Department of Pharmacology

Qualifying Examination (Part I)

July 31 – August 3, 2007

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:

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BEST WISHES FOR YOUR SUCCESSFUL COMPLETION OF THE EXAMINATION!

The reinforcing properties of cocaine are well known to involve interactions of the drug with CNS dopamine (DA) signaling. A number of scientists however have pointed to other systems mediating aspects of cocaine-induced reward. Evidence cited for such a stance includes the fact that DA transporter (DAT) knockout mice exhibit behavioral responses associated with cocaine reinforcement, such as the conditioned place-preference test (CPP), Figure 1, that can be mimicked by administration of fluoxetine (Prozac™).

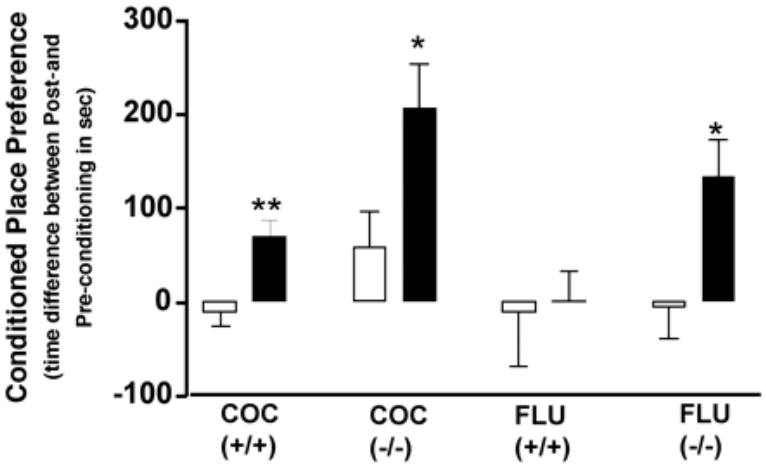


Figure 1: Reinforcing effect of cocaine and fluoxetine in DAT-KO mice. Place preference conditioning of wild-type (+/+) and DAT-KO (-/-) mice to saline (open bars) or to cocaine (COC, 20 mg/kg i.p.) or fluoxetine (FLU, 15 mg/kg i.p.) (filled bars) administration over a four day period. Time scores represent differences between post and pre-conditioning time spent in the saline or drug-paired environment. *, P<.05, **P<.01 comparing drug and saline.

1. **What neurotransmitter system(s) and molecular target(s) are examined via the action of fluoxetine in the CPP test in DAT KO mice? How does the lack of a CPP response for fluoxetine in wildtype (+/+) mice relate to fluoxetine's clinical utility?**

2. **Give two tests that you could utilize to confirm a role for the neurotransmitter system described (in question 1 above) in fluoxetine's positive action in the CPP test in DAT KO mice.**

To investigate further the mechanism of action of fluoxetine in the DAT KO mice, you perform microdialysis experiments, inserting a sampling probe into the nucleus accumbens (NAc) of wildtype mice (open circles) and DAT KO mice (solid circles) (Fig 2). You monitor dialysate DA levels as a function of time before and after injection of cocaine (20 mg/kg i.p.), fluoxetine (15 mg/kg, i.p.), the SERT-selective antagonist s-citalopram (Lexapro™, 10 mg/kg i.p.) or the NET-selective tricyclic agent desipramine, (10 mg/kg i.p.) In Fig 2., for comparison between genotypes, baseline values of extracellular DA have been normalized to 100%. In separate studies desipramine also does not induce a significant response in the CPP test (not shown).

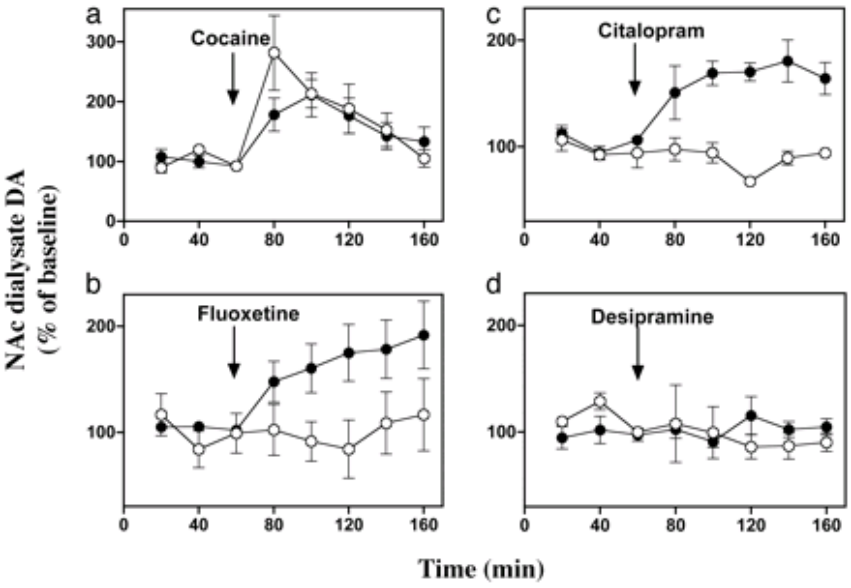


Figure 2: Impact of drug administration on extracellular DA levels in wt and DAT KO mice.

3. **How do the actions of citalopram and desipramine in the DAT KO relate to your hypothesis regarding the neurochemical system participating in fluoxetine's ability to induce CPP?**

4. **Propose a site of action to support the ability of fluoxetine (and citalopram) to elevate nucleus accumbens extracellular DA in the DAT KO mice and thereby trigger CPP. How might you test this mechanism? In your model, why does fluoxetine not trigger elevated extracellular DA or CPP in the wildtype mice.**

Three drugs were administered orally to twenty subjects, before and after they took daily doses of the herbal anti-depression remedy, St John's wort (SJW), for 14 days. The three drugs were given at intervals, so that the previous one was largely eliminated before the next was given:

Midazolam at t = 0, given orally (4mg) **and** intravenously (1mg of heavy isotope-labelled $_{15}N_3$-midazolam). Plasma concentrations were determined by LC-MS.
Fexofenadine at t = 4 hr (180 mg orally). Plasma concentrations measured by HPLC.
Cyclosporine at 24 hr (2.5 mg/kg mg orally). Whole blood levels measured by immunoassay.
[Midazolam is extensively metabolized, fexofenadine is not metabolized, cyclosporine is partly metabolized]

The results for the orally administered drugs are shown in Fig 1.

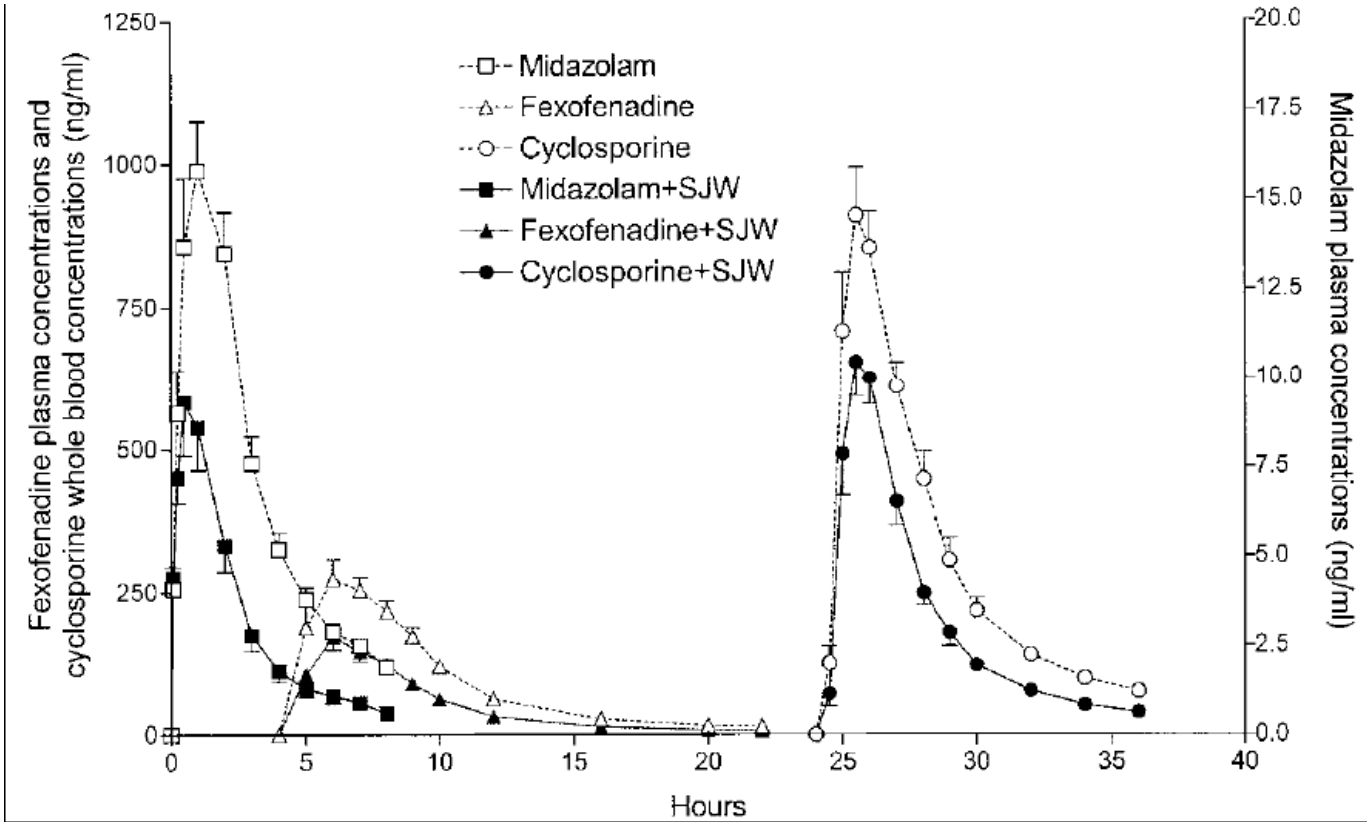


Fig 1. Plasma concentration versus time profile (mean +/- SD) for orally administered midazolam, fexofenadine, and cyclosporine before and after St John's wort (SJW) treatment.

1. **Based on the information given, outline the rationale for the study design, including why the three drugs were used, and why midazolam is given both orally and i.v.?**

2. **Interpret the pharmacokinetic results (Table 1 – see next page), including an explanation of how the parameters were measured or calculated. (Your attention is drawn to the term "oral clearance" used by these authors; its value is a function of systemic clearance and bioavailability).**

3. **Outline a mechanism or mechanisms for the effects of St. John's wort.**

Table I. Pharmacokinetic parameters before and after 14 days of treatment with St John's wort (N = 20)

Parameter	*Control*	*St John's wort*	*Change**
Midazolam			
CLs (mL/min)	383±101	540±113†	1.44±0.20
Vss (L)	75.0±30.2	80.8±26.9	1.12±0.26
t1/2 (h) (intravenous dose)	3.2±1.4	2.6±1.0	0.85±0.2
Cmax (ng/mL)	20.2±6.6	9.6±3.2†	0.51±0.19
CLs/F (mL/min)	1182±346	3063±809	2.68±0.69
F (%)	33.0±5.6	18.2±0.05†	0.56±0.13
E_H	0.36±0.08	0.51±0.10†	1.44±0.20
E_{GI}	0.47±0.13	0.61±0.13‡	1.25±0.30
Fexofenadine			
Cmax (ng/mL)	330±144	202±101†	0.63±0.22
CLs/F (mL/min)	1961±726	3661±1926†	1.94±0.60
t1/2 (h)	4.8±1.3	5.3±2.0	1.14±0.42
Cyclosporine			
Cmax (ng/mL)	1123±162	805±185†	0.72±0.15
CLs/F (mL/min)	728±195	1155±236†	1.63±0.29
t1/2 (h)	3.9±0.6	3.8±1.0	0.98±0.26

CLs, Systemic clearance; Vss, steady-state volume of distribution; t1/2, elimination half-life; Cmax maximum plasma concentration; CLs/F, oral clearance; F, oral bioavailability; E_H, extraction ratio by liver; E_{GI}, extraction ratio by intestinal tract.
*Ratio of parameter value after St John's wort relative to that before (control). †P<0.0001. ‡P< 0.001.

For your information:
E_H was calculated using the equation $E_H = CLs/Q_H$
The hepatic blood flow Q_H was obtained from a reported constant relationship of 25.7 mL/min per kilogram of body weight.
E_{GI} was then calculated by simple arithmetic arithmetic from knowing that F (which is 1 - E) is a product of F_H and F_{GI}. So, $E_{GI} = 1 – F_{GI} = 1 – F/F_H$

You have recently identified a strain of mutant mice that demonstrates sluggishness, bradycardia, fatigue, myxedema and cognitive impairment. You hypothesize that these animals have deficits in the hypothalamic-pituitary-thyroid axis and you subsequently quantify hormone levels to obtain the results presented in Table 1.

Mouse #	TRH (nM)	TSH (nM)	T_4 (nM)
1	17.6	19.6	277
2	12.9	23.4	198
3	14.2	22.1	236
Normal Range	3.0-5.5	7.3-12.1	70-160

Table 1. Serum concentrations of hormones involved in thyroid endocrine status in mutant mice.

1. **Describe the hypothalamic-pituitary-thyroid axis and the cellular and biochemical processes involved in the synthesis of thyroid hormone.**

2. **Develop a hypothesis for a precise molecular target that could be defective to explain both the physiologic and hormone changes observed in mutant mice. Provide strategies for testing your hypothesis.**

Qualifying Exam – July 2007
Question 4

Somatostatin (SS), the ligand for the sstr2-GPCR, potentiates the action of arginine vasopressin (AVP) in increasing intracellular Ca^{2+} and releasing insulin in a model cell line of pancreatic beta cells; SS has no such effect alone (Fig 1).

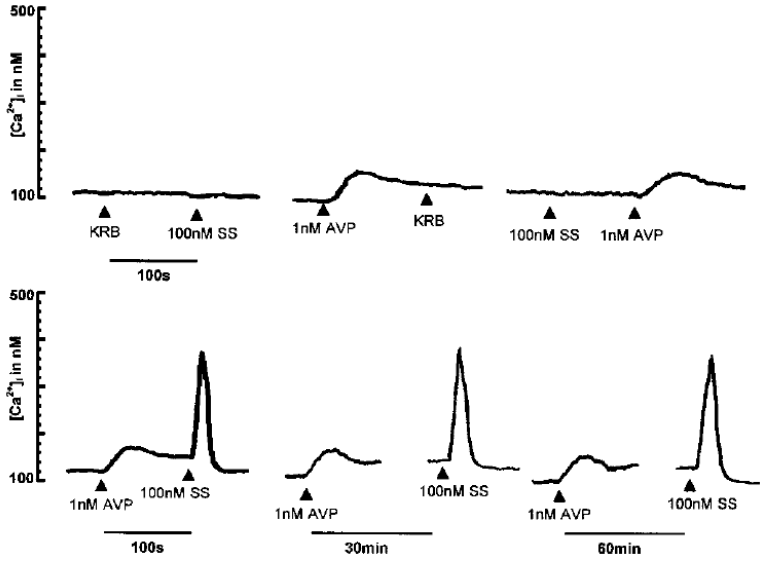


Figure 1 Somatostatin-induced increase in [Ca2+]i in the presence of AVP
Upper panel : the effect of somatostatin (SS) was confirmed by replacing 1 nM AVP with working buffer (KRB). No increase in [Ca2+]i was observed after 100 nM somatostatin treatment (left-hand panel). When KRB was replaced for somatostatin, only the typical increase in [Ca2+]I by AVP was observed (middle panel). Treatment with somatostatin prior to AVP did not induce a further increase in [Ca2+]i by AVP (right-hand panel). Lower panel : in the presence of AVP, somatostatin increased [Ca2+]i (left-hand panel). The increase in [Ca2+]i by somatostatin was observed even after 60 min of AVP treatment (right-hand panel). Traces are representative of four experiments.

The mechanism of the SS-AVP interaction was investigated in the following experiments:

- Microinjection of PIP2 into individual cells, Fig 2.

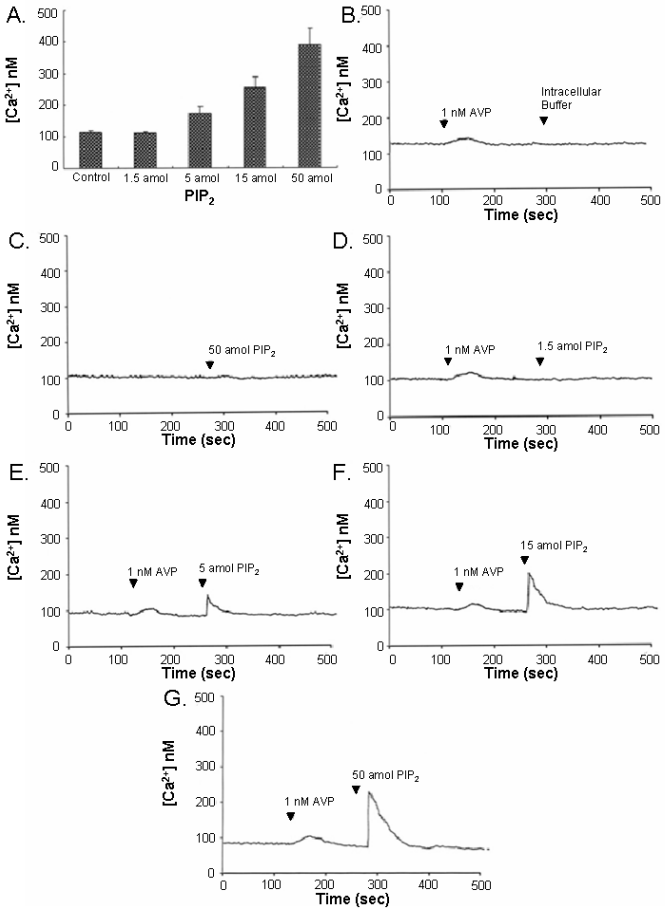
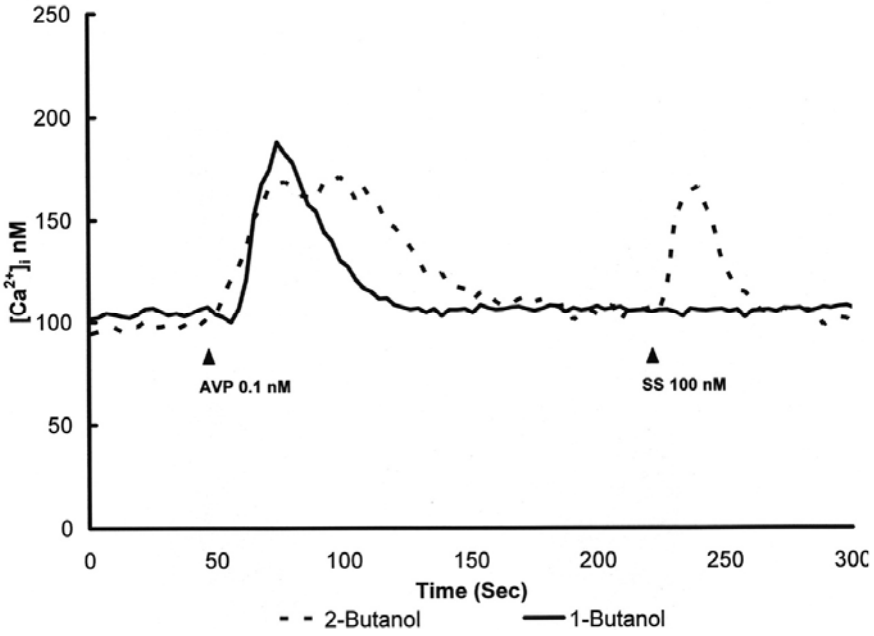


Fig. 2. Effect of PIP2 microinjection on [Ca2+]i in the presence of AVP in HIT-T15 cells. A, administration of PIP2 (1.5–50 amol) into single cells increased [Ca2+]i in a dose-dependent manner after 100 s of AVP (1 nM). Values are the mean ± S.E.; n = 3 independent cell preparations. B, representative calcium trace of HIT-T15 cells microinjected with intracellular buffer after the addition AVP did not increase [Ca2+]i. C, representative calcium trace of HIT-T15 cells microinjected with PIP2 alone at 50 amol did not increase [Ca2+]i. D, Representative calcium trace of HIT-T15 cells microinjected with 1.5 amol of PIP2 after the addition of AVP did not increase [Ca2+]i. E–G, representative calcium traces of microinjected PIP2 (5, 15, and 50 amol) did increase [Ca2+]i, while in the presence of AVP, in a dose-dependent manner.

- Pretreatment of the cells with pertussis toxin blocked the potentiating activity of SS, yet had no effect on AVP-stimulated Ca^{2+} levels or insulin release (data not shown).

- The mechanism of the SS-AVP interaction was further investigated by pretreatment of cells with 1-butanol or 2-butanol (Fig. 3)

Fig. 3. Effect of 1-butanol and 2-butanol on somatostatin (SS)-induced (100 nM) increase in [Ca2+]i in the presence of AVP (1 nM). HIT-T15 cells were pretreated with 1-butanol or 2-butanol (0.5%) 5 min before treatments. Cells treated with 1-butanol or 2-butanol were exposed to AVP (1 nM) for 150 s before the addition of somatostatin. Each line depicts the mean from 8 to 14 cells. The lines are representative of four independent experiments.



1. Present a hypothesis that can explain the molecular basis of the SS-AVP interaction in these cells and account for the effects of the different agents tested.

2. To further test your hypothesis, you plan to measure the lipid levels in these cells throughout the course of the SS-AVP interaction.
   Outline, in general terms, the methodology you would employ. Indicate which lipids you would measure, and make predictions (based on your hypothesis) of the results you might expect to find.

3. Present two additional experimental approaches that you would use to examine the mechanism and/or physiological significance of this SS-AVP interaction. These experiments could make use of this cell line or employ a different experimental system.

Normotensive and hypertensive patients were examined for alterations in the regulator of G-protein signaling 2 (RGS2) gene sequence. A heterozygous mutation was found to be correlated with hypertension and fibroblasts were isolated from normotensive patients lacking the mutation and hypertensive patients containing the mutation. The data below were generated using these isolated fibroblasts.

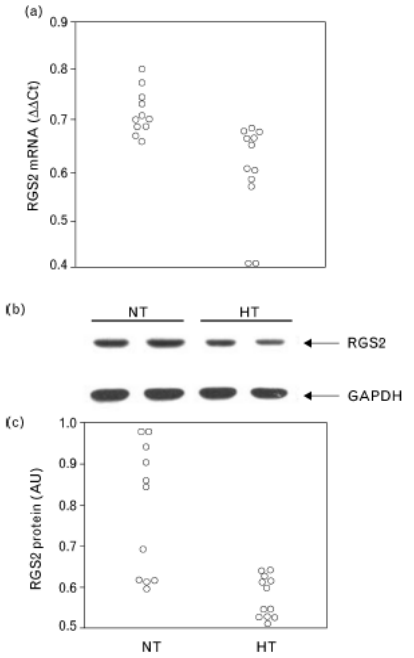


Figure 1. RGS2 gene and protein expression in fibroblasts from normotensive subjects (NT, n=11) and from hypertensive patients (HT, n=12). Skin fibroblasts were cultured in normal growth medium until confluence and made quiescent in serum-free medium for 24 h. **(a)** Quantitative analysis of gene expression. mRNA was extracted and quantitative mRNA RGS2 expression was performed by quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR). **(b)** A representative immunoblot for RGS2 and for glyceraldehyde-3- phosphate dehydrogenase (GAPDH) in two NT (left-hand lanes) and two HT (right-hand lanes) patients. Proteins were extracted and immunoblots for RGS2 and GAPDH were performed. **(c)** Quantitative analysis of protein expression (each point represents the RGS2/GAPDH ratio of a single patient).

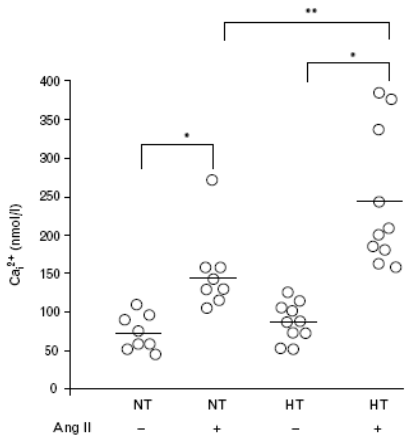


Figure 2. Free intracellular Ca^{+2} (Ca_i^{+2}) in quiescent fibroblasts at baseline and after acute angiotensin II (Ang II) stimulation (100 nmol/l, for 2 min) in normotensive subjects (NT, n=8) and hypertensive patients (HT, n=10). *P<0.01 versus unstimulated fibroblasts, **P<0.01 versus NT.

1. **Describe the data and outline at least one hypothesis to explain how alterations in RGS2 levels may result in hypertension in humans.**

2. **Describe a series of *in vivo* and *in vitro* experiments to test your hypothesis and confirm a role for RGS2 in hypertension.**

Qualifying Exam – July 2007
Question 6

In a recent paper, Aardvark and colleagues reported experimental observations that reveal a novel regulatory mechanism affecting the renal microcirculation. Their study was based on anatomic evidence that the short segment of the renal tubule connecting the distal convoluted tubule with the cortical collecting duct (a segment they refer to as "CNT") folds back on the afferent arteriole in superficial cortical nephrons. They hypothesized that there might exist 'cross-talk' between the CNT and the afferent arteriole that could affected glomerular filtration rate (GFR).

They used an experimental preparation of isolated, perfused rabbit cortical nephrons as illustrated in Fig. 1. Using this preparation, the investigators perfused the glomerulus under constant pressure and monitored the diameter of the afferent arteriole while they perfused the CNT segment with different solutions. Because isolated afferent arterioles have very little vascular tone, they also included norepinephrine in the bath solution to pre-constrict the vessel. Their results are illustrated in Fig. 2-4.

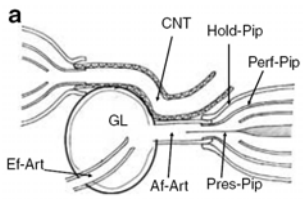


Fig 1 – Illustration of experimental preparation used in the study of CNT segment. Abbreviations: GL, glomerulus; Ef-Art, efferent arteriole; Af-Art, afferent arteriole; Hold-Pip, holding pipette; Perf-Pip, perfusion pipette; Pres-Pip, pressure pipette.

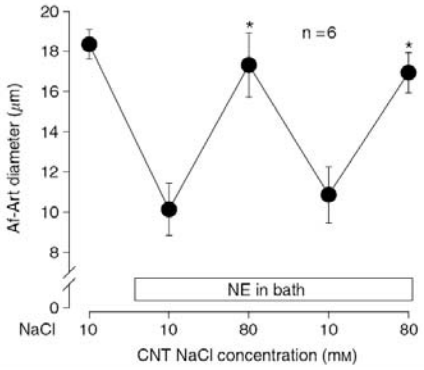


Fig 2 – Effect of perfusing CNT with low or high NaCl concentration. *, p < 0.05, high vs low NaCl.

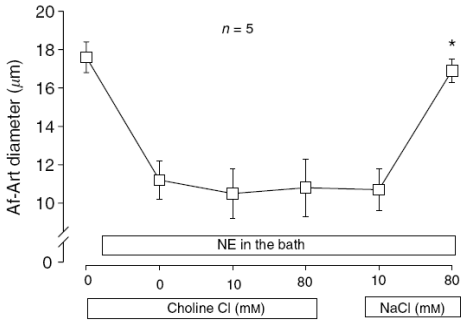


Fig 3 – Effect of perfusing CNT with choline chloride vs NaCl. *, p < 0.05, high vs low NaCl.

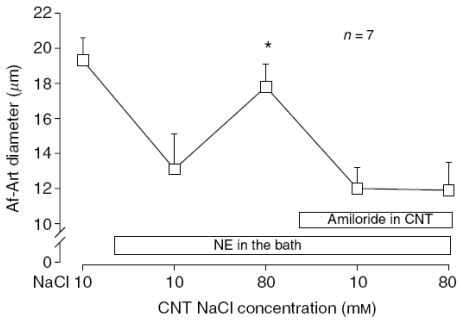


Fig 4 – Effect of adding amiloride to the CNT lumen. *, p < 0.05, high vs low NaCl.

1. **Propose at least one hypothesis or model to explain these data.**

2. **What conclusions can you draw regarding the ionic basis for this effect?**

3. **Propose 2 candidate mediators of this phenomenon and explain how you would experimentally test your hypothesis.**

Qualifying Exam – July 2007
Question 7

A recent study examined the role of β-arrestins in Gq-coupled M1 muscarinic receptor signaling. The investigators of this study demonstrated that β-arrestin 1 and 2 (βarr1 and βarr2) physically interact with active diacylglycerol kinases (DGKs). To investigate the consequences of βarr-DGK complexation in cells, the investigators monitored carbachol-induced phosphatidic acid (PA production in cells overexpressing or lacking βarr1/2 (Fig. 1) In addition, the investigators examined the effects of a dominant negative βarr (βarr-dn) on carbachol-induced DGK translocation to the M1 muscarinic receptor and PA production (Fig. 2).

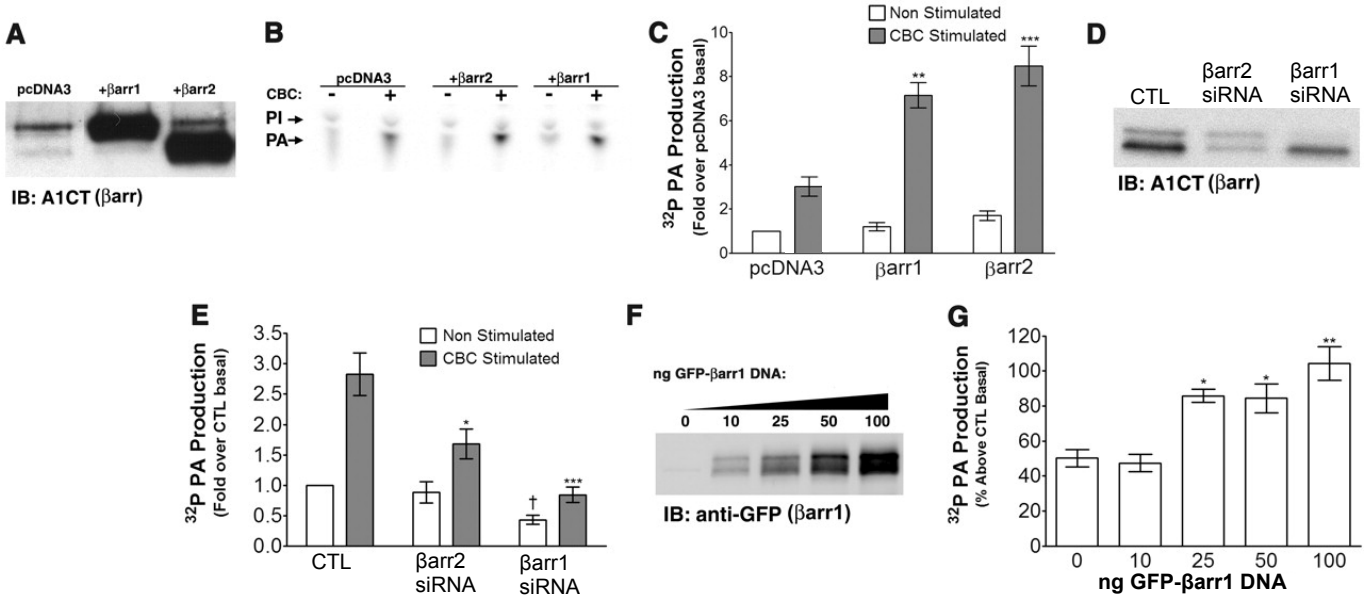


Fig. 1. (A) Immunoblot (IB) for β-arrestins from HEK293 cells transfected with pcDNA3, FLAG- βarr1, or FLAG- βarr2. (B) Phosphorimager screen image of radiolabeled lipids extracted and separated by thin-layer chromatography from β-arrestin– and control-transfected cells with and without 50 μM carbachol stimulation of endogenous M1Rs for 5 min (CBC, carbachol; PI, phosphatidylinositol; PA, phosphatidic acid). (C) Quantification of carbachol-stimulated [^{32}P]PA normalized to nonstimulated controls. Values shown represent the mean ± SE from eight independent experiments. Statistical significance was determined by one-way ANOVA (**P < 0.01 versus stimulated pcDNA3; ***P < 0.001 vs. stimulated pcDNA3). (D) Western blot of β-arrestins from HEK293 cells treated with control (CTL) or β-arrestin–specific siRNA oligonucleotides. (E) Summary data of carbachol-stimulated [^{32}P]PA production in cells treated with CTL, βarr2, or βarr1 siRNA. Values shown represent the mean ± SE of five independent experiments. Statistical significance was determined by one-way ANOVA (*P < 0.05 versus stimulated control cells; ***P < 0.001 versus stimulated control cells; P < 0.01 versus nonstimulated control cells). (F) Anti-GFP immunoblot showing expression of GFP-tagged rat β-arrestin 1 in HEK293 cells treated with siRNA specific for human β-arrestin 1. (G) Summary data of rescued [^{32}P]PA production normalized to nonstimulated control siRNA transfections. Values shown represent the mean ± SE of three independent experiments. Statistical significance was determined by one-way ANOVA (*P < 0.05 versus nonstimulated cells; **P < 0.01 versus nonstimulated cells).

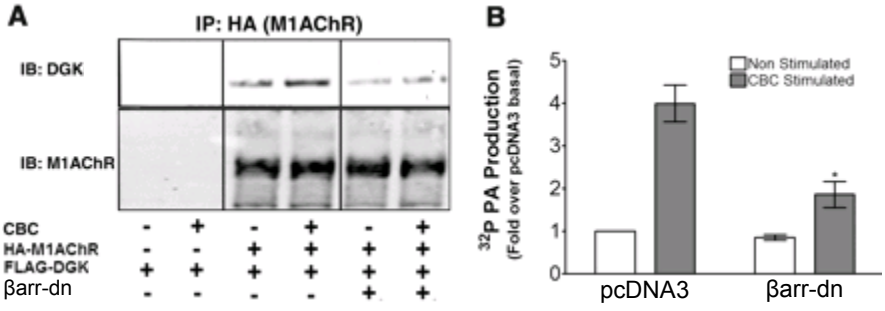


Fig. 2. (A) Immunoblots for DGK and M1AChR of anti-HA immunoprecipitates (IP) from HEK293 cells transfected with HA-M1AChR, FLAG-DGK, and βarr-dn (as indicated), and incubated with or without 50 μM carbachol for 5 min. βarr-dn does not bind agonist-stimulated M1AChR but does compete with endogenous β-arrestins for binding to DGK. Thus, βarr-dn functions as a dominant negative protein to inhibit DGK activity. (B) Summary data of three experiments showing the effect of βarr-dn overexpression on agonist-induced [^{32}P]PA production. Statistical significance was determined by a paired t test (*P < 0.05 versus stimulated pcDNA3).

1. **Develop a hypothesis to explain the findings depicted in figures 1 and 2. Describe how the results of these experiments support your hypothesis.**
2. **Design two independent experiments to test your hypothesis.**