Department of Pharmacology

Qualifying Examination (Part I)

July 20-24, 2009

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.

BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

Qualifying Exam – July 2009
Question 1

AMPA glutamate receptors of different subunit composition are expressed in the spinal cord. This study investigated the role of the GluR2 AMPA receptor subunit in persistent inflammatory pain. GluR2 subunit determines the properties of the AMPA receptor including Ca^{2+} permeability (GluR2-containing AMPA receptors are Ca^{2+}-impermeable, whereas AMPA receptors lacking GluR2 are permeable for Ca^{2+}).

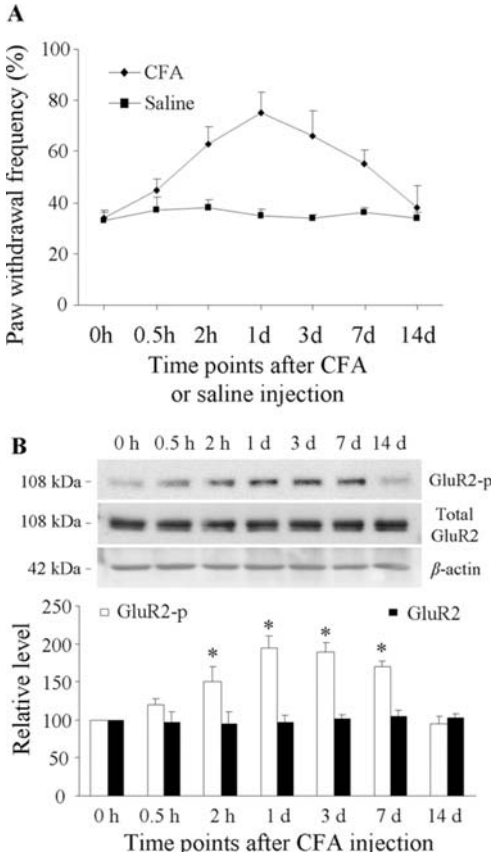

Figure 1. Injection of complete Freund's adjuvant (CFA) into the hind paw induces inflammation and nociceptive hypersensitivity, which is seen as increased frequency of the paw withdrawal in response to repeated mechanical stimulation. (**A**) The time course of paw withdrawal frequency after CFA or saline injections. (B) Corresponding time-dependent changes in the level of GluR2 phosphorylation at Ser 880. No changes in the total level of GluR2 were observed (middle blot). β-Actin is shown as loading control. The graph shows quantification for the level of phosphorylated GluR2 (GluR2-p). *, $p<0.05$ as compared to 0 h .

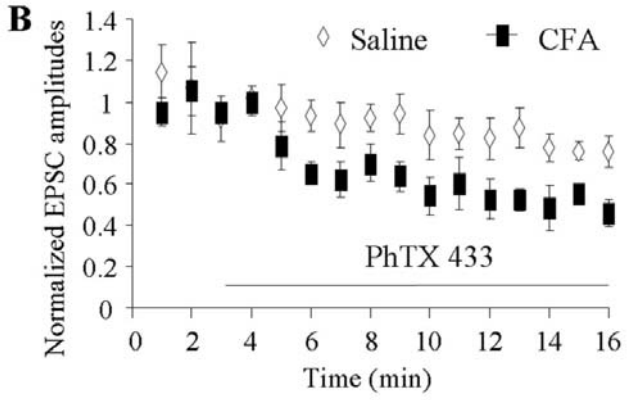

Figure 2. The graph shows AMPA receptor-mediated EPSCs (excitatory postsynaptic currents) recorded in the spinal cord neurons in animals treated with saline or CFA (1 day after the injections) in the presence of PhTX433, a selective blocker of **Ca^{2+}-permeable** AMPA receptors. In the presence of PhTX433 the difference in EPSC between saline and CFA treated animals was statistically significant (however, there was no difference in the amplitude of the AMPA-mediated EPSCs between the saline and CFA-treated groups before the PhTX433 administration).

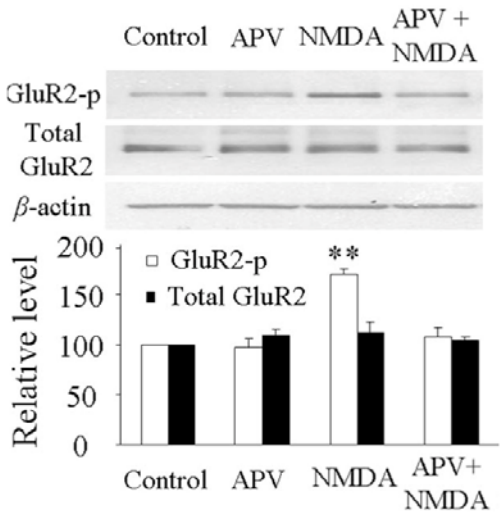

Figure 3. The effect of NMDA receptor stimulation on the level of GluR2 phosphorylation at Ser 880. The *in vitro* culture of spinal cord neurons was treated with NMDA receptor agonist (NMDA), NMDA antagonist (APV), or a combination (NMDA+APV). The Western blot shows the level of phosphorylated GluR2 (GluR2-p; upper panel) and total GluR2 (middle panel). The graph below shows quantification of the Western blot data for both total and phosphorylated GluR2. **, p<0.01 as compared to control.

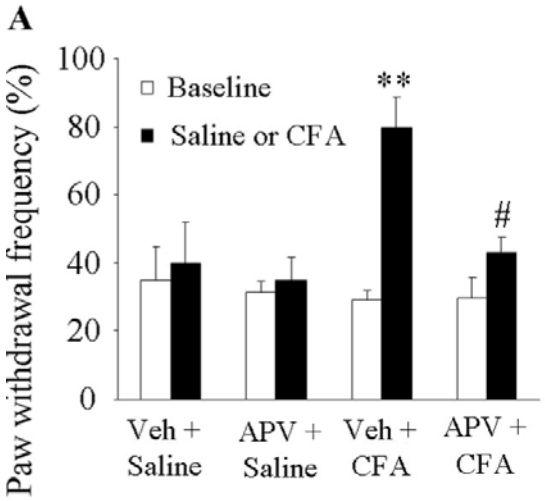

Figure 4. The effect of NMDA receptor inhibition on mechanical hypersensitivity induced by CFA in mice. The NMDA receptor antagonist APV was infused into the spinal cord for 1 day beginning 1 h before the administration of CFA injection. The behavior (paw withdrawal frequency in response to repeated mechanical stimulation) was measured 1 d after CFA administration. **, p<0.01 as compared to Vehicle+Saline; #, p<0.05 as compared to Vehicle+CFA.

(A) Describe the classification of glutamate receptors. Describe the structure of ionotropic glutamate receptors. Summarize functional differences between the AMPA and NMDA receptor subtypes.

(B) Describe the data. Propose a mechanistic model of the nociceptive hypersensitivity in the inflammatory pain and the role of glutamate receptors in this phenomenon that accounts for the data. Suggest experiments to test the model.

Midazolam is classified as an intermediate-to-high clearance drug, which is completely metabolized in the liver. The pharmacokinetics of midazolam were studied in a patient with refractory status epilepticus treated with extraordinary doses of midazolam and who was treated concomitantly with phenytoin and carbamazepine over a period of 2 months in intensive care. The patient received very high doses (at times up to 4 mg/min) of intravenous midazolam (1 – 2.5 mg total dose i.v. is used for sedation in normal healthy subjects).

The kinetics of midazolam (MDZ) were assessed at steady state (infusion of 1 mg/min for days). Plasma MDZ levels were measured by LC-MS and the free (unbound) fraction measured after ultrafiltration of plasma. The dose was tapered down over three days, the infusion stopped, and the levels monitored during the period of elimination.

Table 1 summarizes the PK data obtained in the patient, compared with values taken from the literature (normals and patients).

TABLE 1. Pharmacokinetic Parameters of Midazolam*

Parameter	Patient	Values from Literature
Css (ng/mL)	860	116–2817
fu (%)	58	3–6
Clss (mL/min)	1160	240–620
Clint (mL/min/kg)	138	13.3
Vss (L/kg)	33	1.0–3.3
Ehep	0.76	0.25–0.44
ke (h–1)	0.03	0.09–0.19
t1/2 (h)	24	1.8–3.5, 3.7–9.4

*Steady-state values were determined at a midazolam dose rate of 1 mg/min, i.v.

Css, steady-state plasma concentration; fu, free fraction in plasma; Clss, total blood clearance in steady state; Clint, intrinsic hepatic clearance; Vss, volume of distribution in steady state; Ehep, hepatic extraction ratio; ke, terminal elimination constant; t1/2, terminal elimination half-life.
Comparative values are values published from studies with healthy volunteers or with patients.

Additional information that you are not required to derive: Because the renal clearance of midazolam is negligible, Cl_{hep} equals Clss. By using an estimated value for liver blood flow (Q), the intrinsic hepatic clearance was calculated (using an equation not needed here). Finally, taking into account that Cl_{hep} = Clss, the hepatic extraction ratio (Ehep) of midazolam was calculated using:

$$E_{hep} = \frac{Clss}{Q}$$

Question

1. Give an overview of the PK data and present a hypothesis that can explain the disposition of midazolam in this patient, in comparison to normal healthy subjects.

2. How could your hypothesis/explanations be tested?

Qualifying Exam – July 2009
Question 3

A young couple is seen in a fertility clinic because they have been unable to conceive. Examination of the man reveals gynecomastia (enlarged breast tissue), and he is found to have a very low sperm count.

Based on your knowledge of gonadal endocrinology, you hypothesize that the patient has a defect in either A) testosterone production or B) sensitivity to testosterone.

1. For each of these possible explanations, indicate the likely result in the measured level of the hormone listed (high, low, or no change). If there is more than one possibility, note this and explain.

	Testosterone	**LH**	**FSH**
A			
B			

2. Choose either condition A or B and indicate the potential molecular mechanisms of the defect and how you would test for them.

3. List 3 additional clinical manifestations that might be seen for both (A) and (B). Specify when manifestations indicate a defect in testosterone production or sensitivity during a particular developmental period.

Background. You are asked by the CDC to serve as an expert consultant in cellular signaling. An outbreak in what is thought to be a food borne pathogen has occurred. The source of the pathogen has not been identified and appears to come from a variety of sources. A multidisciplinary team of experts has been looking at samples for several days and reached the following preliminary conclusions:

Preliminary Findings.
-The pathogen is a gram-positive bacteria, but appears to be some new strain as standard ELISAs have failed to identify the specific infectious agent.
-The primary cell infected is macrophages.
-The life cycle of the pathogen consists of the following- internalization of the agent into the macrophage phagocytes, escape from the phagosome (3-6 hrs post infection), intracellular mobility by means of bacterially induced host actin polymerization, and cell-to-cell spread.

A series of studies shows that "escape from the phagosome" is the critical step in the life cycle of the infectious agent and subsequent studies are conducted to characterize that process. Preliminary analysis suggests that "phagosome escape" begins around 3 hrs post-infection and peaks around the 6 hr time point, although the process continues throughout the infection life cycle.

Intracellular cAMP levels are normal throughout the time course until 18hrs when there are general signs of macrophage degeneration. Intracellular calcium levels are not perturbed in infected macrophages. Both resting and patterns of calcium mobilization following stimulation with ligands are the same as in uninfected cells. However, it was noted that treatment of macrophages with calcium channel blockers lowered the resting levels of calcium and this substantially inhibited the "phagosome escape" step (suggesting that calcium is somehow necessary).

Extensive analysis of the bacterial proteins in the isolated phagosomes indicates that two factors appear to be secreted by the infectious agent at the onset of the "escape from phagosome" phase. The first, **Factor A**, is generated in large quantities and has been identified as resembling other known bacterial proteins that are pore-forming cytolysin listerolysin O (i.e., proteins that form small pores in intracellular vesicles). The second, **Factor B**, is generated in very small quantities. On gel filtration chromatography **Factor B** co-elutes with proteins that have apparent molecular weights around 35kD. The chromatographic properties of these factors are sensitive to heat and proteases.

Intact macrophages and phagocytic vesicles are isolated from affected vs non-affected people. A number of biochemical and pharmacological parameters were measured. Naïve macrophages are exposed to the infectious agent over an established time course. The lipid composition is shown to systematically change in the infected patients as illustrated in Fig 1. By contrast in a parallel set of the uninfected these same lipid species do not change over the same time course. In addition, very small quantities of isolated **Factor B** can be added directly to phagosomes from uninfected macrophages and the same pattern of lipid changes are observed.

Question 1- What is the most parsimonious identification of Factor B being generated by the infectious agent? Describe in detail how your answer explains the pattern of species changes in Fig 1.

A series of experimental drugs were tested in a screen on the macrophages. Several interesting observations were noted. Although the mechanism is unknown an experimental antibiotic apparently inhibited transcription of **Factor B**. Factor A was still produced, but in the absence of **Factor B** the "phagosome escape" step appears to have been blocked, new infectious agent was not produced, and macrophages appeared healthy at the 6hr time point. However, at the 9hr time point a new **Factor (C)** appeared and the same set of pathological events followed with a delayed onset. **Factor C** has an apparent molecular weight of 29kD and further examination of properties (e.g., isoelectric point, chromatography, protease sensitivity) strongly suggests it is distinct from **Factor B**. It was the consensus of the CDC group that **Factor C** was some type of resistance factor that was transcribed in response to macrophages that showed initial resistance. The lipid analysis of phagosomes where **Factor C** was expressed had a differential pattern of changes illustrated in **Fig 2**.

Question 2- What is the most parsimonious identification of Factor C? Justify your answer based on the data.

Subsequent studies suggested that some host cell protein is essential to the "phagosome escape" step. A host cell protein of approximately 75kD was observed to translocate to the recycling endosome compartment shortly after the initial appearance of either **Factors B or C** at their respective time points. Several parameters were noted regarding the bacterial factors and translocation of the host cell protein, including (i) the bacterial factors were heat-sensitive and addition of heat inactivated factors resulted in no lipid changes nor translocation of the 75kD host protein, (ii) compounds that lower intracellular calcium prevented translocation, and (iii) treatment of normal macrophages with phorbol esters did not cause the exact same lipid changes as the bacterial factors but did result in the translocation of what appeared to be the same 75kD protein.

Question 3- What western blot should you initially run to test your hypothesis as to the identity of the 75kD host protein? Explain how these two different bacterial factors are regulating the same host cell protein.

Qualifying Exam – July 2009
Question 4

Figure 1 (Factor B)

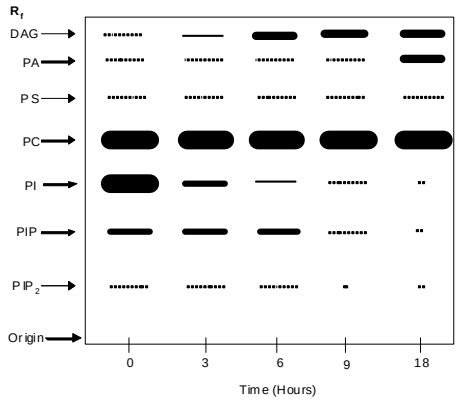

Figure 2 (Factor C)

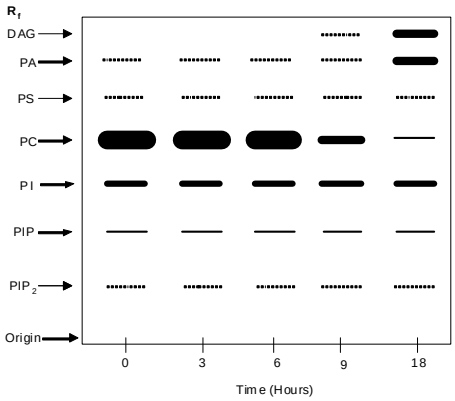

Fig 1 and 2. Phagocytic vesicles were rapidly isolated at the indicated times (hrs post-infection). Lipids were extracted using standard methodologies and analyzed by thin-layer chromatography. Chemically defined lipid standards were shown to migrate at the indicated relative front (Rf) values. The intensities of the bands may be assumed to be linear and differences represent real changes in the amounts of lipid species. The following lipid species were measured: diacylglycerol (DAG), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), polyphosphatidylinositols (PIP and PIP_2). No changes were observed in phosphatidylethanolamine, phosphatdiylglycerol, or cardiolipin (not shown)

You are evaluating several small molecules as potential anti-hypertensive therapies. Initial screens demonstrate several compounds that provide dose dependent decreases in blood pressure after oral administration. Screening of these compounds reveals a group of compounds that lack activity at β-adrenergic receptors and Ca^{++} channels. Further screening of these compounds fails to identify interactions with known targets of diuretics in the tubules of the kidney.

To further evaluate these compounds you administer them to conscious, freely moving marmosets and measure blood pressure continuously by telemetry. Representative data for one such compound (VU-14U2C) is depicted in Figure 1. To address the mechanism of action of these compounds you measure Bradykinin, Angiotensin I, and Angiotensin II levels in marmosets given each compound twice a day for two weeks. Based on the results of these experiments, the 7 compounds were divided into one of three groups (Table 1).

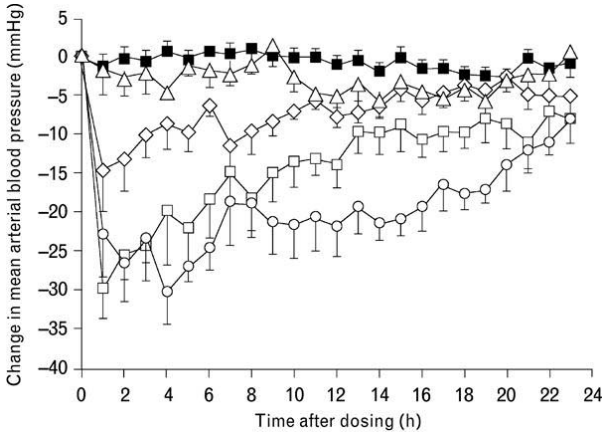

Figure 1. Changes in mean arterial blood pressure (BP) following administration of a single oral dose of VU-14U2C, 0.3 mg/kg (open triangles), 1 mg/kg (open diamonds), 3 mg/kg (open squares) or 10 mg/kg (open circles), or vehicle control (filled squares) to sodium-depleted marmosets. BP was continuously measured by telemetry in conscious, freely-moving marmosets as described in Methods. Data presented as mean ± standard error of the mean. Mean baseline arterial BP values in each group as follows: vehicle (BP, 80 ± 2 mmHg); 0.3 mg/kg VU-14U2C (BP, 78 ± 4 mmHg); 1 mg/kg VU-14U2C (BP, 80 ± 4 mmHg); 3 mg/kg VU-14U2C (BP, 81 ± 6 mmHg); and 10 mg/kg VU-14U2C (BP, 73 ± 5 mmHg).

Table 1. Measurements of Bradykinin, Angiotensin 1, and Angiotensin II levels.*

	Group 1	Group 2	Group 3 (includes VU-145U2C)
Bradykinin	Increased	No change	No change
Angiotensin I	Increased	Increased	Decreased
Angiotensin II	Decreased	Increased	Decreased

*All changes in levels are when compared to control

State hypotheses that address the possible mechanisms of action of compounds in each group. Outline strategies using both *in vitro* and *in vivo* approaches to test your hypotheses.

Given that there is more than one compound in each group, outline strategies to compare the activities of each compound within a group.

Qualifying Exam – July 2009
Question 6

A recent publication in *Int J Pharm Qual Exams* by Kühldudes, *et al*. demonstrated that adenosine-mediated afferent arteriole vasoconstriction can be modulation by low concentrations of angiotensin-II (Ang-II). Their experimental system utilized isolated, perfused rat kidney afferent glomerular arterioles monitored for luminal diameter as a proxy for the degree of vascular tone, and intracellular Ca^{2+} levels in vascular smooth muscle cells by using a fluorescent reporter (Fura-2). All drugs and hormones were administered in the bath solution. The following observations were made.

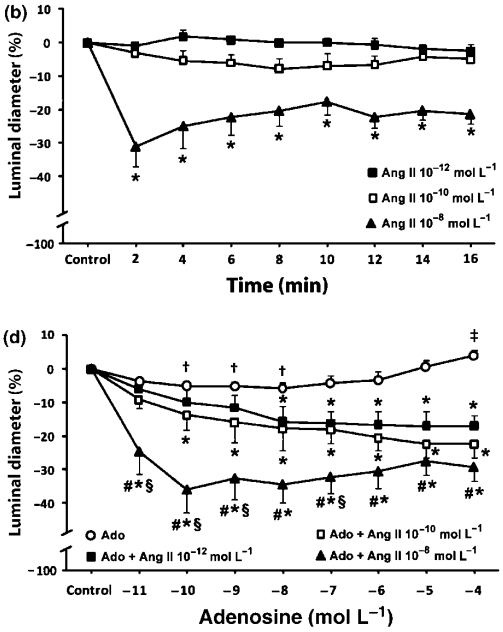

Figure 1 – (b) Time course of the relative change in luminal diameter (compared with pre-treatment control period) in response to treatment with three different Ang-II concentrations. * $p < 0.05$ compared with 10^{-12} M.

(d) Effect of adenosine (Ado) with or without Ang-II on relative luminal diameter. Symbols represent significant differences ($p < 0.05$) for the following comparisons:

* comparisons with Ado alone
# comparisons with Ado + Ang-II 10^{-12} M
† significant contraction compared with control
‡ significant dilation compared with control
§ comparisons with Ado + Ang-II 10^{-10} M

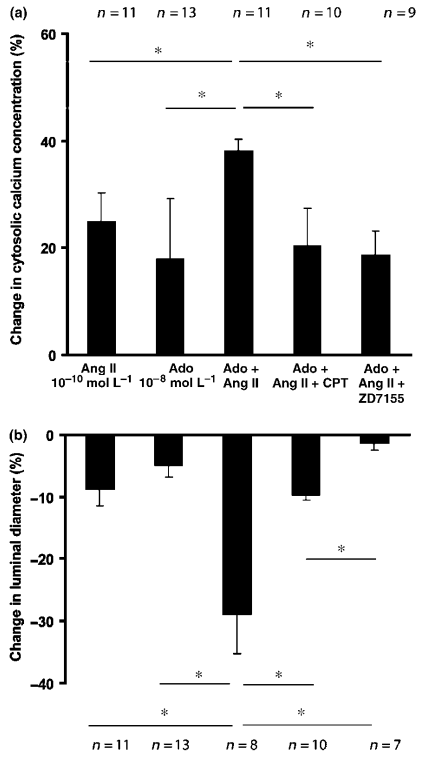

Figure 2 – (a) Relative change in cytosolic Ca^{2+} compared with pre-treatment control. CPT is a selective adenosine A_1 receptor antagonist. ZD7155 is a selective Ang-II AT_1 receptor antagonist.

(b) Relative change in luminal diameter compared with pre-treatment control.

*$p < 0.05$

Qualifying Exam – July 2009
Question 6

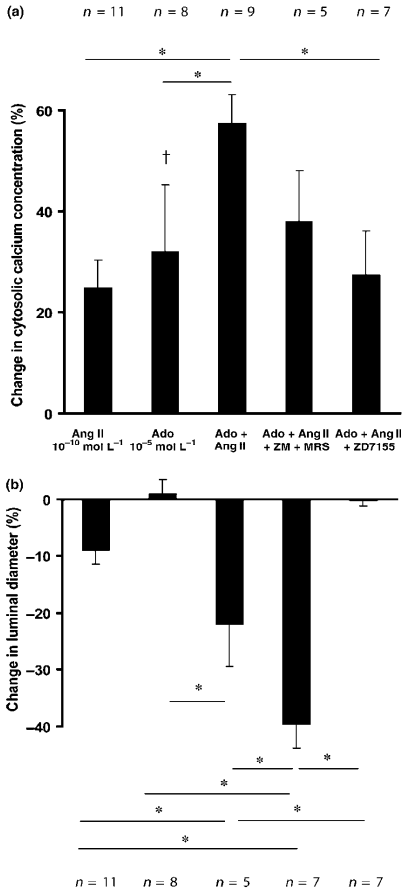

Figure 3 – (a) Relative change in cytosolic Ca^{2+} compared with pre-treatment control. ZM and MRS are selective adenosine A_{2a} and A_{2b} receptor antagonists, respectively. ZD7155 is a selective Ang-II AT_1 receptor antagonist.

(b) Relative change in luminal diameter compared with pre-treatment control.

*$p < 0.05$

Questions:

1. Explain the physiological importance for the effects of adenosine and Ang-II on renal function.

2. Provide a hypothesis that accounts for the combined effects of adenosine and Ang-II on the afferent arteriole. How would you test your idea?

3. Explain why 10^{-4} M adenosine causes vasodilation (Figure 1).

Qualifying Exam – July 2009
Question 7

G protein-coupled receptor kinase-5 (GRK5) deficiency has recently been linked to early Alzheimer's disease (AD), but the mechanism by which GRK deficiency may contribute to AD pathogenesis has remained elusive. Accumulating evidence indicates that reduced hippocampal acetylcholine (ACh) release and cholinergic hypofunction is one of the key neurochemical changes in AD. In hippocampal memory circuits, the M2 and M4 muscarinic acetylcholine receptors (M2R and M4R) are the primary presynaptic autoreceptors that inhibit ACh release. The current study was undertaken to investigate the impact of GRK5 deficiency on ACh release and desensitization of mAChR subtypes using GRK5 deficient models both *in vitro* and in hippocampal slices from GRK5 knockout (GRK5KO) mice.

To investigate the impact of GRK5 on muscarinic receptors, the authors of this study initially exploited HT22 cells, which are immortalized murine hippocampal neuronal precursor cells that possess functional cholinergic neuronal properties. HT22 cell lines stably expressing empty vector (EV), wild type GRK5 (wtGRK5), or a dominant-negative form of GRK5 (dnGRK5) were established. Western blot data confirmed that the exogenous wtGRK5 and dnGRK5 were expressed at similar levels in the corresponding cell lines; the expression of muscarinic receptors (M1, M2, M4) were also found to be similar among the cell lines (data not shown). After the *in vitro* models were established, the investigators examined high K^+-evoked ACh release from these cell lines (Fig. 1). The authors also examined agonist-induced internalization of mAChRs in these cell lines (Fig. 2).

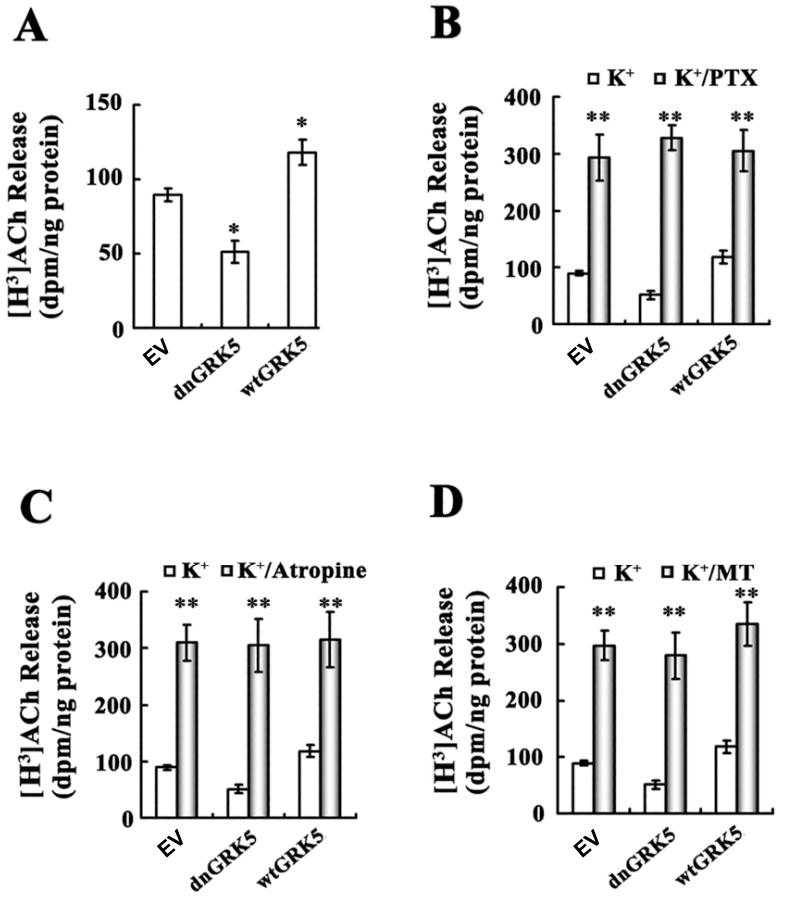

Figure 1. Effects of pertussis toxin and muscarinic antagonists on high potassium-evoked ACh release. *A*, high K^+-evoked [^{3}H]ACh release in the EV, dnGRK5, and wtGRK5 cell lines. A single optimal concentration of potassium (50 mM) was used to evoke the [^{3}H]ACh release. **P*<0.05, as compared to the EV cells. *B*, *C*, and *D* show the effects of PTX, atropine, and methoctramine tetrahydrochloride (MT) on high K^+-evoked [^{3}H]ACh release in the EV, dnGRK5, and wtGRK5 cell lines. Atropine is a non-selective muscarinic antagonist and MT is a selective M2/M4 antagonist. The open bars represent high K^+-evoked [^{3}H]ACh release in the three cell lines and are the same as depicted in *A*; the shaded bars represent high K^+-evoked [^{3}H]ACh release following treatment with indicated agent. ***P*<0.001, as compared to the potassium-alone treated cells.

Qualifying Exam – July 2009
Question 7

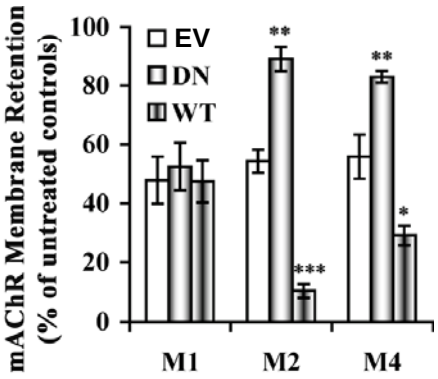

Figure 2. Membrane retention of M1, M2, and M4 receptors in the three HT22 cell lines following agonist treatment. The EV, dnGRK5 (DN), and wtGRK5 (WT) cells were treated with a saturating concentration of the non-selective muscarinic agonist oxotremorine for 20 min. Plasma membrane fractions were prepared and subjected to Western analysis using M1-, M2-, and M4-specific antibodies. Depicted in the figure is the quantitation of the Western blotting data. The data were expressed as the percentage of the receptors remaining in membrane fraction after oxotremorine treatment (the treated divided by the untreated). **P*<0.05, ***P*<0.01, and ****P*<0.001, as compared to the corresponding EV control cells.

To validate the physiological relevance of their *in vitro* findings, the investigators performed experiments in an *ex vivo* setting, using acute hippocampal slice cultures from young adult GRK5KO mice and wild type (WT) littermates. Western analysis of hippocampal slices from GRK5KO and WT littermates revealed similar levels of cholinergic and synaptic markers in these animals (data not shown), thus demonstrating that the hippocampal cholinergic system in the GRK5KO mice is normal at the structural level. To determine cholinergic function, the investigators measured high K^+-evoked acetylcholine release from the hippocampal slices (Fig. 3).

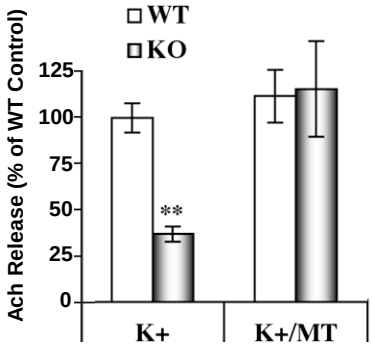

Figure 3. High potassium-evoked ACh release in hippocampal slices of WT and GRK5KO mice. The high K^+-evoked [^{3}H]ACh release from hippocampal slices of 3-month old GRK5$^{-/-}$ (KO, *n*=3) and GRK5$^{+/+}$ (WT, *n*=5) mice were measured using a standard assay. Slices were pretreated with a saturating concentration of carbachol to desensitize the muscarinic receptors, and then stimulated with high potassium (K+) in the presence or absence of MT (a selective M2/M4 antagonist). ***P*<0.01, as compared to K^+-alone treated WT controls.

QUESTION:

A) Describe the molecular components of the signaling pathway that is under study here. Include a schematic.

B) Develop a hypothesis to explain these data. Describe how the experimental results support your hypothesis.

C) Design two additional, independent experiments to test your hypothesis.