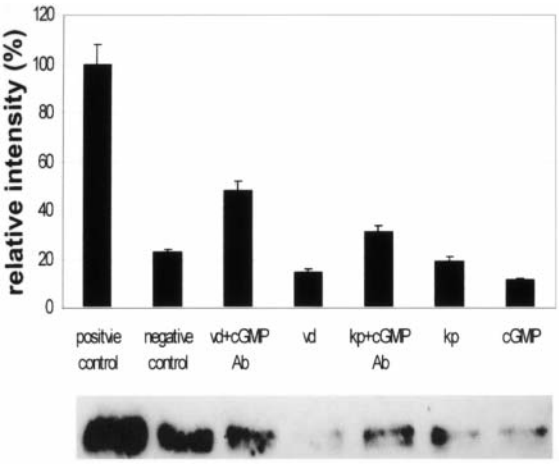

Figure 4. *Cyclic GMP inhibits the phosphorylation of Ras 89% and the cyclic GMP antibody (Ab) blocks vessel dilator and kaliuretic peptide's inhibition of Ras which were both significant at p=0.0015 when evaluated by ANOVA. Ras-GTP is at 21 kD. The positive control of Ras-GTP is illustrated as 100% intensity.*

decreased from 81% with kaliuretic peptide alone to 61%. The addition of the cyclic GMP antibody blocked the 85% inhibition of phosphorylation of Ras by vessel dilator down to 52% ($p<0.01$ for vessel dilator without *versus* with GMP antibody) (Figure 4).

Discussion

Ras is a small GTPase that cycles between an inactive GDP-bound and an active GTP-bound form (14). A large variety of ligands that stimulate cell surface receptors induce the activation of Ras (14). Ras has become important in cancer research with the finding that in 25-30% of human cancers, Ras protein is constitutively active in the GTP-bound form (3, 4). This is usually due to point mutation in one of three Ras genes, *i.e.* H-Ras, K-Ras or N-Ras which encode for highly similar proteins with molecular weight of 21,000 (15). This point mutation abolishes the intrinsic GTPase activity of Ras protein (15). The present investigation is the first investigation demonstrating that endogenous substances, *i.e.* cardiac hormones, can significantly ($p<0.0001$) inhibit the activity of Ras. Previous studies to prevent the membranes localization of Ras proved unfruitful as tumor cells proved generally impervious to their action (3). Likewise, attempts to target Ras by perturbing interaction with SOS or GRB2 have not yielded any viable drugs because of the inherent difficulties in disrupting protein-protein interactions (3). Thus, the present endogenous inhibitors of Ras are unique in that they markedly decrease the phosphorylation (*i.e.* activation) of Ras and are known to eliminate up to 80% of human pancreatic cancers (7) and two-thirds of human breast cancers (8) in athymic mice.

The present investigation suggests that one of vessel dilator and kaliuretic peptide's metabolic targets for the elimination of cancers is Ras. These cardiac hormones, thus, have three metabolic targets within cancer cells to mediate their effects. The other two metabolic targets in addition to Ras are part of the Ras-MEK 1/2-ERK 1/2 (Ras-MAPK kinase) cascade. Vessel dilator and kaliuretic peptide's second metabolic target on cancer cells is MEK 1/2 (9). These two hormones inhibit up to 98% of MEK 1/2 kinases (9). The third metabolic target in cancer cells is the downstream extracellular-signal regulated (ERK) 1/2 kinases, whose activation is inhibited up to 97% by vessel dilator and kaliuretic peptide (10). In addition to eliminating up to 97% of the basal phosphorylation ERK 1/2, these cardiac hormones completely block mitogens such as epidermal growth factor (EGF)'s ability to stimulate ERK 1/2 (16). It is important to note that vessel dilator and kaliuretic peptide do not inhibit the basal phosporylation of ERK1/2 kinase in normal human cells (17). Thus, with the demonstration in the present investigation that vessel dilator and kaliuretic peptide are very potent ($p<0.0001$) inhibitors of Ras, there are now three known metabolic targets within cancer cells for these hormones to mediate their anticancer effects.

With respect to the mechanism of how vessel dilator and kaliuretic peptide inhibit the phosphorylation of Ras, one of the second messengers of their biological effects, *i.e.* cyclic GMP (11, 12), was found using 8-bromo-cyclic GMP (1 μM) to inhibit the phosphorylation of Ras by 89% in human prostate cancer cells (Figure 4). That cyclic GMP mimicks the effects of vessel dilator and kaliuretic peptide on the activation of Ras in the same human prostate cancer cells suggests that cyclic GMP is one of the mediators of these peptide hormones' ability to cause a 97% decrease in prostate cancer cell number (18). This was further defined in the present investigation where, utilizing a cyclic GMP antibody, it was demonstrated for the first time that this antibody could significantly block vessel dilator and kaliuretic peptide's effects on the activation of Ras (Figure 4). Utilizing this antibody suggests that decrease of the activation of Ras is specifically mediated in part by cyclic GMP.

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