Department of Pharmacology

Qualifying Examination (Part I)

December 12 & 13, 2006

Please remember that this is a closed-book examination. You must be prepared to answer 4 of the 7 questions. Although not necessary, you may prepare written answers, overhead figures, or any type of materials that you think might be useful in the presentation of your answers. You may bring such preparation materials with you to the examination. The oral examination itself will not extend beyond two hours.

If you have any questions regarding the examination, please contact Joey Barnett at:

936-1722 (w)
385-4396 (h)
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BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

In one arm of the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) in hypertension compared the α_1-antagonist to the diuretic chlorthalidone in almost 25,000 men and women with cardiac risk factors. This arm of the trial was stopped prematurely when it was recognized that doxazosin doubled the incidence of heart failure, an effect not seen with other classes of antihypertensive medications. Since α_1-antagonists are enjoying increasing use to treat disorders of smooth muscle such as hypertension and prostate enlargement, whether the effects seen in the ALLHAT trial are due to α_1-antagonism or a nonspecific drug action must be determined. To address this question the effect of cardiac pressure overload (TAC, which is thoracic aortic constriction) was examined in mice null for both α_{1A} and α_{1B} (ABKO, which is A&B knockout) receptors.

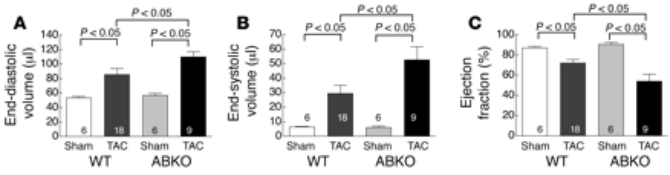


Figure 1. Hemodynamic parameters after TAC by echocardiography. Conscious mice were studied 2 weeks after TAC or sham operation. Ventricular volumes (**A** and **B**) were calculated by the cubed method (volume = 1.047 x LV internal dimension^3), where LV internal dimension is defined as the distance between the LVFW and the IVS on a 2-dimensionally guided M-mode echocardiogram, and ejection fraction (**C**) was determined by the formula (end-diastolic volume – end-systolic volume)/end-diastolic volume x 100. Numbers of mice are indicated.

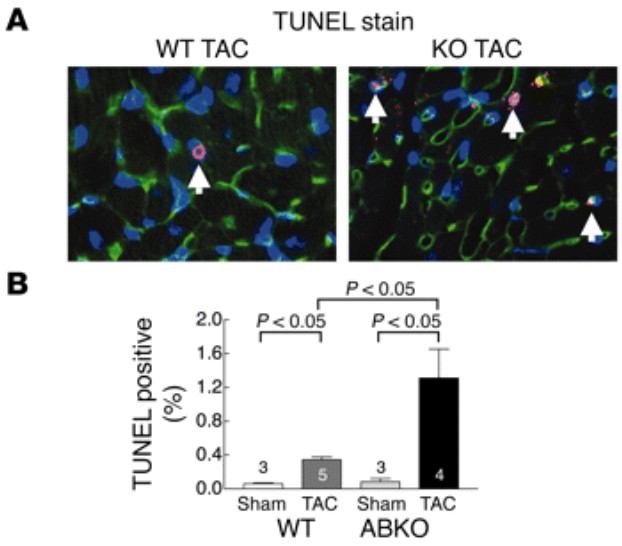


Figure 3. Apoptosis in heart. (**A**) Hearts at 2 weeks after TAC or sham surgery were analyzed by TUNEL staining. TUNEL-positive nuclei in TAC hearts are stained pink (arrows); membranes are green (FITC-conjugated wheat germ agglutinin), and nuclei are blue (Hoechst 33342). Magnification, x400. (**B**) TUNEL staining was quantified in 3,000 nuclei from 10 or more randomly selected fields per heart. Numbers of hearts are indicated, and matched areas from the LV, IVS, and RV were sampled in every heart.

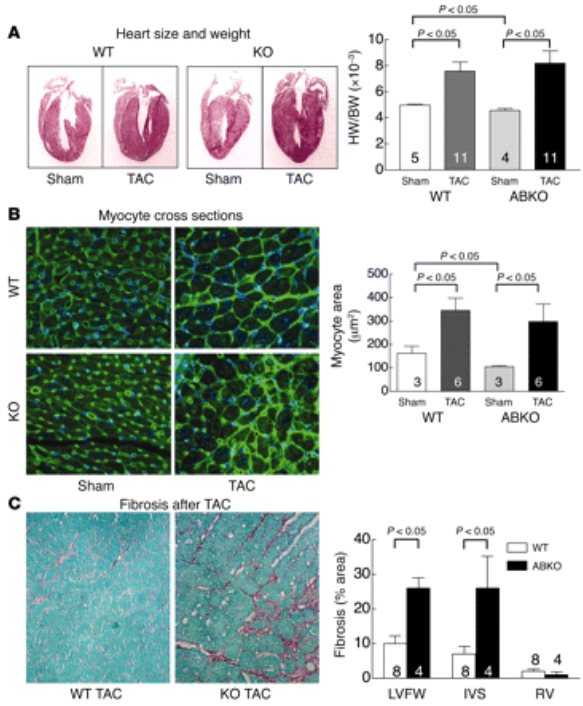


Figure 2. Heart and myocyte size and fibrosis. Measurements were made 2 weeks after TAC or sham surgery. (**A**) Left: Heart coronal sections stained with H&E. Magnification, x25. Right: Heart wet weight/body weight ratio (HW/BW). (**B**) Left: Ventricular sections stained with FITC-conjugated wheat germ agglutinin and Hoechst 33342. Magnification, x400. Right: Myocyte cross-sectional area from at least 200 myocytes per heart in randomly selected fields. (**C**) Left: Ventricular sections stained with fast green and Sirius red for collagen. Magnification, x400. Right: Interstitial fibrosis as a percentage of total microscopic area from at least 12–14 fields per heart. Numbers of hearts are indicated on the bars.

Qualifying Exam – December 2006
Question 1

Describe the data.

State a hypothesis as to the role of alpha adrenergic stimulation in heart failure. Design in vivo and in vitro experiments to test your hypothesis.

Based on your interpretation, comment on the ALLHAT findings.

Caffeine is a known inhibitor of phosphodiesterases and a non-selective adenosine receptor antagonist. Starbucks, *et al.*, recently demonstrated the effects of caffeine on fluid and solute excretion in mice engineered to have a targeted deletion of the adenosine A1 receptor gene (A1AR). Measurements were made on conscious animals living in metabolic cages. Caffeine (45 mg/kg) was administered by oral gavage followed by quantitative urine collections during a 3 hour period while animals had food and water restriction. Figure 1 illustrates their findings.

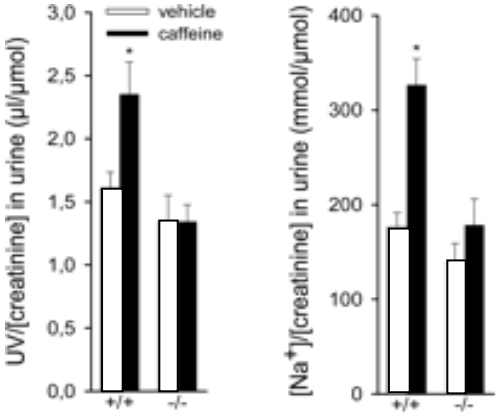


Figure 1 – Urine flow rate (UV) and urinary concentration of Na normalized to urine creatinine concentration in WT mice (+/+) and A1AR knock-out mice (-/-).
*, $p < 0.05$ *vs* the same genotype.

***Questions*:**

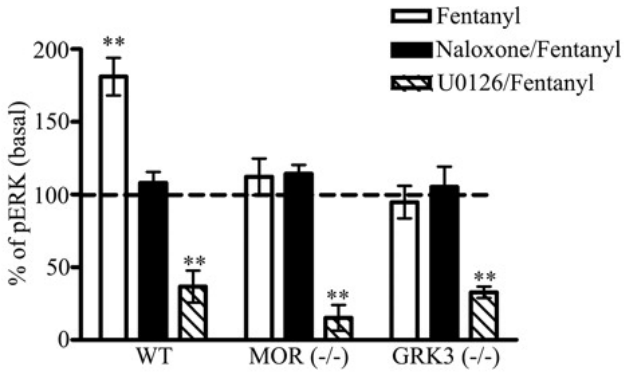
1. **Develop 2 hypotheses to explain these observations. Explain how you would test each hypothesis.**

2. **Make predictions about the potential effects of caffeine on glomerular filtration rate and explain in the context of your hypotheses.**

Qualifying Exam – December 2006
Question 3

A group of investigators is studying the mechanism of ERK1/2 (extracellular-signal-regulated kinase) activation by endogenous mu-opioid receptor (MOR). Using primary cultures of striatal neurons from wild type (WT), MOR knockout (-/-), and GRK3 knockout (-/-) animals they obtained the following results.

FIG. 1. Agonist-induced activation of phospho-ERK1/2 by the endogenous mu-opioid receptor (MOR) in wild type, MOR-/-, and GRK3-/- striatal neurons was assessed. Whole cell lysates prepared from neuronal cultures were used to quantify the levels of phospho-ERK1/2. Fentanyl is a MOR agonist; naloxone is a MOR antagonist. U0126 is a MEK1 (MAPK/ERK kinase) inhibitor. The results are the mean ± S.E., expressed as a percent of basal, untreated striatal neuronal cultures. (**, *p* 0.01 by Dunnett's post hoc comparison, *n* 4–7).



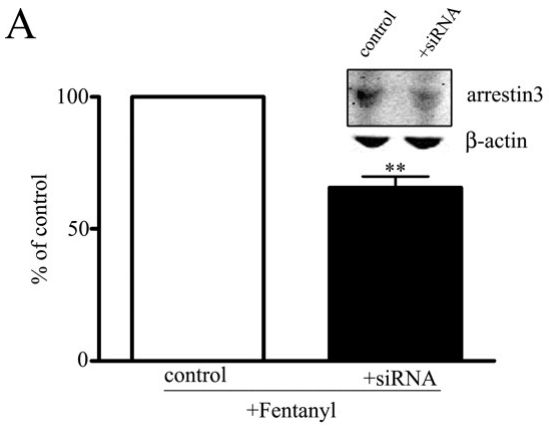


FIG.2. Fentanyl-induced activation of phospho-ERK1/2 by the endogenous MOR in siRNA arrestin3-treated wild type striatal neurons. *A*, immunoreactivity of arrestin3 (55 kDa) was determined in cells transfected with siRNA specific to arrestin3 and control cells. Cells were treated with 100 nM fentanyl for 30 min. (**, *p* 0.01, by Student's *t* test). *A, inset*, a representative immunoblot of arrestin3 in control cells and cells transfected with arrestin3 siRNA (siRNA) is shown. To control for equal loading of protein, membranes were reblotted with b-actin.

1. **Based on these data, propose a hypothetical mechanism of MOR activation-dependent ERK phosphorylation.**

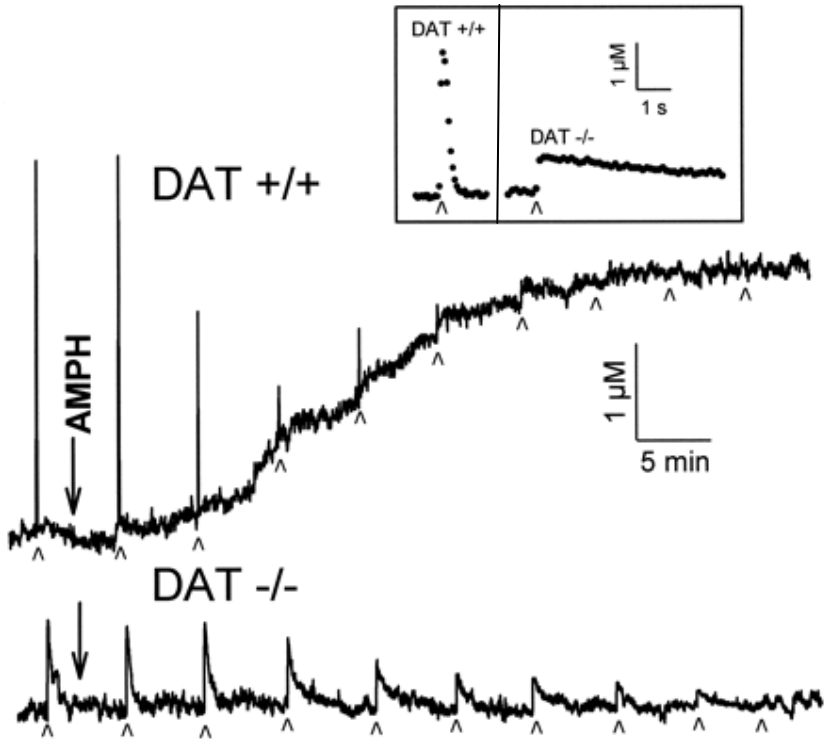
2. **Propose experiments to test your hypothesis, assuming that you can transfect anything you want into the primary neurons harvested from animals with all three genotypes used in Fig.1.**

3. **You have the following constructs: a) dominant-negative GRK3; b) dominant-negative arrestin3 mutant that binds the receptor but does not promote MOR internalization; c) phosphorylation-independent arrestin3 mutant that binds agonist-activated phosphorylated and unphosphorylated MOR equally well; d) MOR mutant that mimics the GRK-phosphorylated state of the receptor; e) MOR mutant that does not internalize regardless of the presence of GRKs and arrestins; f) expression constructs for GRKs 1, 2, 3, 4, 5, 6, or 7; g) siRNA constructs that knockdown GRKs 1, 2, 3, 4, 5, 6, or 7. Which of these tools would help you to test your hypothesis and which are useless for this purpose. Explain your reasoning.**

In order to examine the mechanisms by which electrical activity or amphetamine elevates extracellular dopamine (DA), brain slices derived from wildtype and dopamine transporter (DAT) knockout mouse striatum are stimulated in the presence or absence of amphetamine as shown below. Amperometry is used to record the release of DA.

A) Describe the salient features of the amperometric traces (inset box) seen after electrical stimulation (^) of slices from wildtype (+/+) or DAT KO (-/-) mice, specifically with respect to amplitude and kinetics of the evoked DA response.

B) Propose a mechanism that explains the differential response of wt and DAT KO brain slices to amphetamine application (downward arrow). Your mechanism should also account for the time-dependent changes seen in electrically evoked DA (^), which does not change in wt slices stimulated in the absence of amphetamine (data not shown).



C) The above experiments were conducted with wildtype slices derived from 129Sv/J mice as these are the same strain as used to produce the DAT KO. When slices from Balb/c mice are used as control, amphetamine application triggers a much larger increase in the baseline amperometric signal that can be blocked by cocaine pretreatment. Assessments of DAT protein and DA uptake show no differences with 129Sv/J mice and tissue content of DA appears the same. What genetic differences could account for the elevated response of DA neurons to amphetamine? How might you test your ideas using heterologous and native systems?

The new antifungal, voriconazole, and the antiretroviral agent ritonavir were co-administered as follows. Figure 1 and Table 1 show data from oral administration of voriconazole (400 mg) in combination with placebo (open circles) or 300mg ritonavir twice daily (black squares) to 20 healthy subjects.

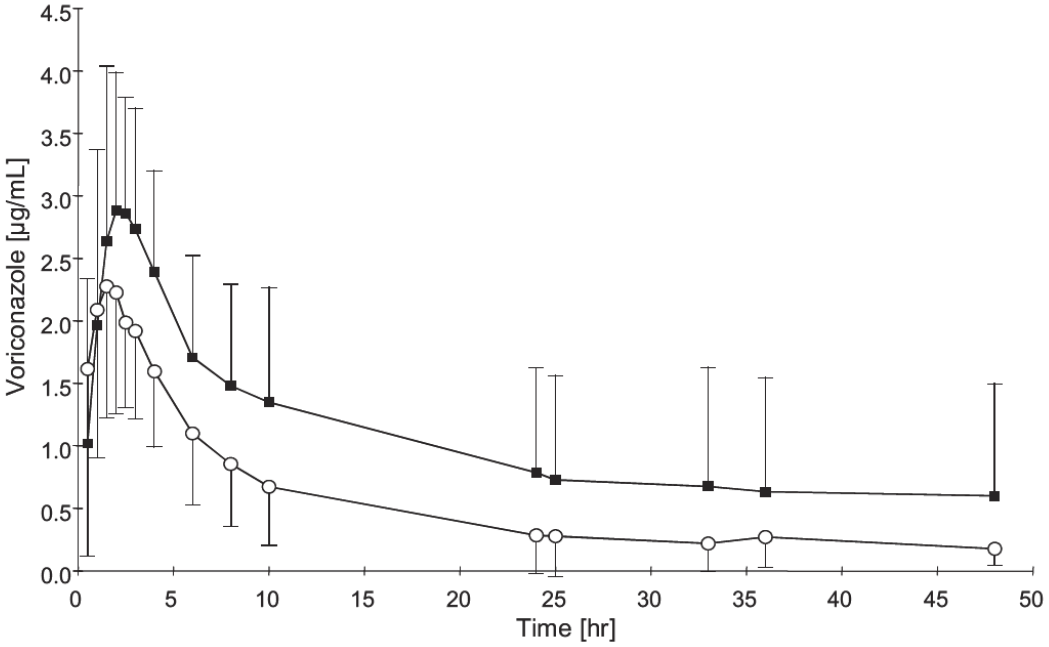


Fig 1. Mean (±SD) voriconazole plasma concentration–time profile after single oral administration of 400 mg voriconazole in combination with placebo (*circles*) or 300 mg ritonavir twice daily (*squares*) to 20 healthy subjects.

Table I. Pharmacokinetic parameters after noncompartmental analysis of voriconazole during placebo and ritonavir administration in 20 healthy individuals

	Placebo	*Ritonavir*	*P value*
t_{max} (h)	1.95 ± 1.31	2.03 ± 0.60	.762
C_{max} (µg/mL)	2.97 ± 1.07	3.49 ± 1.12	.015
$AUC_{0\text{-}48}$ (h · µg/mL)	23.50 ± 17.78	48.16 ± 38.81	.0001
$AUC_{0\text{-}\infty}$ (h · µg/mL)	25.26 ± 17.09	114.80 ± 221.17	.0001
$t_{½}$ (h)	9.5 ± 3.5	28.4 ± 42.6	.0001
MRT (h)	11.5 ± 5.8	39.2 ± 62.4	.0001
$Ae_{0\text{-}48}$ (µg)	1851 ± 1843	3020 ± 1551	.0003
Cl_{renal} (mL/min)	1.41 ± 0.66	1.39 ± 0.76	.475

Data are given as mean +/- SD. Difference between placebo and ritonavir treatment were assessed by use of the Wilcoxon matched pairs signed rank test. t_{max}, Time to reach maximum observed plasma concentration; C_{max}, maximum observed plasma concentration; $AUC_{0\text{-}48}$, AUC from time 0 to last measurable concentration; $AUC_{0\text{-}\infty}$, AUC extrapolated to infinity; $t_{1/2}$, terminal elimination half-life; MRT, mean residence time; $Ae_{0\text{-}48}$, amount excreted in urine from 0 to 48 hours; Cl_{renal}, renal clearance.

Question A. Provide an analysis and review of all the data in Figure 1 and Table 1 (give a point by point illustration of how the data in the Table relate to the data in the figure). What further experiment(s) would you carry out to further understand the pharmacokinetics of the vorinconazole/ritonavir interaction. Outline your interpretation of a plausible mechanism for the drug interaction. (continued)

Figure 2 shows a similar protocol repeated in subjects who are genotyped as normal (1*/1*) for P450 2C19, or as heterozygous (1*/2*) or homozygous (2*/2*) for an inactivating mutation.

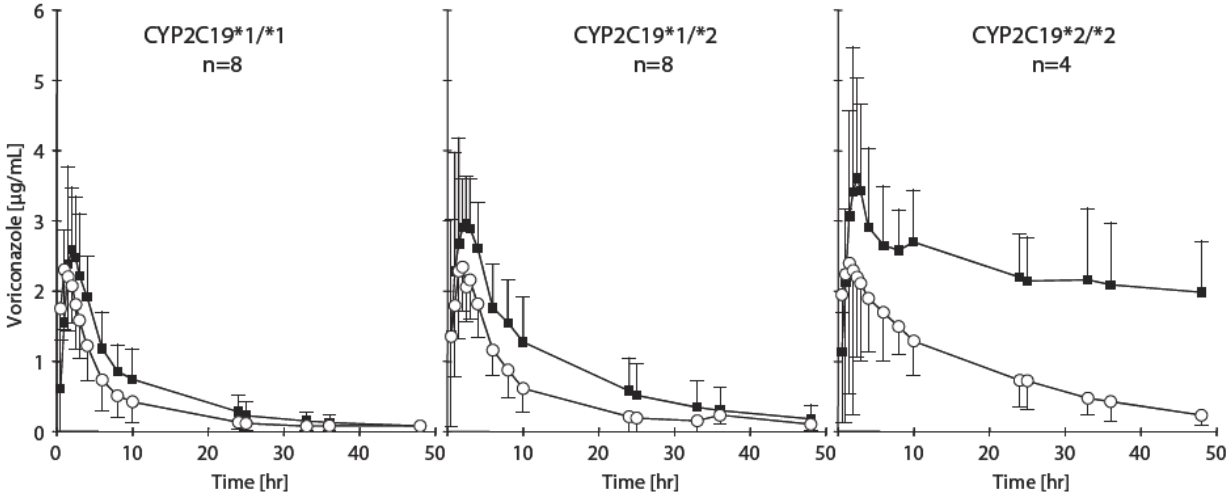


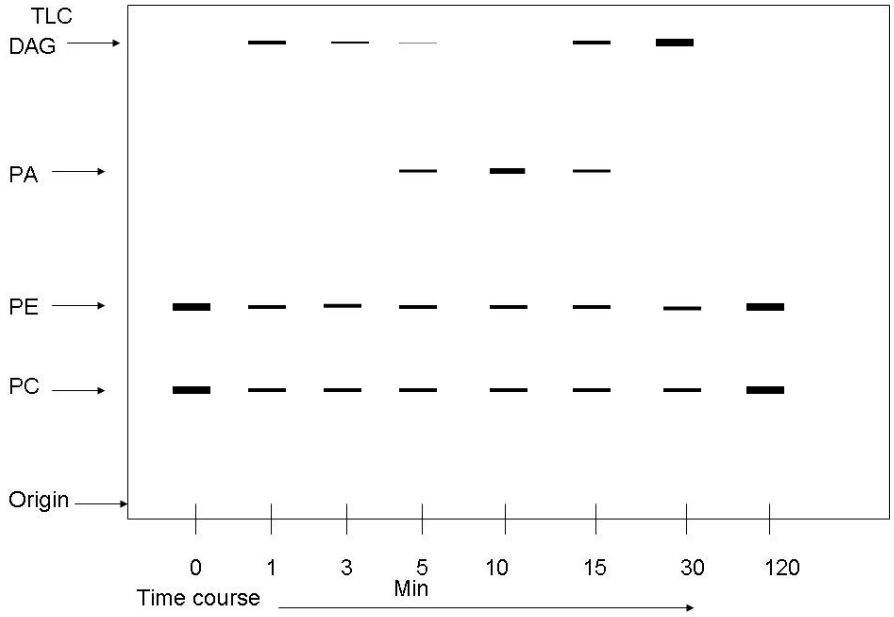
Fig 2. Mean (±SD) voriconazole plasma concentrations after single oral administration of 400 mg voriconazole in combination with placebo (*circles*) and 300 mg ritonavir twice daily (*squares*) to *CYP2C19*1/*1* (n = 8), *CYP2C19*1/*2* (n = 8), and *CYP2C19*2/*2* (n = 4) subjects.

Question B. Based on these data, formulate and rationalize a proposal regarding the mechanisms underlying the metabolic disposition of voriconazole.

Question C. Explain how you would go about testing the validity of your proposal.

Agonist-mediated activation of GPCRs, such as metabotropic glutamate receptors, lead to signal transduction events including production of lipid second messengers and lipid remodeling. You label a primary astrocyte with ^{3}H-stearic acid (18:0) for 6 hours in serum free media and then challenge for the indicated time with a stable glutamate analogue. You observe the changes in ^{3}H-lipid species as shown in Figure 1 (TLC below). Answer the following questions:

Fig. 1

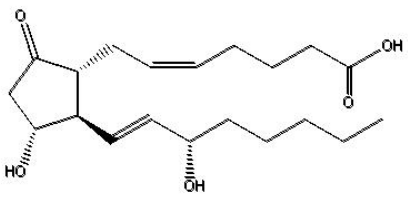


Question A:
On the TLC you observe a temporal change in two well known lipid second messenger, diacylglycerol (DAG) and phosphatidic acid (PA). Explain and discuss the most likely lipid signaling events to explain the temporal changes in these shown.

Question B:
What are the two products of PLC-beta enzyme activity and briefly discuss what each of these second messengers does in the cell?

Question C
In a parallel experiment using unlabelled cells, one of your colleagues identifies the species shown in Figure 2 (below) as also being generated following the activation of the metabotropic glutamate receptor. **What is this species?**

Fig. 2



You have recently identified ten unrelated families in which some family members display polydipsia and polyuria. Sequence analyses of genomic DNA from affected individuals revealed missense mutations in the coding region of the gene encoding the arginine vasopressin-neurophysin II (AVP-NPII) prohormone as shown in Figure 1. Two families had no mutations within the coding region of the AVP-NPII precursor and further analyses identified no mutations within the entire AVP-NPII gene.

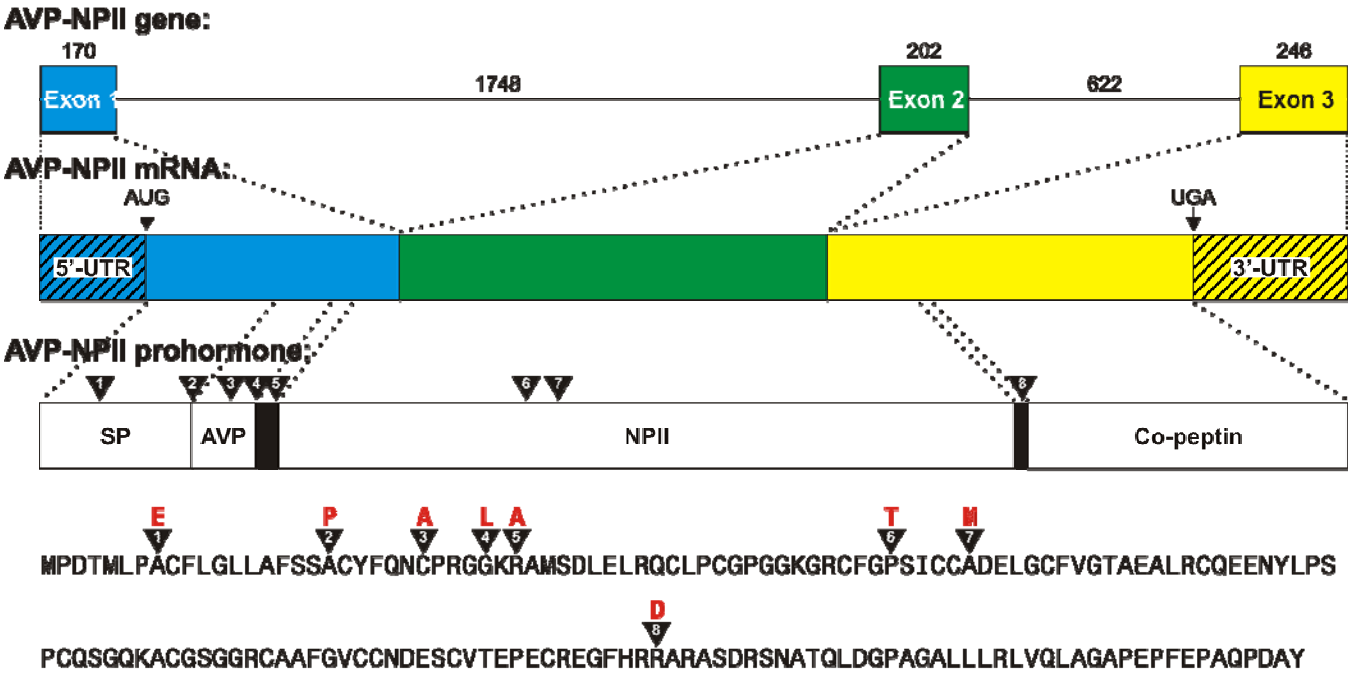


Figure 1. Identification of familial mutations in the human arginine vasopressin-neurophysin II gene. A schematic diagram of the organization of the human AVP-NPII gene is presented indicating the sizes (bp) of introns and exons along with the corresponding structure of the AVP-NPII mRNA and prohormone; UTR, untranslated region; SP, signal peptide, AVP, arginine vasopressin; NPII, neurophysin II. The relative position of identified mutations in the AVP-NPII prohormone for eight affected families is shown; the specific amino acid alterations resulting from these identified missense mutations are indicated in red.

1) Describe the physiological processes by which mutations in the AVP-NPII gene lead to polydipsia and polyuria.

2) For mutations 1, 3, 5 and 7 (indicated in Figure 1), describe how a mutation in these regions of the prohormone would affect AVP synthesis and function. Where possible, describe how these specific amino acid changes could result in decreased AVP synthesis and function.

3) For the two families in which no mutations were identified in the AVP-NPII gene, propose two additional candidate genes in which mutations could give rise to a similar phenotype. Rather than sequencing these genes directly, propose other experimental approaches that would support the selection of your candidates.