Vessel Dilator and Kaliuretic Peptide Inhibit ERK 1/2 Activation in Human Prostate Cancer Cells

YING SUN[1], EHRENTRAUD J. EICHELBAUM[1], HAI WANG[2] and DAVID L. VESELY[1]

[1]*Departments of Internal Medicine, Molecular Pharmacology and Physiology University of South Florida Cardiac Hormone Center and James A. Haley Veterans Medical Center Tampa, Florida 33612;*
[2]*Department of Interdisciplinary Oncology, Molecular Oncology Program H. Lee Moffitt Cancer Center and Research Institute Tampa, Florida 33612, U.S.A.*

Abstract. *Background: Vessel dilator and kaliuretic peptide have anticancer effects in human prostate adenocarcinomas. Materials and Methods: Vessel dilator, kaliuretic peptide and cyclic GMP's effects on ERK 1/2 kinase were examined in human prostate adenocarcinoma cells. Results: Vessel dilator and kaliuretic peptide decreased the activation of ERK 1/2 over a concentration range of 0.01 μM to 1 μM. Vessel dilator and kaliuretic peptide (each 1 μM) inhibited the phosphorylation of ERK 1/2 kinase 96% ($p<0.0001$) and 70% ($p<0.001$), respectively. Both had significant effects within five minutes at their 0.01 μM concentrations. The inhibition of ERK 1/2 lasted for at least two hours secondary to both. Their ability to inhibit ERK 1/2 was decreased by cyclic GMP antibody and cyclic GMP itself decreased ERK 1/2 phosphorylation. Conclusion: Vessel dilator and kaliuretic peptide both inhibit ERK 1/2 kinase mediated via cyclic GMP as part of their anticancer mechanism(s) of action.*

Extracellular-signal regulated kinase (ERK) 1/2 is a mitogen activated protein kinase (MAP kinase) important for the growth of cancers (1, 2). Growth factors such as epidermal growth factor (EGF), fibroblast growth factor, platelet derived growth factor and vascular endothelial growth factor (VEGF) work after binding to their specific receptor tyrosine kinases *via* ERK 1/2 kinase to cause proliferation (1). EGF, for example, when it binds to its EGF receptor, causes its autophosphorylation on tyrosine residues and recruits the Grb2-Sos complex to turn on membrane-associated Ras, which then activates the Raf-Mek 1/2 – ERK 1/2 kinase cascade (1).

Cardiac natriuretics are a family of peptide hormones that have significant anticancer effects on human prostate, breast, colon and pancreatic adenocarcinoma cells (3-6), as well as small cell and squamous lung carcinoma cells *in vitro* (7, 8). One gene in the heart of this peptide hormones family synthesizes a 126 amino acid prohormone, which with proteolytic processing results in four peptide hormones consisting of: 1) long acting natriuretic peptide, LANP (*i.e.,* the first 30 a.a. of this prohormone); 2) vessel dilator (VDL; a.a. 31-67); 3) kaliuretic peptide (KP, a.a. 79-98); and 4) atrial natriuretic peptide (ANP), a.a. 99-126 of the 126 a.a. prohormone (Figure 1). Vessel dilator appears to have the strongest anticancer effects decreasing up to 97% the number of human prostate and colon cancer cells within 24 hours (5, 6). *In vivo*, vessel dilator decreases the volume of human pancreatic adenocarcinomas growing in athymic mice the most (49% decrease in tumor volume in one week), while all four peptide hormones stop the growth of this cancer *in vivo* (9).

It has been found that a modified kaliuretic peptide (*i.e.*, a.a. 73-102) decreases phosphorylation of ERK 1/2 in human alveolar type II epithelial cells (ATCC A549) (10). The present investigation is to determine if an unmodified kaliuretic peptide, *i.e.,* the natural hormone, and vessel dilator, the peptide hormone with the most significant anticancer effects (3-9), could directly inhibit ERK 1/2 kinase in prostate cancer cells. Since both vessel dilator and kaliuretic peptide were demonstrated to inhibit ERK 1/2 kinase in dose-response and time-sequenced experiments, it was 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

Correspondence to: David L. Vesely, MD, Ph.D., Professor of Internal Medicine, Molecular Pharmacology and Physiology, Director, USF Cardiac Hormone Center, J. A. Haley Veterans Medical Center-151, 13000 Bruce B. Downs Blvd., Tampa, Florida 33612, U.S.A. Tel: (813) 972-7624, Fax: (813) 972-7623, e-mail: david.vesely@med.va.gov

Key Words: Adenocarcinoma, prostate neoplasm, natriuretic peptides, vessel dilator, kaliuretic peptide, ERK 1/2 kinase.

purchased from American Type Culture Association (ATCC), Manassas, VA, USA. This prostate cancer cell line was derived in 1978 by K.R. Stone *et al.* (13) from a 69 year old man. These homogenous cells when injected into athymic mice form moderately 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 with 2 mM/L-glutamine adjusted with addition of 1.5 g/L sodium bicarbonate, 90% 10 mM HEPES, 1 mM 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, 5% CO_2 as recommended by the ATCC. The cells were subcultured every 6-8 days. The growth medium was changed every three days. The peptide hormones were from Phoenix Pharmaceuticals, Inc., Belmont, CA, USA.

Research protocol. The human prostate cancers were incubated for 5, 10, 15, 30 and 45 minutes, or 1 hour and 2 hours, respectively, in dose-response curves with 0.01 μM to 1 μM of kaliuretic peptide or vessel dilator. After the respective time periods the prostate cancer cells were placed on ice, washed with cold PBS, and lysed in lysis buffer (1% Triton X-100, 50 mM Tris-Cl, PH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 25 mM NaF, 0.5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 μg/ml pepstanin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mg/ml phenylmethanesulfonyl fluoride [PMSF]). The cell lysates were centrifuged at 13,400 rpm for 15 minutes at 4˚C. Protein concentrations in cell lysate supernatants were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Thirty μg of each protein sample was utilized for SDS-polyacrylamide gel electrophoresis and immunoblotting analysis.

Immunoblotting analysis. After heating (100˚C) in 1xSDS loading buffer for 5 minutes, the proteins 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 minutes at 110 volts in transfer buffer. The membranes were washed four times with 1xTris-Buffered Saline (TBS), blocked with 5% non-fat dry milk for 1 hour with gentle rocking, washed three more times with 1xTBS, and incubated overnight at 4˚C with gentle rocking. These membranes were washed three times (8 min each time) with TBS. The membranes were then immediately incubated with goat anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) at a dilution of 1:1000 for extracellular-signal-related kinase 1 and 2 (ERK 1/2) for 102 hours at room temperature. The membranes were washed again and then examined by the chemicuminescent method. The primary antibody (P-ERK [E-4]) for measuring the detection of ERK 1 and ERK 2's phosphorylation at Try-204 and the antibody for measurement of total ERK 1/2 (K-23) were both utilized at a 1:1000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Cyclic GMP effects on ERK 1/2 kinase. Cyclic GMP is one of the known mediators of the biologic effects of these peptide hormones (11, 12). For the mechanism of action of these peptide hormones' ability to inhibit ERK 1/2 kinase in prostate adenocarcinoma cells, 1 μM of 8-bromoguanosine 3',5'-cyclic monophosphate (*i.e.*, 8-bromo-cyclic GMP, Sigma Chemical Company), the cell-permeable analog of cyclic GMP was utilized.

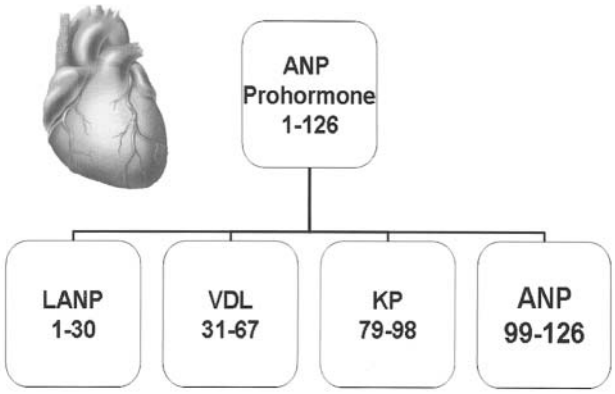

Figure 1. *The atrial natriuretic peptide gene in the heart synthesizes a 126 amino acid (a.a.) prohormone with which proteolytic processing results in the formation of four cardiac hormones. These four cardiac hormones, i.e., 1) long acting natriuretic peptide (LANP) consists of the first 30 amino acids of the 126 a.a. prohormone, 2) vessel dilator (VDL), a.a. 31-67 of the prohormone, 3) kaliuretic peptide (KP), a.a. 79-98 of this prohormone and 4) atrial natriuretic peptide (ANP), consisting of a.a. 99-126 of the 126 a.a. prohormone.*

Do these peptide hormones' ability to inhibit ERK 1/2 in prostate adenocarcinoma cells specifically involve cyclic GMP? To determine if the inhibition of ERK 1/2 kinase in prostate adenocarcinoma cells was cyclic GMP specific, these peptide hormones (1 μM each) and the specific cyclic GMP antibody in 1:80 dilution (Sigma Chemical Co.) were incubated together for 30 minutes followed by the above Research Protocol and 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 ERK 1/2 kinase by 92% ($p<0.00001$) within ten minutes at its 1 μM concentration (Figure 2). At 30 minutes, there was a 96% decrease ($p<0.00001$) in phosphorylation of ERK 1/2 secondary to 1 μM of vessel dilator (Figure 2). Even after two hours there was a 69% decrease ($p<0.001$) in the activation of ERK 1/2 secondary to 1 μM of vessel dilator (Figure 2). Decreasing the concentration of vessel dilator 10-fold in dose-response studies to 0.1 μM resulted in a 55% ($p<0.001$) decrease in the activation of ERK 1/2 at 30 minutes and a 28% decrease ($p<0.05$) in the phosphorylation of ERK 1/2 at 45 minutes (Figure 3). After 45 minutes the ability of 0.1 μM vessel dilator to decrease the activation of ERK 1/2 was no longer

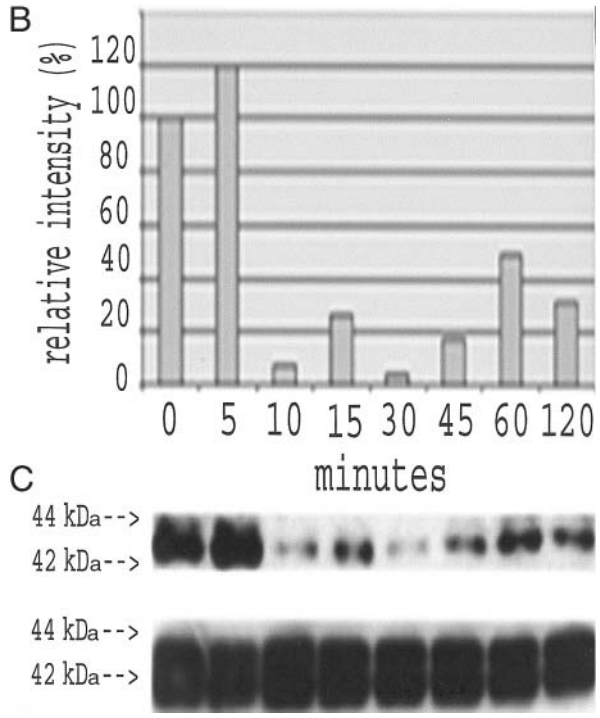

Figure 2. *Vessel dilator at 1 μM inhibits 96% of the phosphorylation of extracellular-signal regulated kinase (ERK) 1/2 at 30 minutes which was significant at p<0.0001 when evaluated by analysis of variance (ANOVA). At two hours there was still a significant (p<0.001) 69% decrease in the activation of ERK 1/2 kinase when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK2 is at 42 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1 and 2. The relative intensity in the bar graphs is a comparison against untreated ERK 1/2 (100% intensity).*

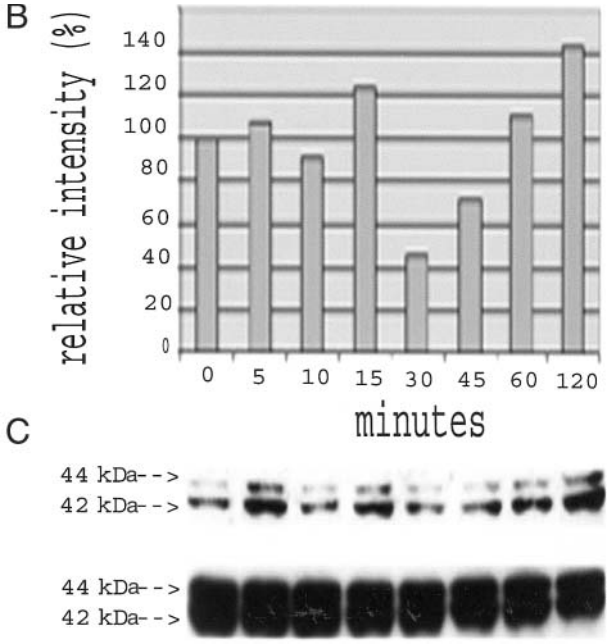

Figure 3. *Ten-fold decrease in vessel dilator to 0.1 μM resulted in a 55% decreased phosphorylation of ERK 1/2 at 30 minutes which was significant at p<0.001 when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kD) while ERK2 is at 42 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1/2.*

significant (Figure 3). A 100-fold decrease in vessel dilator in these dose-response experiments to 0.01 μM revealed that this concentration vessel dilator caused a maximal decrease (67%, $p<0.001$) at 10 minutes, but there was a significant decrease (37%, $p<0.05$) of the activation of ERK 1/2 as early as five minutes (Figure 4). As with the 10-fold higher concentration, *i.e.,* 0.01 μM of vessel dilator resulted in a significant (51%, $p<0.001$) decrease in the phosphorylation of ERK 1/2 at 45 minutes. At 60 and 120 minutes this lower concentration of vessel dilator became not significant (Figure 4).

The natural hormone kaliuretic peptide (1 μM) as opposed to the modified hormone examined previously, decreased the activation of ERK 1/2 in human prostate adenocarcinoma cells by 30% ($p<0.01$) within 10 minutes and caused a 51% ($p<0.001$) decreased phosphorylation at 30 minutes (Figure 5). With continuing exposure to 1 μM of kaliuretic peptide for longer periods, there was 70% ($p<0.0001$) decrease at 45 minutes and a 65% ($p<0.001$) decrease in ERK 1/2 phosphorylation at 60 minutes (Figure 5). Kaliuretic peptide at its 1 μM concentration continued

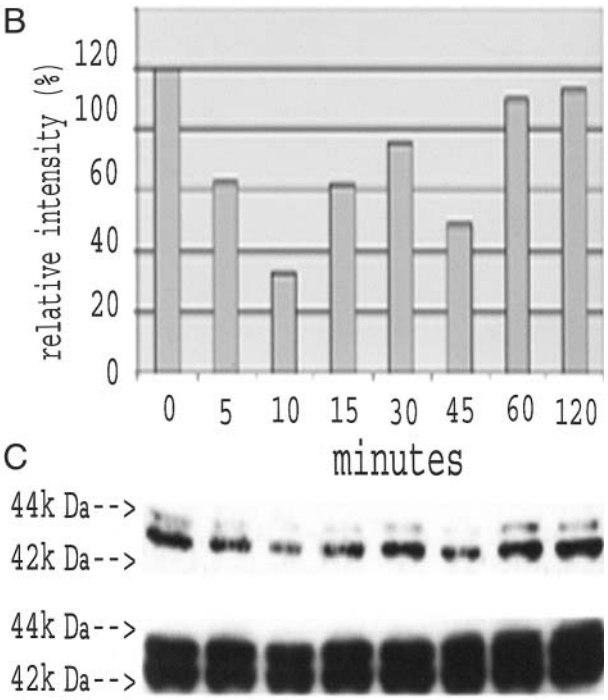

Figure 4. *0.01 μM of vessel dilator decreased (37%) the activation of ERK 1/2 as early as 5 minutes with maximal decrease (67%) of the phosphorylation occurring at 10 minutes. (p<0.05 and p<0.001, respectively, when evaluated by ANOVA). ERK 1 is at 44 kilo Daltons (kDa) while ERK2 is at 44 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1 and 2.*

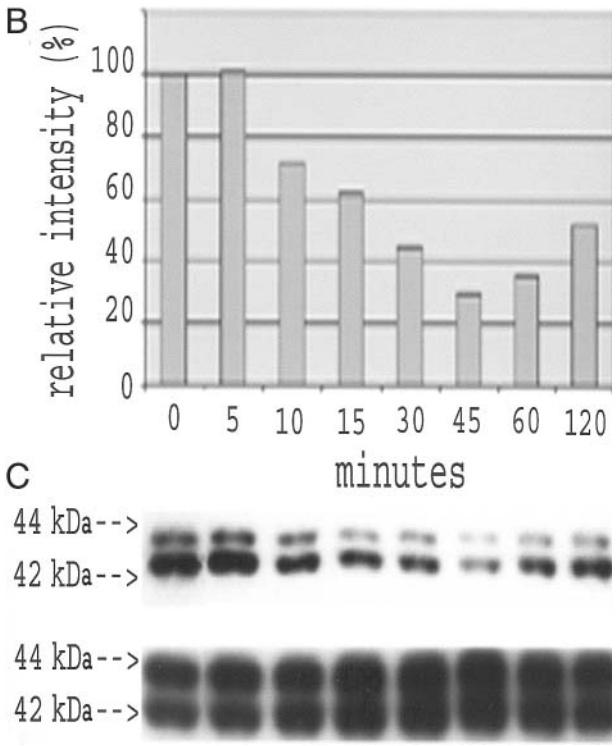

Figure 5. *Kaliuretic peptide (1 μM) caused a 51% decrease at 30 minutes and a 70% decrease at 45 minutes in the phosphorylation of ERK 1/2, $p<0.001$ and $p<0.00001$ (ANOVA). ERK 1 is at 44 kilo Daltons (kDa) while ERK2 is at 42 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1 and 2.*

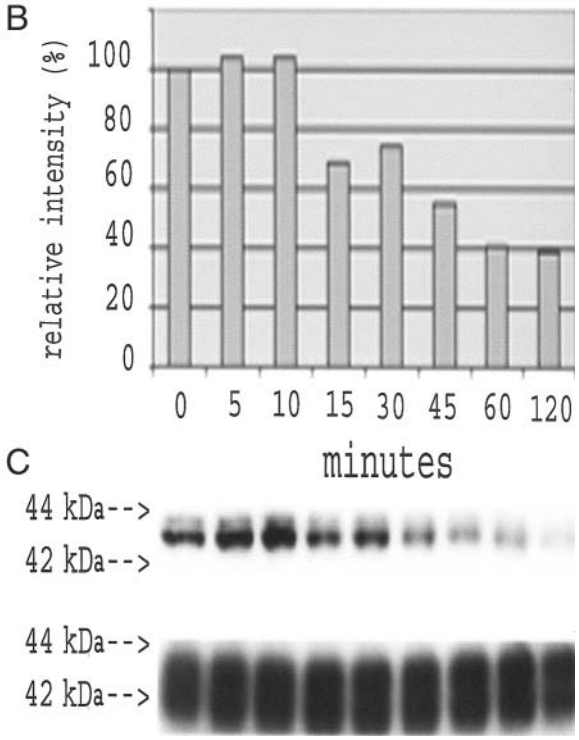

Figure 6. *Delayed onset of decreasing the activation of ERK 1/2 with decreasing the concentration 10-fold to 0.1 μM of kaliuretic peptide. Maximal decrease in the phosphorylation of ERK 1/2 was at 60 minutes (60% decrease) and two hours (61% decrease) which were both significant at $p<0.001$ hen evaluated by ANOVA. ERK 1 is 42 kilo Daltons (kDa) while ERK2 is 44 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1 and 2.*

to decrease (48%, $p<0.001$) the activation of ERK 1/2 for two hours (Figure 5). When the concentration of kaliuretic peptide was decreased to 0.1 μM, the inhibition of phosphorylation of ERK 1/2 did not begin until 15 minutes (32% decrease, $p<0.05$) (Figure 6). Kaliuretic peptide (0.1 μM) had its most significant decrease in activation of ERK 1/2 in a delayed fashion with the amount of decrease at 60 minutes and 2 hours being 60% and 61%, respectively ($p<0.001$ for both) (Figure 6). When kaliuretic peptide's concentration was decreased another 10-fold to 0.01 μM, its decrease in the activation of ERK 1/2 began as early as 5 minutes (15% decrease, $p<0.05$), but its maximal inhibition of ERK 1/2 of 30% ($p<0.05$) at 30 minutes was less than half of that obtained with 0.1 μM and 1 μM (Figure 7).

Cyclic GMP antibody specifically blocks vessel dilator and kaliuretic peptide's inhibition of ERK 1/2 kinase. Cyclic GMP (1 μM) inhibited the phosphorylation of ERK by 36%, while kaliuretic peptide and vessel dilator (1 each at μM) in the same experiment inhibited the phosphorylation of ERK 1/2 by 45% and 48% (Figure 8). When cyclic GMP antibody was added to kaliuretic peptide there was no inhibition of the activation of ERK 1/2, rather there was a 14% increase in the phosphorylation of ERK 1/2 (Figure 8). The addition of the cyclic GMP antibody decreased the inhibition of phosphorylation of ERK 1/2 by vessel dilator in half, from a 48% inhibition to a 24% inhibition (Figure 8).

Discussion

The results of this investigation define a part of the mechanism(s) of action of these peptide hormones in cancer cells. This is the first investigation demonstrating that the native peptide hormone, kaliuretic peptide, can decrease the activation of ERK 1/2 whose phosphorylation decreased up to 70%. It is also the first demonstration that kaliuretic peptide can decrease the activation of ERK 1/2 in any cancer cell. Vessel dilator was investigated for the first time for its' ability to inhibit phosphorylation of ERK 1/2 and it inhibited the phosphorylation ERK 1/2 by 96% (Figure 2) at its 1 μM concentration in a dose-response and time-sequenced manner. In the dose response-curves (Figures 2-

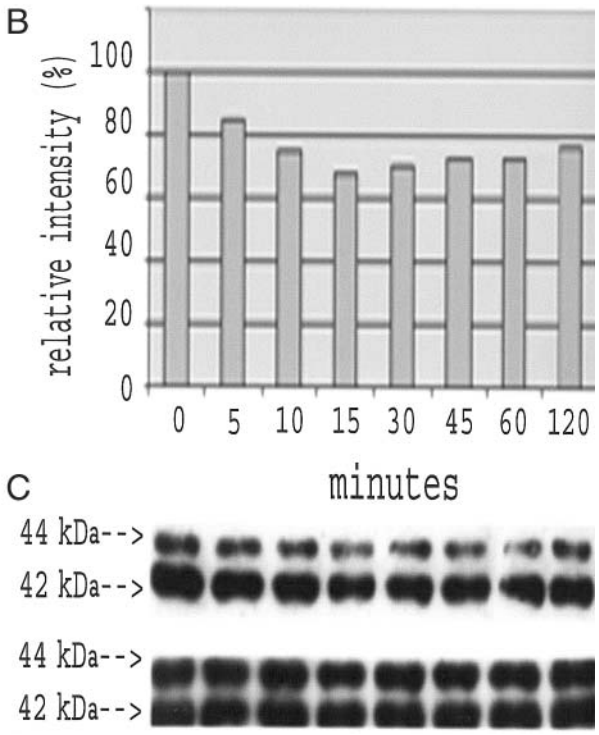

Figure 7. *The maximal inhibition of the activation of ERK 1/2 was only 30% by kaliuretic peptide at 0.01 μM. This decrease was significant at $p<0.05$ when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK2 is at 42 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1 and 2.*

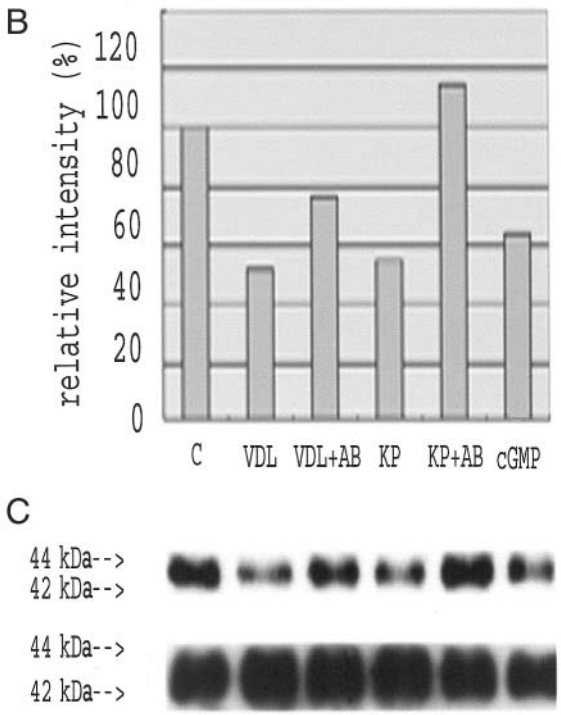

Figure 8. *Cyclic GMP inhibits the phosphorylation of ERK 1/2. Kaliuretic peptide's activation of ERK 1/2 was blocked completely by the cyclic GMP antibody (Ab) while vessel dilator's activation of ERK was decreased in half by the cyclic GMP antibody which was significant at $p<0.0001$ and $p<0.001$ when evaluated by ANOVA. ERK 1 is at 44 kilo Daltons (kDa) while ERK2 is at 42 kDa. B = Phosphorylation of ERK 1 and 2 at Try-204. C = Total ERK 1 and 2.*

6) of the present investigation vessel dilator was a stronger inhibitor of ERK 1/2 than kaliuretic peptide at every concentration. This information is consistent with vessel dilator's ability to decrease (*i.e.,* eliminate) more of human prostate adenocarcinoma cells in cell culture within 24 hours (5). Vessel dilator eliminates up to 97% of human prostate cancer cells in 24 hours, which is consistent with the present demonstrated ability to inhibit 96% of the activation of ERK 1/2. Kaliuretic peptide kills 88% of human prostate cancer cells in 24 hours (5), which is also consistent with its ability to decrease the activation of ERK 1/2 by 70% in the present investigation, as kaliuretic peptide also inhibits DNA synthesis in human prostate cancer cells as part of its mechanism of action (5). The present investigation suggests that the inhibition of the phosphorylation of ERK 1/2 is one of the mechanisms involved in vessel dilator and kaliuretic peptide's ability to decrease the number of viable cancer cells *in vitro* (3-8), and their ability to stop the growth and decrease the volume of human cancers *in vivo* (9). Thus, in addition to inhibiting DNA synthesis within cancer cells (3-8), vessel dilator and kaliuretic peptide's anticancer effects involve their ability to decrease the activation of extracellular-signal regulated kinase (ERK) 1/2, a cancer growth promoting peptide that translates from the extracellular membrane to the nucleus of the cell to promote growth (1, 2).

With respect to the mechanism of how vessel dilator and kaliuretic peptide inhibit the phosphorylation of ERK 1/2, one of the second messengers of their biologic effects, *i.e.,* cyclic GMP (11, 12), was found using 8-bromo-cyclic GMP (1 μM) to inhibit the phosphorphylation of ERK 1/2 by 36% in human prostate cancer cells similar in that experiment to kaliuretic peptide and vessel dilator. Cyclic GMP's mimicking the effects of vessel dilator and kaliuretic peptide on the activation of ERK 1/2 in the same human prostate cancer cells suggests that cyclic GMP is one of the mediators of these peptide hormones' ability to decrease cancer cell number (5) and inhibit the activation of ERK 1/2 in prostate adenocarcinoma cells. 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 block all of kaliuretic peptide's effects on the activation of ERK 1/2 and decrease in half vessel dilator's ability to inhibit the phosphorylation of ERK 1/2 (Figure 8).

Utilizing this antibody suggests that cyclic GMP's effects of decreasing the activation of ERK 1/2 are specific for kaliuretic peptide, *i.e.,* not due to some other mediator and important for vessel dilator's inhibition of the activation (*i.e.*, phosphorylation) of ERK 1/2.

Acknowledgements

We thank Charlene Pennington for her excellent secretarial assistance. This work was supported in part by the Department of Veterans Affairs Merit Review Grant Award (DLV, USA).

References

1 Schlessinger J: Cell signaling by receptor tyrosine kinases. Cell *103*: 211-225, 2000.
2 Davis RJ: Signal transduction by the JNK group of MAP kinases. Cell *103*: 239-252, 2000.
3 Vesely BA, McAfee Q, Gower WR Jr and Vesely DL: Four peptides decrease the number of human pancreatic adenocarcinoma cells. Eur J Clin Invest *33*: 998-1005, 2003.
4 Vesely BA, Song S, Sanchez-Ramos J, Fitz SR, Solivan SM, Gower WR Jr and Vesely DL: Four peptide hormones decrease the number of human breast adenocarcinoma cells. Eur J Clin Invest *35*: 60-69, 2005.
5 Vesely BA, Alli AA, Song S, Gower WR Jr, Sanchez-Ramos J and Vesely DL: Four peptide hormones specific decrease (up to 97%) of human prostate carcinoma cells. Eur J Clin Invest *35*: 700-710, 2005.
6 Gower WR Jr, Vesely BA, Alli AA and Vesely DL: Four peptides decrease human colon adenocarcinoma cell number and DNA synthesis via guanosine 3'5'-cyclic monophosphate. Int J Gastrointestinal Cancer *36*: 77-88, 2005.
7 Vesely BA, Fitz SR, Gower WR Jr and Vesely DL: Vessel dilator: Most potent of the atrial natriuretic peptides in decreasing the number and DNA synthesis of squamous lung cancer cells. Cancer Lett *232*: 226-231, 2006.
8 Vesely BA, Song S, Sanchez-Ramos J, Fitz SR, Alli A, Solivan SM, Gower WR Jr and Vesely DL: Five cardiac hormones decrease the number of human small-cell cancer cells. Eur J Clin Invest *35*: 388-398, 2005.
9 Vesely DL, Clark LC, Garces AH, McAfee QW, Soto J and Gower WR Jr: Novel therapeutic approach for cancer using four cardiovascular hormones. Eur J Clin Invest *34*: 674-682, 2004.
10 Hellermann G, Kong X, Gunnarsdottir J, San Juan H, Singam R, Behera S, Zhang W, Lockey RF and Mohapatra SS: Mechanism of bronchoprotective effects of a novel natriuretic hormone peptide. J Allergy Clin Immunol *113*: 79-85, 2004.
11 Waldman SA, Rapoport RM and Murad F: Atrial natriuretic factor selectively activates membranous guanylate cyclase and elevates cyclic GMP in rat tissues. J Biol Chem *259*: 14332-14334, 1984.
12 Vesely DL: Signal transduction: Activation of guanylate cyclase-cyclic guanosine-3'-5' monophosphate system by hormones and free radicals. Am J Med Sci *314*: 311-323, 1997.
13 Stone KR, Mickey DD, Wunderli H, Mickey GH and Paulson DF: Isolation of a human prostate carcinoma cell line (DU-145). Int J Cancer *21*: 274-281, 1978.

Received July 3, 2006
Accepted July 12, 2006