Department of Pharmacology

Qualifying Examination (Part I)

December 19, 2013

ALL EXAMS TAKE PLACE IN THE BASS CONFERENCE ROOM, 436 RRB

Exam Schedule:	
Thursday, December 19th	10:00 am – 12:00 pm - Exam #1
	12:00 pm - Meeting to determine results
	12:30 pm - Results to Students in Pharm South Conf. Room (449 PRB)

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.

The reinforcing effect of psychostimulants such as cocaine and amphetamine involves the interaction of the drugs with the dopamine (DA) signaling. However, recent data suggest that other neurotransmitter systems may also be involved in mediating aspects of the psychostimulant-induced reward.

The study examined behavioral responses associated with psychostimulant reinforcement in the conditioned place preference (CPP) paradigm in mice lacking DA (dopamine-deficient mice, DD). DD mice have no DA but normal norepinephrine and serotonin. Figure 1 the result of the CPP experiments.

One line of evidence in support of this notion is that mice lacking DA (dopamine-deficient mice, DD; mice have no DA but normal norepinephrine) display behavioral responses associated with psychostimulant reinforcement such as conditioned place preference (CPP).

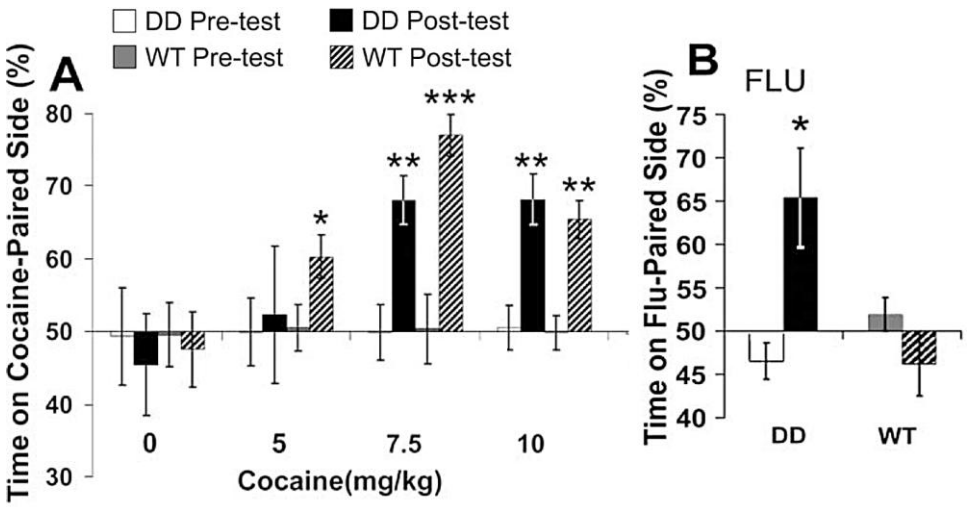


Figure 1. Conditioned place preference in mice without dopamine. During the pre-testing session, the mice were tested for the time spent in each of the two (black and white) chambers of the conditioned place preference apparatus without any drugs. During the training period, the mice received a dose of the drug in one chamber and saline in another (pairing). During the post-testing session, the mice did not receive any drugs but were tested for the time spent in the drug- and saline-paired chambers. The difference between the time spent in the drug-paired chamber during pre-testing and post-testing session is indicative of CPP. (**A**) CPP response to varying doses of cocaine in dopamine-deficient (DD) and wild type (WT) mice. * - $p<0.05$; ** - $p<0.01$ as compared to the pre-test. (**B**) Fluoxetine (5 mg/kg) produces CCP in DD but not WT mice. * - $p<0,05$ to pre-test.

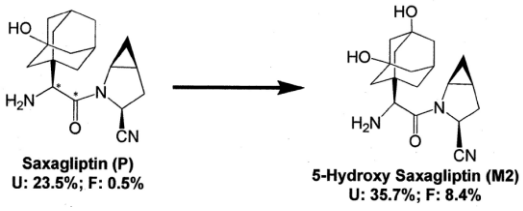
1. **Describe the data presented in Fig. 1. How do mice lacking DA compare to wild type mice in their ability to form CPP to cocaine and amphetamine? How do they compare in they ability to form CPP to fluoxetine?**

2. **What neurotransmitter system(s) and protein(s) are targeted by fluoxetine? What disease(s) fluoxetine is used to treat? What does the absence of CPP to fluoxetine in wild type animals is indicative of in terms of the drug's clinical utility?**

3. **Devise two tests that could confirm that the neurotransmitter system in question 1 above plays the critical role in the fluoxetine-mediated CPP in DD mice.**

Saxagliptin is an inhibitor of dipeptidyl peptidase-4 (the enzyme that degrades GLP-1, glucagon-like peptide-1) under development for the treatment of type-2 diabetes. The pharmacokinetics and disposition of [^{14}C]-saxagliptin were evaluated in healthy male volunteers following a single 50 mg (91.5 µCi) dose and found to be partially converted to a metabolite designated as **M2** (Figures 1 and 2; Table 1). Importantly, prior to the clinical development DMPK scientists determined that saxagliptin was primarily metabolized by CYP3A (Figure 3).

Figure 1



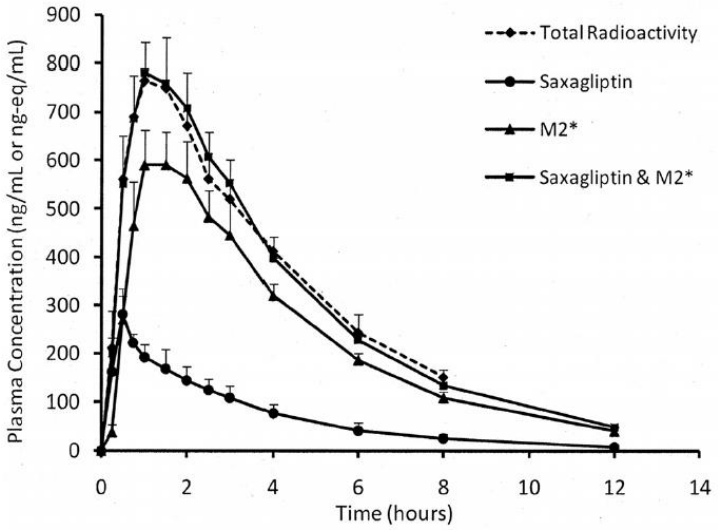
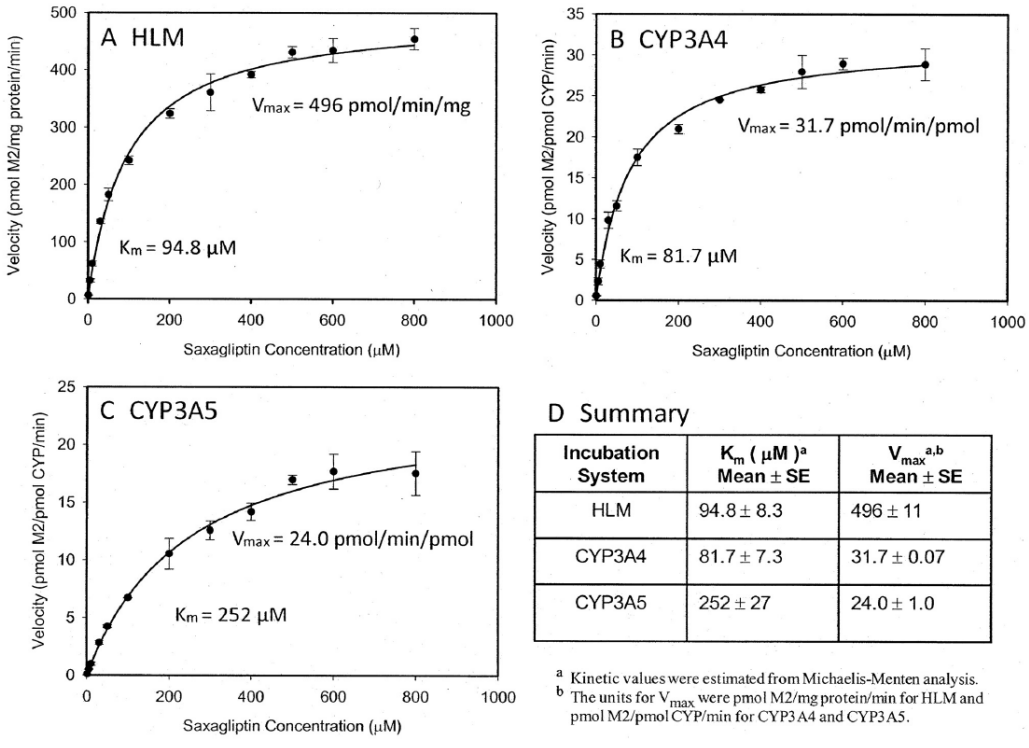


FIG. 2. Mean concentration versus time profiles of total radioactivity, saxagliptin, M2, and the summed contribution of saxagliptin and M2, in plasma from healthy male subjects after oral administration of 50 mg (91.5 µCi) of [14C]saxagliptin.

FIG. 3. Concentration-dependent formation of M2 from saxagliptin in incubations with HLM (A), CYP3A4 (B), and CYP3A5 (C); and summary of kinetic parameters (D).
Data were fitted to a Michaelis-Menten substrate binding curve: $V = (V_{max} \times S)/(K_m \times S)$. The values of K_m and V_{max} are shown.



D Summary

Incubation System	K_m (µM)[a] Mean ± SE	V_{max}[a,b] Mean ± SE
HLM	94.8 ± 8.3	496 ± 11
CYP3A4	81.7 ± 7.3	31.7 ± 0.07
CYP3A5	252 ± 27	24.0 ± 1.0

[a] Kinetic values were estimated from Michaelis-Menten analysis.
[b] The units for V_{max} were pmol M2/mg protein/min for HLM and pmol M2/pmol CYP/min for CYP3A4 and CYP3A5.

Table 1
Mean pharmacokinetic parameters for saxagliptin, M2, and radioactivity after administration of a single oral dose of [^{14}C]saxagliptin.
Following administration of a single 50-mg (91.5 μCi) oral dose of [^{14}C]saxagliptin to healthy male subjects, the mean cumulative recovery of radioactivity over the study duration (168 h) was ≈97% (Table 1). The majority of the radioactivity (mean value, ≈75%) was excreted in the urine. Approximately 22% was recovered in the feces.

Mean, %CV (17) (7) (9)

PK Parameter s	**Saxagliptin**	**M2**	**Radioactivity**
*C*max, ng/ml (%CV)	279 (13)	606 (11)	779 (10)
$AUC_{0\text{-}INF}$, ng . h/ml (%CV)	845 (17)	2943 (7)	3874 (9)
T_{max}, h	0.50	1.50	1.50
t½ (plasma half-life), h (S.D.)	2.45 (0.31)	2.69 (0.31)	2.77 (0.22)
Renal Clearance, ml/min (S.D**)**	234 (23)	102 (10)	165 (14)
% Urinary excretion (S.D.)	23.5 (3.1)	35.7 (3.0)	74.9 (5.1)
% Fecal excretion (S.D.)	0.5 (NA)	8.4 (NA)	22.1 (9.5)
Bioavailability (from a separate study)	51%		

%CV = % coefficient of variation
S.D. = standard deviation
NA = not available

Questions:

1. **Using the data provided, give a summary of the most important factors influencing the disposition of Saxagliptin in healthy subjects.**

2. **Describe how you would determine the plasma clearance of Saxagliptin?**

3. **Considering the *in vitro* metabolism data provided in Figure 3, offer a prediction as to the impact of CYP3A4 or CYP3A5 genetic polymorphisms on the clearance of saxagliptin *in vivo*.**

4. **Briefly discuss the renal clearance calculated for saxagliptin, specifically as it compares to GFR. Considering that renal impairment is associated with type-2 diabetes, what impact could this have on saxaglpitin's disposition in patients?**

A young man is seen by an endocrinologist for weight loss and hypoglycemic (low blood sugar) episodes.

1) **You test his blood insulin level, and it is appropriately low given his low blood sugar. What other endocrine system may be dysfunctional? What laboratory finding(s) would confirm dysfunction in the system you suspect is involved?**

2) **How could you differentiate between a problem in the central nervous system and a problem outside of the CNS to explain his clinical findings? In particular, describe the regulation of the level of the hormone you suspect is involved by the CNS and the measurements you could make to determine the cause. How would you determine if the endocrine gland is responding appropriately?**

3) **This person is also found to have hypotension (low blood pressure). You are concerned that a second hormone system could be dysfunctional. Name a hormone that regulates blood pressure. Can a CNS problem explain deficiencies in the hormone discussed in #1 as well as this one, or do you think an adrenal problem is most likely?**

The α–adrenergic receptors are important for regulating the vascular tone of the vascular smooth muscle in human. A single nucleotide polymorphism, G247R, in the human α1a receptor was recently identified in patients with severe hypertension. The location of G247R substitution occurs in the third cytoplasmic loop of the α1a receptor. To explore the functional outcome associated with this substitution, a full-length cDNA corresponding to the mutant receptor (247R) was generated and transfected into cells. Using thymidine incorporation as the readout, the effect of the mutant receptor on proliferation of cells was evaluated (Figure 1). To investigate the mechanism underlying the proliferative phenotype upon the expression of modified α1a receptor, several pharmacological agents were employed and results were shown in Figure 2.

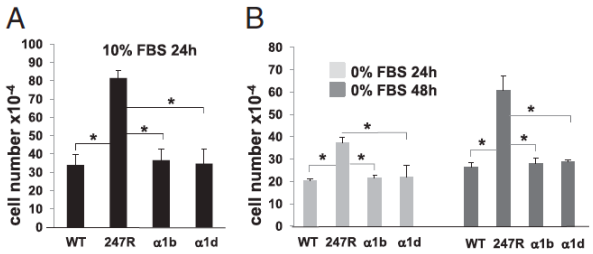


Fig 1. Constitutive expression of 247R confers serum-independent cell proliferation. Data are presented as mean ± SEM from four independent experiments. *p< 0.05
FBS, Fetal bovine serum.

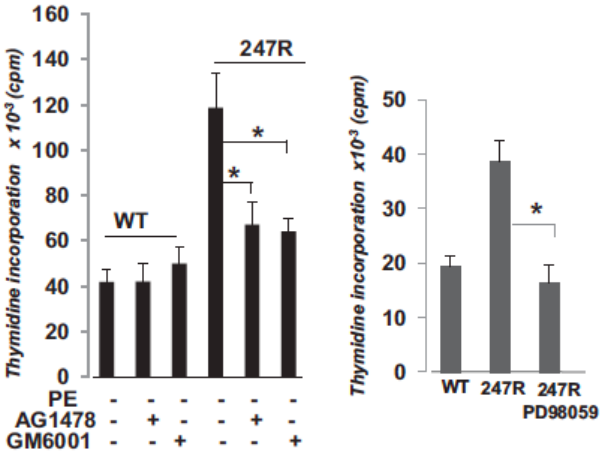


Fig. 2. Effects of inhibitors on the proliferative activity of 247R receptors. AG1478, EGFR inhibitor; GM6001, MMP (Matrix metalloproteinase) inhibitor; PD98059, MEK inhibitor; PE, phenylephrine (α1 agonist)

1. **(a) Explain the results in Figures 1 and 2.**

   (b) What is your conclusion based on these findings?

   (c) Propose a couple of experiments to support your conclusion in (b).

2. **(a) Provide a scheme to illustrate potential mechanism linking 247R to increased cell proliferation.**

   (b) Is transactivation of EGFR dependent on G-protein?

   (c) Design two critical experiments for supporting the proposed transactivation mechanism.

Chronic Heart Failure is associated with cardiac β-adrenergic receptor dysfunction due to increased desensitization and downregulation, which is specific for β1-adrenergic receptors along with G-protein uncoupling. This significantly decreases cardiac adrenergic and inotropic response. Given these observations, β1-arrestin (β1arr) null mice were examined in a model of myocardial infarction (MI)-induced heart failure.

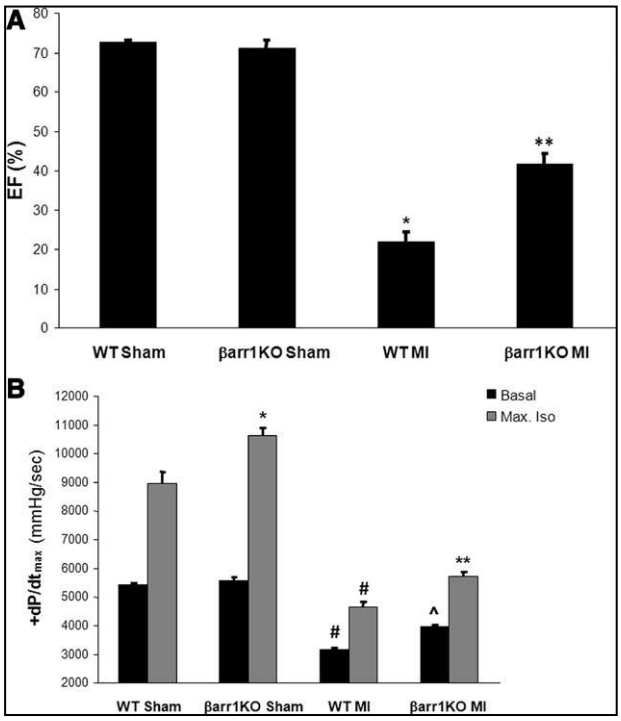


Figure 1. A, Ejection fraction (EF) % of sham-operated (sham) or of 4-week post–myocardial infarction (MI) β-arrestin-1 knockout (βarr1KO) and wild-type (WT) mice. **P*<0.05 vs either Sham; ***P*<0.05 vs WT MI; n=7 mice/group. **B**, Basal and maximal dose (333 ng/kg) of isoproterenol (Max. Iso)-stimulated +dP/dtmax responses of these mice. **P*<0.05 vs WT Sham-Max. Iso; #*P*<0.05 vs either Sham; ***P*<0.05 vs WT MI-Max. Iso; ^*P*<0.05 vs WT MIBasal; n=5 mice/group.

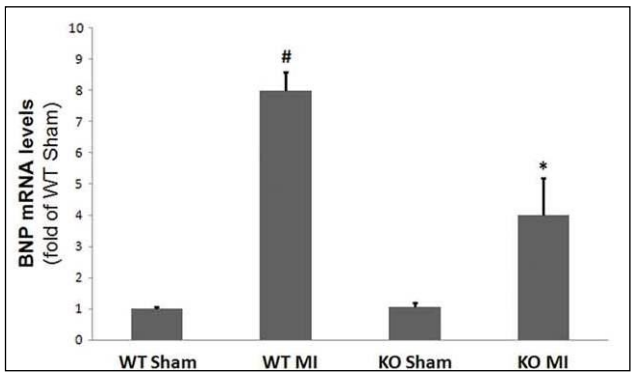


Figure 2. Heart mRNA levels of brain natriuretic peptide (BNP), a marker of heart failure, in these mice. #*P*<0.05 vs either Sham; **P*<0.05 vs WT MI; n=6 hearts/group.

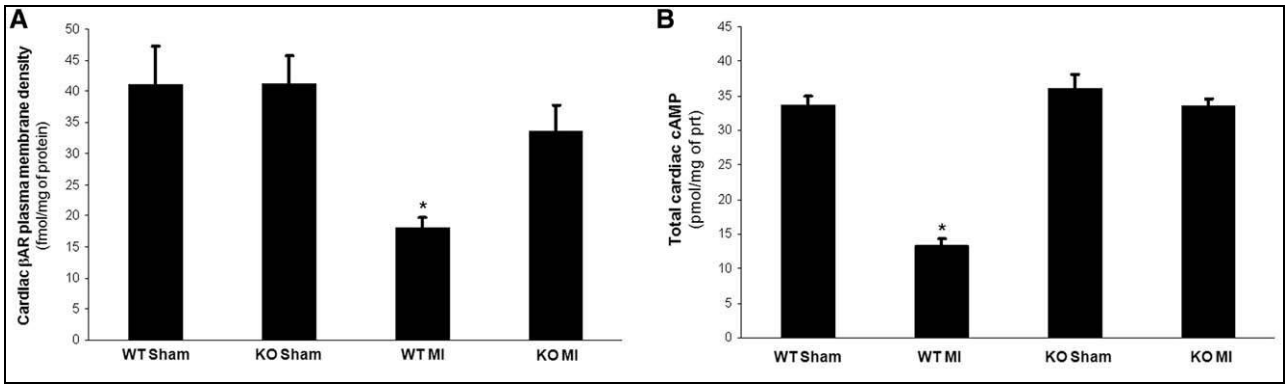


Figure 3. β-Adrenergic receptor (βAR) density in cardiac plasma membranes of sham-operated (Sham) or of 4-week post–myocardial infarction (MI) β-arrestin-1 knockout (KO) and wild-type (WT) mice. **P*<0.05 vs all other groups; n=5 hearts/group. **B**, Steady state total cAMP levels in cardiac homogenates purified from these mice. **P*<0.05 vs all other groups; n=5 hearts/group.

In addition to the above data, it was noted that β1arr null mice have increased survival when compared to wildtype controls. Please address the following points based on the data above.

1. **Diagram how the β1-adrenergic receptor regulates cardiac contractility. Indicate the role of β1arr in this mechanism.**

2. **Interpret the data above and propose a hypothesis to address the role of β1arr in the regulation of the β1-adrenergic receptor and the response to MI-induced heart failure.**

3. **Using the diagram from question 1, indicate where 3 different classes of heart drugs act to alter cardiac contractility and whether the absence of β1arr would be predicted to alter the drug effect.**

A pharmaceutical company in Kuala Lumpur recently reported discovery of a novel agent (called '**Compound A**') that targets the ROMK channel in the kidney. The effects of **Compound A** on ROMK channels expressed in HEK-293 cells exhibited a concentration response illustrated in Figure 1. Oral administration of **Compound A** to adult rats caused dose-dependent changes in urine output and urinary sodium excretion (Figure 2). The time course and magnitude of these effects resembled those of a thiazide diuretic agent (HCTZ).

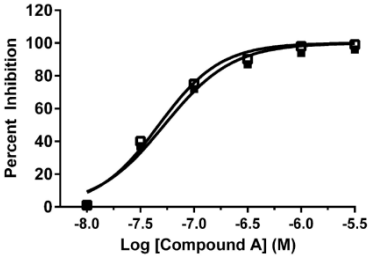


Figure 1 – Concentration response curve for the effect of Compound A on ROMK channels expressed in HEK-293 cells. The different symbols and fitted lines represent distinct cell lines stably expressing ROMK.

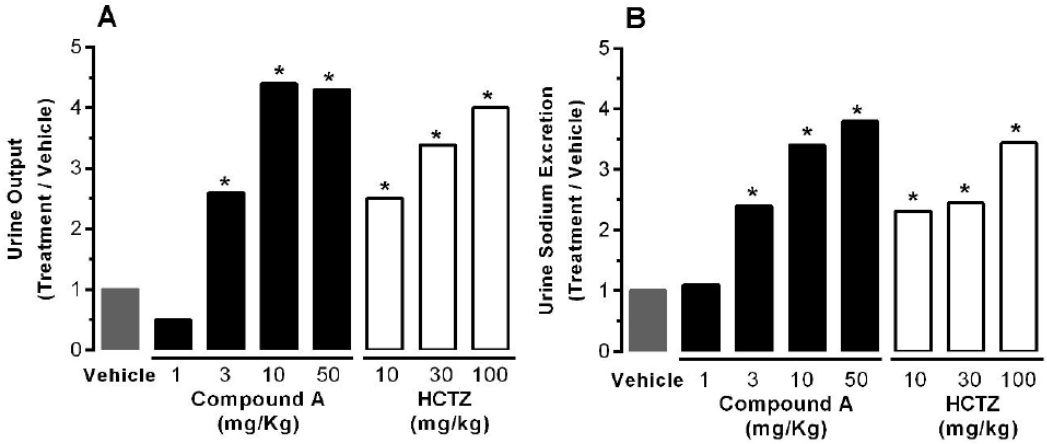


Figure 2 – Effect of Compound A and HCTZ on urine output and urinary sodium excretion in rats given oral dosing. * indicates $p < 0.05$ compared with the vehicle group.

Questions

1. **Formulate a hypothesis to explain the effects of Compound A on renal function at the cellular and molecular levels. What additional experiments could you do to support your hypothesis?**

2. **Predict the effects of Compound A on renal potassium excretion and explain your prediction at the cellular and molecular levels. Contrast your prediction with what you expect for a thiazide diuretic.**

3. **Hypothesize what effects Compound A might have on urinary concentrating ability.**

AKAPs bring PKA and other signaling proteins together with their substrates to facilitate cell signaling. This arrangement is critical for the phosphorylation and regulation of the transient receptor potential vanilloid 1 (TRPV1) channel, a calcium-permeable ion channel that responds to chemical stimuli (e.g., capsaicin, the pungent component of hot chili peppers) or thermal changes (>42°C) for nociceptive signaling. TRPV1 is also a key target of inflammatory mediators such as prostaglandins, and is essential for the development of inflammatory thermal hyperalgesia (i.e., increased sensitivity to pain in response to inflammation). Like many channels and receptors, TRPV1 is desensitized upon continuous activation or in response to repeated exposures of capsaicin. Although the exact mechanisms for desensitization are still unclear, recent studies have revealed that PKA phosphorylation of TRPV1 on Ser116 blocks TRPV1 desensitization. To further investigate AKAP79 regulation of TRPV1 channels, a group of investigators performed the following experiments.

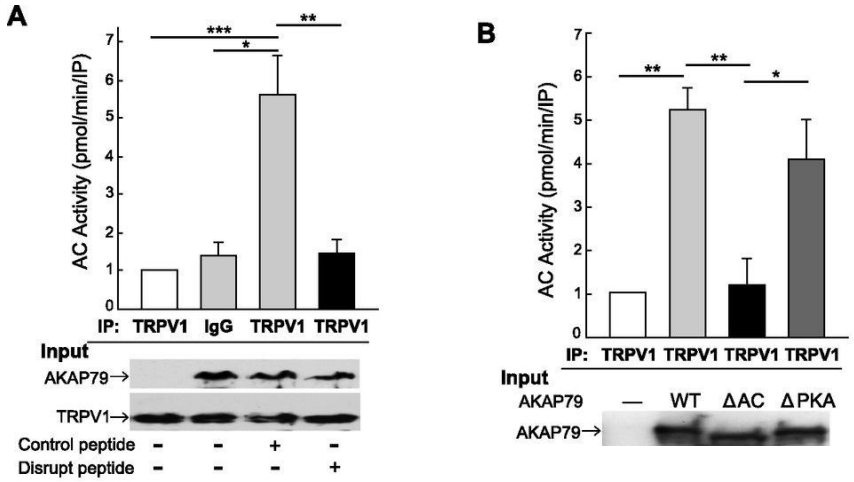


FIGURE 1. **Analysis of TRPV1 complexes.** *A*) HEK293 cells were transfected with adenylyl cyclase 5 (AC5) and TRPV1 +/- AKAP79. Samples were immunoprecipitated (*IP*) with anti-TRPV1 or control IgG and assayed for AC activity with 50 nM $G\alpha_s$ and 100 μM forskolin. Where indicated, control polypeptide ($AKAP79^{109–290}$) or AC-AKAP79-disrupting polypeptide ($AKAP79^{77–153}$; 5 μM) was included during homogenization. Data were normalized to control (TRPV1 IP without AKAP79) and are presented as the mean ± S.E., *n* = 4–5. AKAP79 and TRPV1 expression was confirmed by Western blotting. *B*) HEK293 cells were transfected with AC5 and TRPV1 ± AKAP79 or AKAP79 with a deletion of the AC binding site (*ΔAC*) or PKA binding site (*ΔPKA*). IP-AC assays were performed with anti-TRPV1 antibody as in *A* (*n* = 3). AKAP79ΔAC does not interfere with TRPV1 binding as anti-TRPV1 pulls down both AKAP79 and AKAP79ΔAC when co-expressed in HEK293 cells (data not shown).

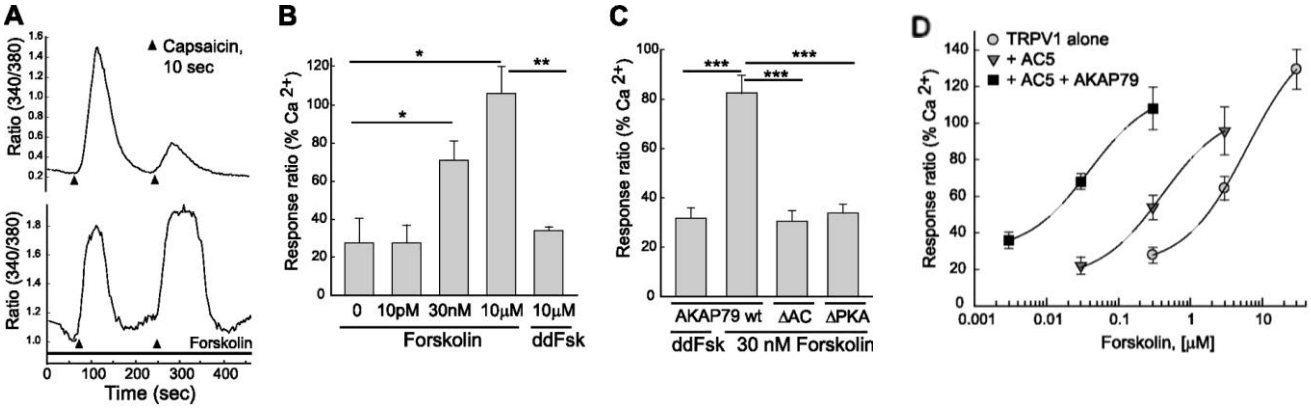


FIGURE 2. **Analysis of TRPV1 sensitivity to forskolin.** HEK293 cells were transfected with TRPV1, AC5, and AKAP79. *A*) Intracellular Ca^{2+} transients were evoked by two applications of capsaicin (300 nM for 10 s; indicated by *arrowheads*) separated by 3 min. Cells were continually perfused under control conditions (*top*) or in the presence of 300 nM forskolin (*bottom*). *B*) Plot of the response ratio (amplitude peak 2/amplitude peak 1) as a function of forskolin concentrations. Inactive dideoxyforskolin (*ddFsk*) was used as a control. Data are presented as mean ± S.E. and were obtained from 11–26 cells for each concentration point. *C*) HEK293 cells were transfected with AC5 and TRPV1 ± AKAP79 or AKAP79 with deletion of the AC (*DAC*) or PKA (*DPKA*) binding site. Intracellular Ca^{2+} transients were measured in the presence of 1,9-dideoxyforskolin or forskolin (30 nM), and the response ratio was plotted. *D*) The response ratio for intracellular Ca^{2+} transients in HEK293 cells expressing TRPV1 alone or with AC5 ± AKAP79 was measured at the indicated forskolin concentrations (mean ± S.E., *n* = 3–4 experiments). *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ relative to the indicated controls.

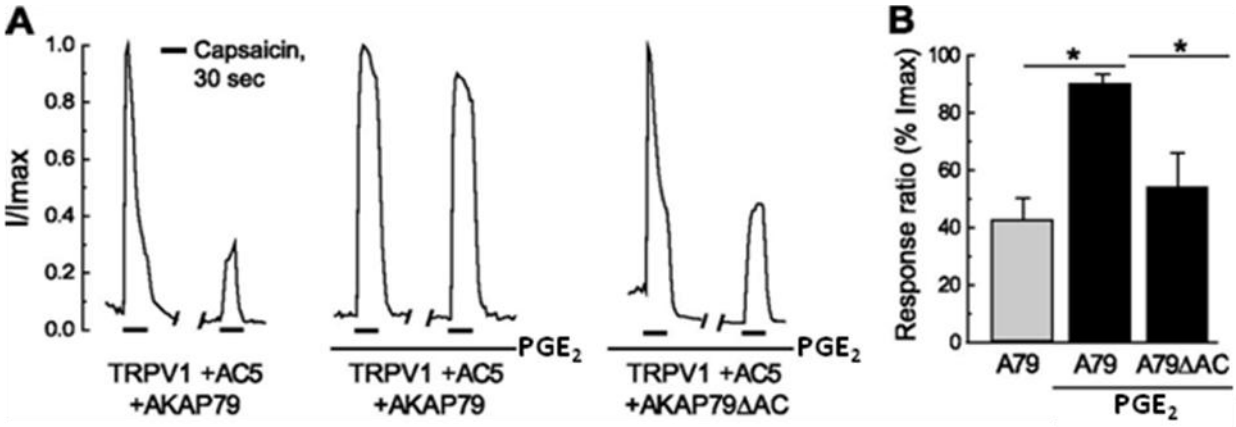


FIGURE 3. **Effects of PGE_2 receptor (Gs-coupled receptor) stimulation on TRPV1 currents.** *A*) Time courses of I_{TRPV1} recorded at +100 mV evoked by two successive applications of capsaicin (300 nM, 30 s) separated by a 3-min interval in HEK-293 cells transfected with TRPV1, AC5, and AKAP79 or AKAP79ΔAC in the presence or absence of prostaglandin E_2 PGE_2, 1 μM). Data are normalized by the peak amplitude of the first capsaicin-evoked response. *B*) Quantification of I_{TRPV1} desensitization. Cells expressed TRPV1 and AC5 plus AKAP79 (*A79*) or AKAP79ΔAC (*A79ΔAC*) as indicated (mean ± S.E., n = 3–5 in each group). *, $p < 0.05$, relative to the indicated controls.

A) Develop a hypothesis that might explain the data shown in Figs. 1-3. Describe how the experimental results support your hypothesis.

B) Design two independent experiments that would allow you to further test your hypothesis.

C) Based on the data presented, what protein-protein interaction would you target in an attempt to ameliorate the effects of chronic TRPV1 activation under inflammatory conditions? Why?