Department of Pharmacology

Qualifying Examination (Part I)

June 22-23, 2004

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) or 385-4396 (h).

BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

Question 1

In examining a colony of wild-type mice that are being used for your thesis studies, you notice a single male animal that has spontaneously developed excessive thirst (polydipsia) and excessive urine production (polyuria). You become quite interested in this phenotype (despite the protest of your thesis advisor) and begin to breed this "mutant" mouse to generate a colony with the pedigree indicated in Fig. 1. Based upon the observed pedigree, you hypothesize that the spontaneous mutation is X-linked and you vaguely recall that there are forms of *diabetes insipidus* resulting from mutations in the V2 vasopressin receptor (V2R) gene on the X chromosome.

DNA sequence analysis of the V2R cDNA from affected mice reveals a premature stop codon (E242X) near the normal carboxyl-terminus of the protein (Fig. 2A). To examine the functional consequences of this mutation, you assess the signaling properties of the V2R mutant by introducing either wild-type or mutant cDNAs into COS-7 cells, treating with arginine-vasopressin (AVP) and measuring changes in intracellular cAMP levels after 30 minutes (Fig. 2B).

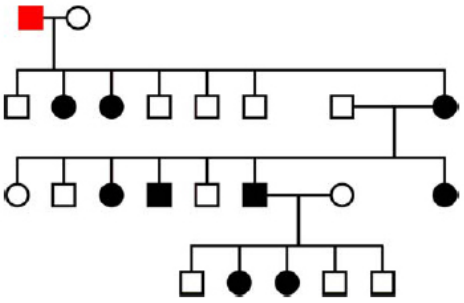


Figure 1. Pedigree of *diabetes insipidus* phenotype in mouse test colony. Healthy (open symbols) and affected (filled symbols) animals, and male (squares) and females (circles) are indicated; the original spontaneous mutant male is indicated in red.

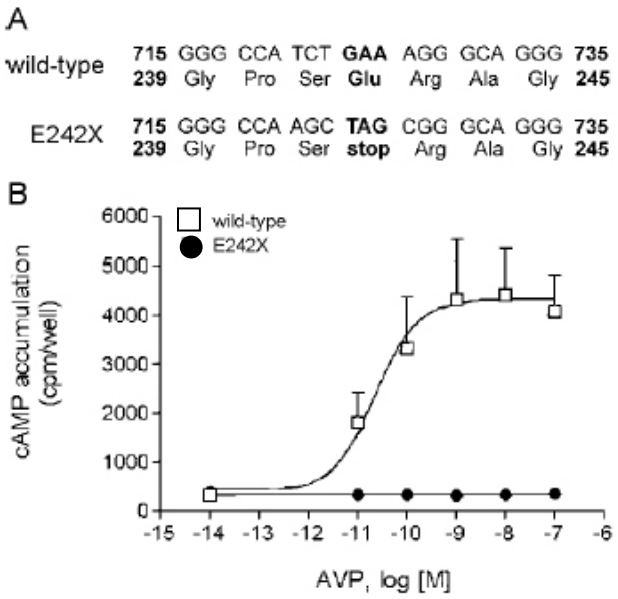


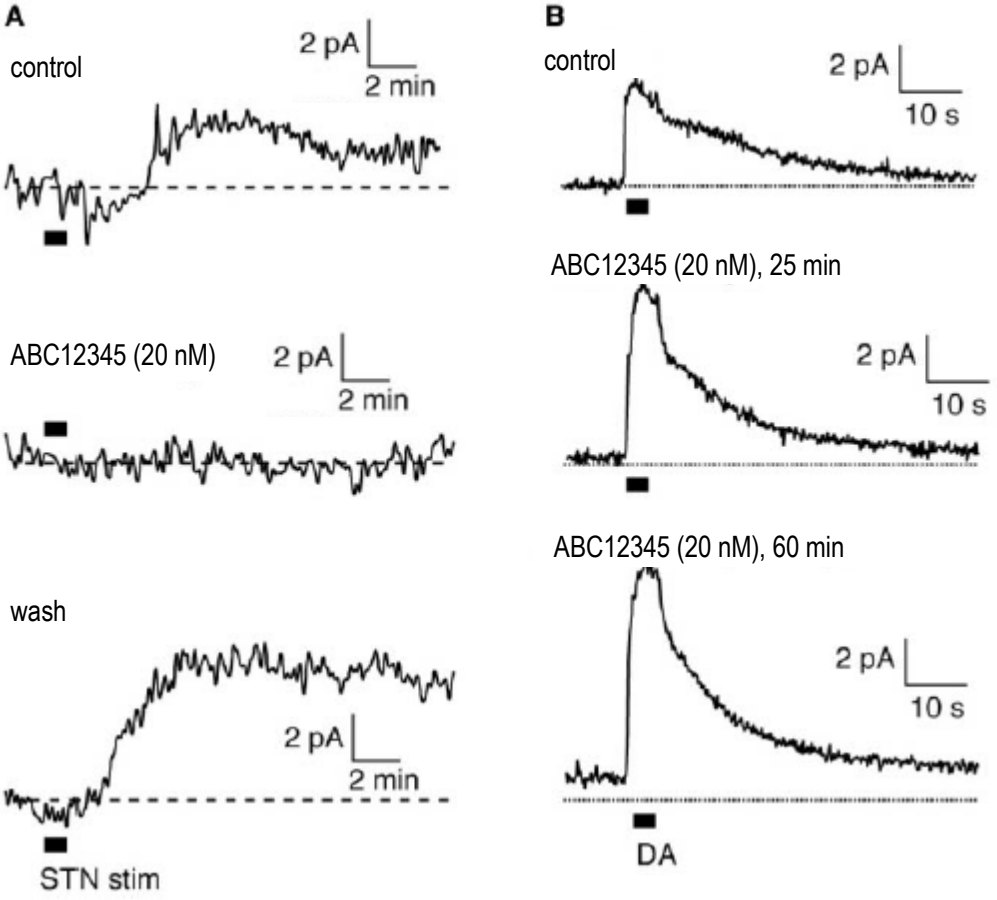
Figure 2. Functional alterations in V2R function in a spontaneous mouse mutant. **A)** Partial nucleotide and DNA sequence analysis of the V2R from wild-type and mutant (E242X) mice. **B)** Concentration response analysis for cAMP production in COS-7 cells transiently transfected with wild-type and mutant (E242X) V2R cDNAs

A) Based upon the pedigree in Fig. 1, explain your rationale for thinking that the spontaneous mutation is X-linked. What additional information can you derive from this pedigree?

B) Based upon all of the presented data, develop a hypothesis for a specific defect in the V2R (E242X) mutant that gives rise to the observed phenotype. Design a series of experiments to test your hypothesis.

Question 2

Stimulation of the subthalamic nucleus (STN) in a rat brain slice that also contains the substantia nigra (SN) reveals a stimulation-evoked elevation of extracellular dopamine (DA) several minutes later from dopaminergic neurons, as measured with carbon fiber amperometry (Fig 1A). In studies not shown, the elevation of extracellular DA is found to be blocked by a metabotropic glutamate receptor antagonist but not by antagonists of NMDA, AMPA or kainate receptors. Moreover, application to the slice of the cocaine-like substance ABC12345 blocks this elevation in extracellular DA in a reversible manner. In contrast, ABC12345 leads to an elevation of extracellular DA when DA is applied directly to the slice (Fig 1B), again monitored by carbon fiber amperometry. In other data not shown, a PKC antagonist blocks the ability of STN stimulation to elevate DA but does not influence glutamate release.



1. Considering monosynaptic innervation of the SN by glutamatergic projections from the STN, formulate a model whereby the delayed response triggered by STN stimulation, the glutamate receptor pharmacology and the properties of ABC12345 on evoked and applied DA can be explained. In your model, why does PKC blockade attenuate the elevation in extracellular DA.

2. What additional experiments, either in this preparation or in heterologous expression systems, would you like to see performed to validate this model?

Question 3

Familial hyperkalemic hypertension (a.k.a. Gordon's syndrome) is an autosomal dominant disease characterized by severe hypertension, elevated plasma potassium concentration (hyperkalemia) and normal renal function. Mutations in two related kinase genes, *WNK1* and *WNK4* (named for "with no lysine" at a key catalytic residue) cause the disease. Mutations in WNK1 are predicted to increase expression levels of this gene, while WNK4 mutations are predicted to cause loss-of-function. Both kinases are expressed in distal nephron segments (distal convoluted tubule through collecting duct).

One target of WNK4 is the Na-Cl co-transporter (NCCT) expressed in the distal convoluted tubule. WNK4 normally inhibits the activity of NCCT, and mutations relieve this inhibition leading to increased NaCl reabsorption, expansion of extracellular fluid volume, and hypertension. However, this does not explain high plasma potassium in this disease.

Recently, a group of really talented investigators examined effects of WNK4 on activity of a cloned human renal potassium channel (ROMK) heterologously expressed in *Xenopus* oocytes (Figure 1). They also determined that ROMK is expressed in thick ascending limb and cortical collecting duct cells.

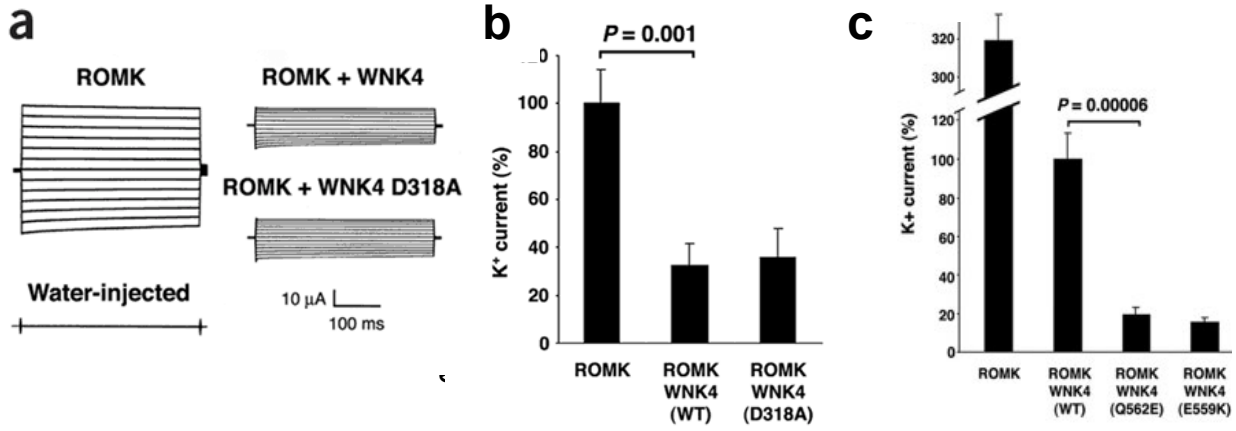


Figure 1 – Functional properties of ROMK co-expressed with WNK4. **a**) Representative voltage-clamp current recordings from *Xenopus* oocytes injected with mRNA encoding ROMK with or without WNK4. **b**) Current amplitudes (mean ± SEM; normalized to ROMK alone). The mutation D318A in WNK4 destroys its kinase activity. **c**) Current amplitudes (mean ± SEM; normalized to ROMK + WNK4-WT). WNK4 Q562E & E559K cause Gordon's syndrome.

Questions:

1. Propose two cellular/molecular mechanisms to explain these data. Your proposed mechanisms should involve **direct** and **indirect** effects of WNK4 on ROMK. Experiments designed to test your hypotheses should be described.

2. Develop physiological hypotheses to explain how *WNK4* mutations associated with Gordon's syndrome cause hyperkalemia. Explain how you might test these ideas.

Question 4

In a study to determine the interaction between a new drug X and verapamil – a drug administered as a racemate and resulting in cardiovascular effects, including prolongation of the PR-interval – the following data were obtained in 8 healthy subjects.

The experimental design involved the chronic administration of 120 mg racemic, unlabeled verapamil daily. On Days 5 to 16 the new drug X was given. Pharmacokinetic and pharmacodynamic measures were obtained on Days 4 and 16, when, in addition to the oral verapamil dose, 10 mg deuterated racemic verapamil was given simultaneously by the intravenous route.

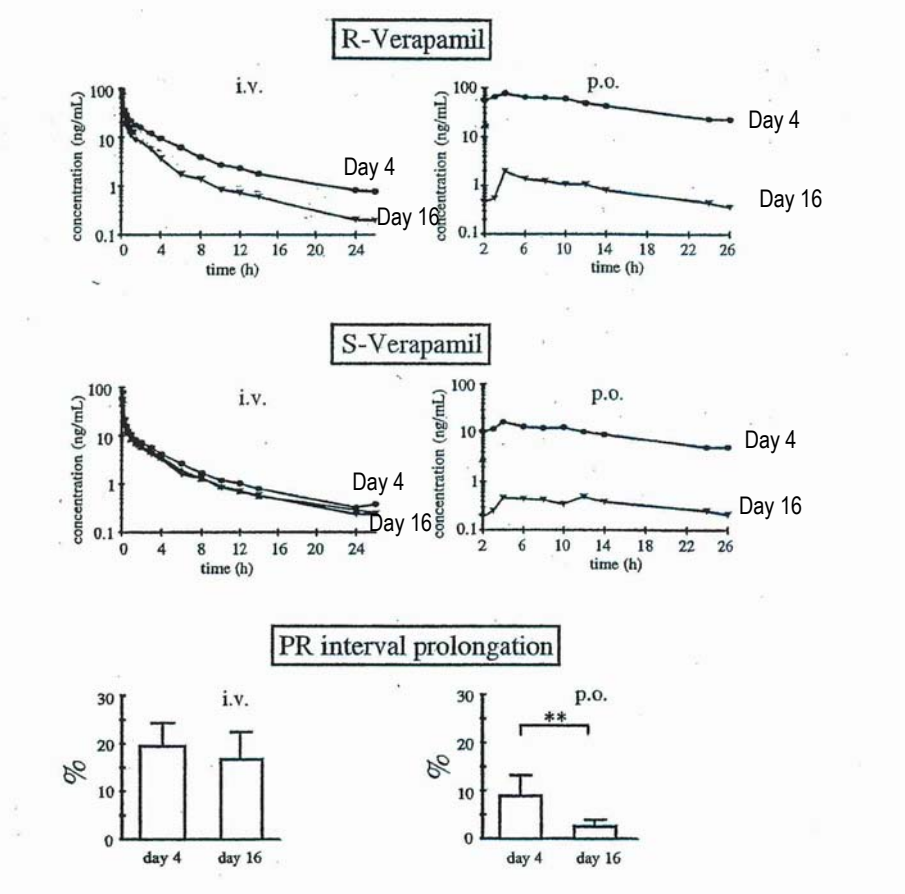


Figure 1. Mean serum concentration-time curves of S- and R-verapamil and maximum PR interval prolongation after co-administration of 10 mg deuterated S/R verapamil intravenously and of 120 mg unlabeled S/R-verapamil orally to eight volunteers before (day 4, ●) and during (day 16, ▼) treatment with new drug X (**P <.01). Pharmacokinetic data (with SD) are shown in Table 1.

Table 1. Pharmacokinetic parameters (mean ± 1 SD) of S- and R-verapamil after administration of 10 mg deuterated S/R-verapamil intravenously and of 120 mg unlabeled S/R-verapamil orally to eight volunteers before (day 4) and during (day 16) treatment with a new drug X.

	S-Verapamil		R-Verapamil	
	Day 4	Day 16	Day 4	Day 16
AUC_{iv} (ng·min/mL)	4,673* (881)	3,651 (568)	9,238* (2,353)	4,610 (638)
AUC_{po} (ng·min/mL)	9,098* (4,690)	299 (135)	43,480* (20,196)	859 (539)

*P <.001 day 4 *versus* day 16

- How do you account for the observed pharmacokinetic and pharmacodynamic observations?
- Describe experiments that would support your mechanistic interpretations.

Question 5

A Drosophila mutant, nos (no seven), was identified in a screen for UV light dependent phototaxis. Unlike wild-type flies, nos flies fail to display UV-dependent phototaxis. Further characterization of the nos mutant revealed that in the compound eye one class of photoreceptors, R7 cell, was missing.

It appears that the nos gene product is essential for differentiation of R7 cells, your colleagues went about to identify the nos gene. Based on the mapping information in the chromosome and the available genome sequence data, your colleagues quickly identified the nos gene. The nos gene product is a polypeptide that shows similarity to insulin-like growth factor.

Your mentor learned that you are "expert" in knowledge related to receptor protein tyrosine kinase signaling (as you took the receptor course the past summer), and asks your help with cloning of the putative nos receptor. Based on your understanding of RPTK, design a series of experiments to identify the unknown receptor that mediates the effect of the nos gene product in controlling differentiation of R7 cells.

Provide at least one experimental strategy that consists of a series of experiments to (1) identify the nos gene (product), (2) show relationship between the nos receptor and the known nos gene product, (3) show the consequence of the receptor activation in vivo and in vitro.

Question 6

Over the counter weight loss supplements represent 3 billion doses at a cost of over 7 billion dollars a year. Consumers are often drawn to herbal preparations due to their nonprescription status, direct to consumer advertising, and the perception that natural products are innately safe. However, perceived safety is often the result of a lack of data.

Go-Go is an example of such an herbal preparation. The purported active ingredient in Go-Go has been isolated, Compound X (CmpdX). Concerns about potential cardiovascular side effects of CmpdX led to the following randomized, double blind, crossover study in humans (Drug or Placebo---7 day washout---Drug or Placebo; half subjects start with drug, half with placebo) Subjects were administered 20 mg of CmpdX (the same dose contained in Go-Go) or placebo orally in a crossover fashion with a 7-day washout period between doses.

Data from this study is presented below.

Maximum Postdosing Electrocardiographic and Hemodynamic Values*

Parameter	Baseline	Placebo	CmpdX	P value
Heart Rate, beats per minute	79.2 (9.1)	73.8 (12.5)	71.5 (8.5)	.51
Systolic blood pressure, mm Hg	117.3 (7.3)	118.9 (9.6)	123.5 (10.9)	.009
Diastolic blood pressure, mm Hg	73.9 (8.3)	73.7 (6.43)	75.1 (6.96)	.22
Stroke Index, mL/m^2	38.7 (4.3)	39.3 (4.76)	42.4 (6.88)	.003
Ejection Fraction	-	No change	30% increase	.001

*Data are presented as mean (standard deviation)

In addition, all subjects receiving CmpdX reported nonspecific symptoms such as jitteriness and queasiness.

1. Your supervisor hypothesizes that CmpdX is a sympathomimetic. Review the subject data and explain how the data are consistent or inconsistent with this hypothesis. Design *in vitro* and *in vivo* experiments to test the hypothesis that CmpdX is a sympathomimetic.

Additional analysis yielded the following data.

Maximum Postdosing Electrocardiographic Values*

Parameter	Baseline	Placebo	CmpdX	P value
Average Interval (milliseconds)				
PR	177.2 (26.1)	182.3 (28.5)	184.3 (22.0)	.74
QRS	94.5 (28.4)	90.5 (9.7)	94.0 (11.1)	.18
QT	342.6 (20.9)	348.9 (30.9)	372.6 (22.0)	.005

*Data are presented as mean (standard deviation)

2. Assuming the effect noted on the electrocardiogram is due to a direct action on the heart, state a hypothesis to explain the electrocardiographic data. Outline experiments to test your hypothesis. Based on the data above, do you have any concerns about the safe use of CmpdX?

Question 7

The signaling unit for G protein-coupled receptors (GPCRs) has been classically defined as consisting of the receptor, a heterotrimeric G protein, and enzymatic or channel effectors controlling second messenger production or ion fluxes. More recently it has become clear that GPCRs may also stimulate a variety of signaling systems that regulate cell proliferation and differentiation, including the extracellular signal-regulated kinase 1/2 (ERK1/2). In the experiments depicted below, a group of investigators used HEK293 fibroblasts stably expressing the β_2 adrenergic receptor (β_2AR) to compare the effects of different β_2AR ligands on the classical Gαs-mediated activation of the adenylyl cyclase (AC) pathway and the MAPK cascade.

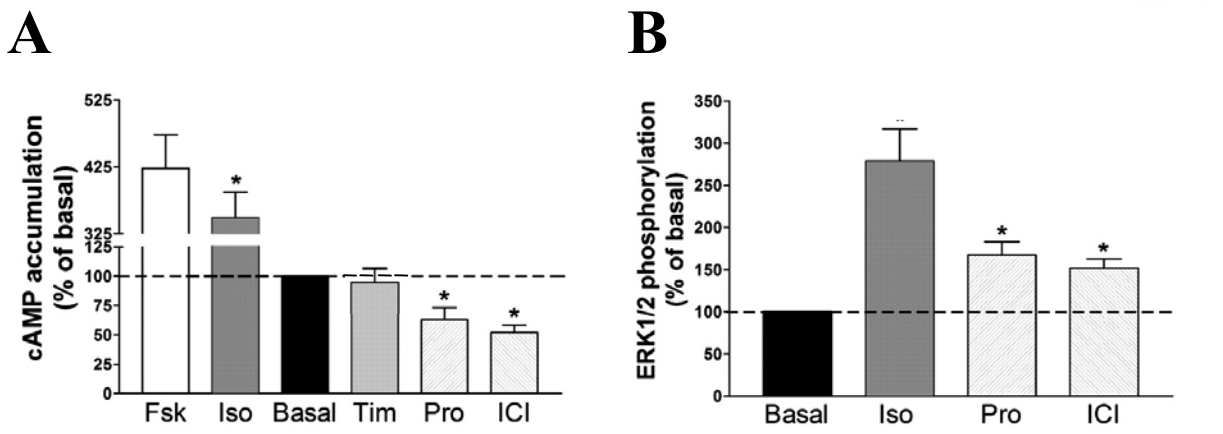


Figure 1. Signaling efficacy of β_2AR ligands. HEK293 cells stably expressing the β_2AR were treated with the indicated drugs (1 μM), and cAMP accumulation (*A*) and ERK1/2 phosphorylation (*B*) were assessed after a 5-min incubation. Results are expressed as percentage of the value obtained in nonstimulated cells and represent the mean +/- SEM of five to eight independent experiments. *, $P < 0.01$ compared with basal. FSK, forskolin; Iso, isoproterenol; Tim, timoolol; Pro, propranolol; ICI, ICI118551

1. What conclusions can be drawn from these data? Propose a mechanism to explain the differential effects of Iso versus Pro/ICI on AC and MAPK activity.

2. Design two independent experiments to test your model.