Update on Developments in Flux Assays- ASICs, SACs, and Na^+, K^+ ATPase

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Need for HTS

- **Ion Channels**
  - Important therapeutic targets
  - Pore forming biopesticides
  - Pore forming antibiotics
- **Need for HTS Bio-assays**
  - Large chemical / biochemical libraries
    - Combinatorial chemistry
    - CAD modelling & designing
  - New ion channel targets
    - Genomics & proteomics
    - Disease models
  - HTS screening bottlenecks

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Aurora Biomed's ICR Technology

An Overview

Aurora Biomed's ICR Series

- HTS Instrument
  - ICR 8000

  - ICR 12000

Parameters	ICR 12000
Sensitivity	0.05mg/L (0.05ppm)
Sampling Volume	100 µL @ ~5% CV 50µL @ ≤10% CV
Throughput/ 8hr day	24,000 data points
Cost/well	≤ $0.04/well
Fuel	natural gas - compressed air
Add-on Options	included: plate stacker, bar code reader
Plate format	96/384-well Plates
Bench space required	120cm X 95cm

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Surrogate Ions

- Potassium ion channels: Rb^+ for K^+ (George Terstappen)
- Na^+,K^+ ATPase: Rb^+ for K^+
- Sodium ion channels: Li^+ for Na^+
- Acid sensing ion channels: Li^+ for Na^+
- Chloride ion channels:
  - Ag^+ titrated with Cl^- in the samples
  - Ag^+ precipitates as AgCl
  - Ag^+ remaining free is measured in the samples
- K^+,Cl^- co-transporter: Ag^+
- Calcium ion channels: Sr^{2+} or Ca^{2+}

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Expression Systems

- Endogenously expression
- Stable or transient expression
  - Mammalian cell lines
    - HEK293 cells
    - Mouse L cells
    - CHO cells
  - Non-mammalian
    - *Xenopus* Oocytes
    - *C. elegans*
    - Yeast

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Targets Screened Using ICR

Ion Channel	Associated Diseases
hERG	Long-QT syndrome, drug-induced arrhythmias
KCNA3 (Kv1.3)	Multiple sclerosis, obesity, diabetes
KCNQ2/3	Epilepsy
KCNA5 (Kv1.5)	Pulmonary hypertension
BK_{Ca} SK_{Ca}	Erectile dysfunction Incontinence
KCNA4 (Kv1.4)	Ventricular diseases
KCNA1 (Kv1.1)	Episodic ataxia
Stretch-activated K^+ channels	Muscle damage / cardiomyocytes

(Next)

Targets Screened Using ICR (cont.)

Ion Channel	Associated Diseases
Na^+/K^+-ATPase	Congestive heart failure
K-Cl co-transporter	Sickle cell diseases
$Na_v1.7$	Pain
$Na_v1.5$	Long-QT syndrome
$Na_v1.2$	Multiple sclerosis, seizure disorders
CFTR	Cystic Fibrosis
Cl_{Ca}	Asthma

To date, these targets have been studied using ICR, and more are being developed and optimized

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Acid Sensing Ion Channel (ASICs)

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Therapeutic Importance

- Extracellular acid has important effects on neuron function
- In central and peripheral neurons, ASICs have emerged as key receptors for extracellular protons
  - Belong to sodium channel superfamily- ENaC/DEG
  - Activated by an increase in tissue acidosis
    - Happens during acute and chronic pain conditions
      - Including inflammation
      - Angina
      - Stroke
      - Ischemic heart disease
      - Arthritis
      - Cancer and
      - Traumatic injuries

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Therapeutic Importance (contd)

- Six different isoforms of ASIC channels have been identified:
  - ASIC1a, ASIC1b
  - ASIC2a, ASIC2b
  - ASIC3 and
  - ASIC4
- The most attractive targets for the treatment of pain
  - ASIC1 (1a/1b) and
  - ASIC3
  - Highly enriched in primary sensory neurons that detect and signal painful sensations to the brain

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Search for ASICs Blockers

- Currently known blockers
  - A spider toxin (PcTx1) for ASIC1a and
  - A toxin from sea anemone (APETX2) for ASIC3
  - A small molecule blocking several ASIC channels shown to decrease pain in various animal models
- Search for blockers of ASICs is being actively pursued
    - Clone A: expressing ASIC 2a+3 subunits
    - Clone B expressing ASIC 3
    - Clone C nNative cell line
    - Clone D: expressing ASIC1a

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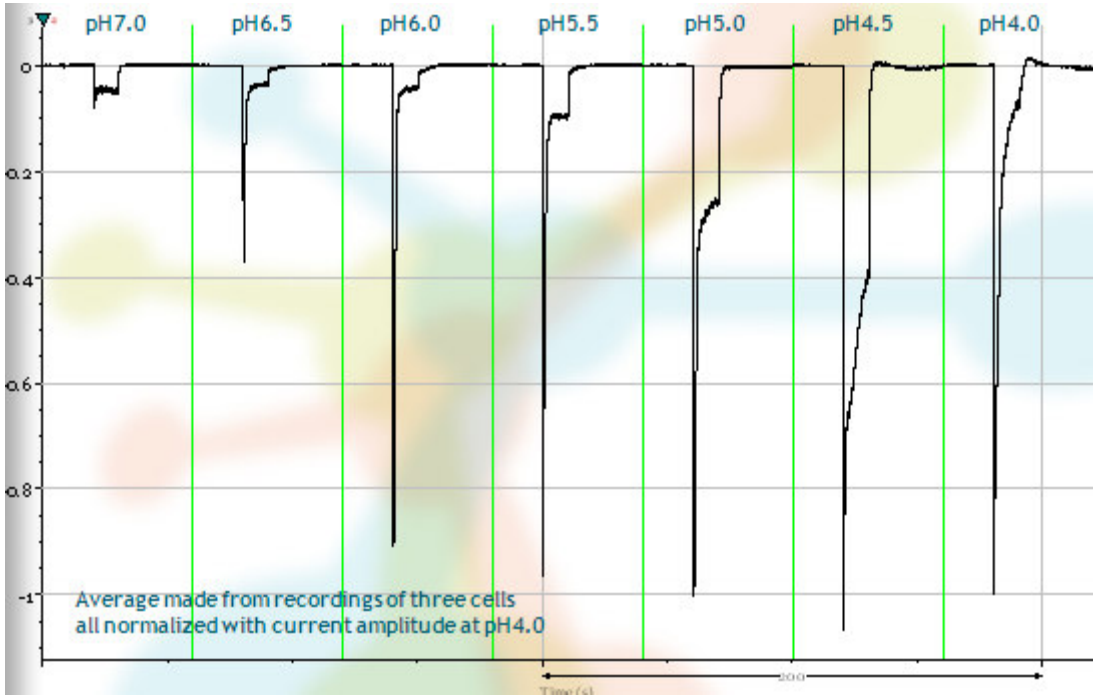
EP Profile of ASIC2a+3

Stimulation of CHO clone A at decreasing pH from 7.5



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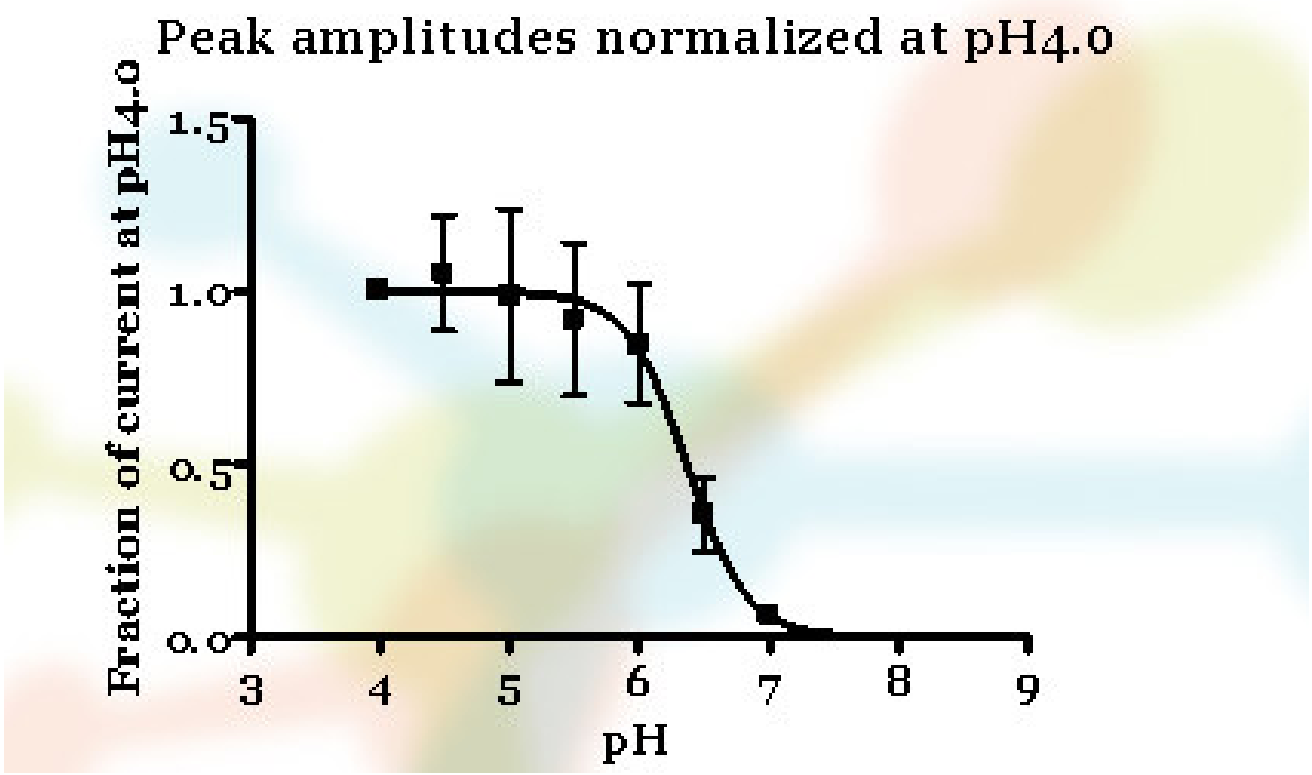
EP Profile of ASIC2a+3

Clone A has a pH_{50} of 6.4



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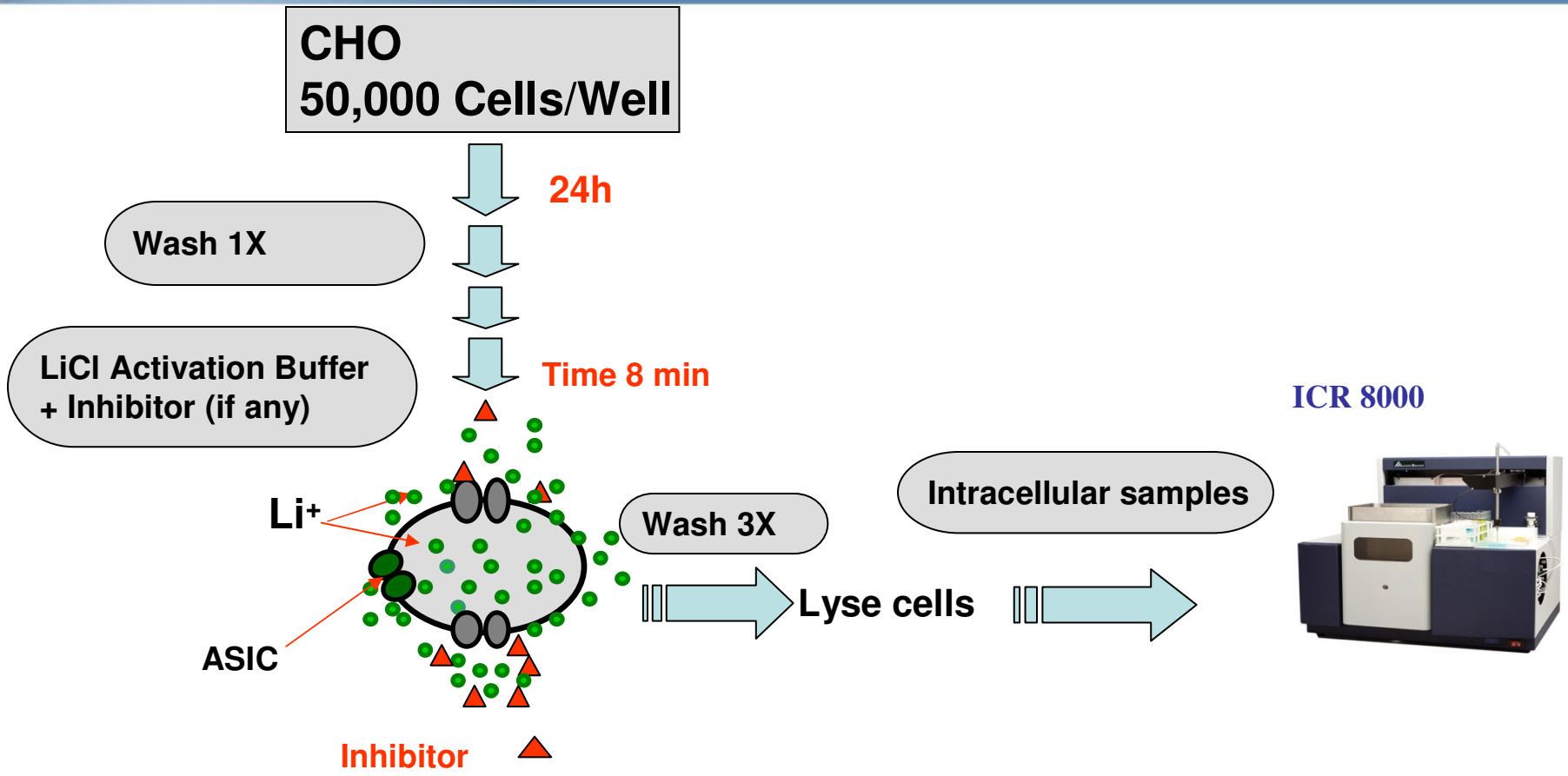
ASICs Flux Assay Steps



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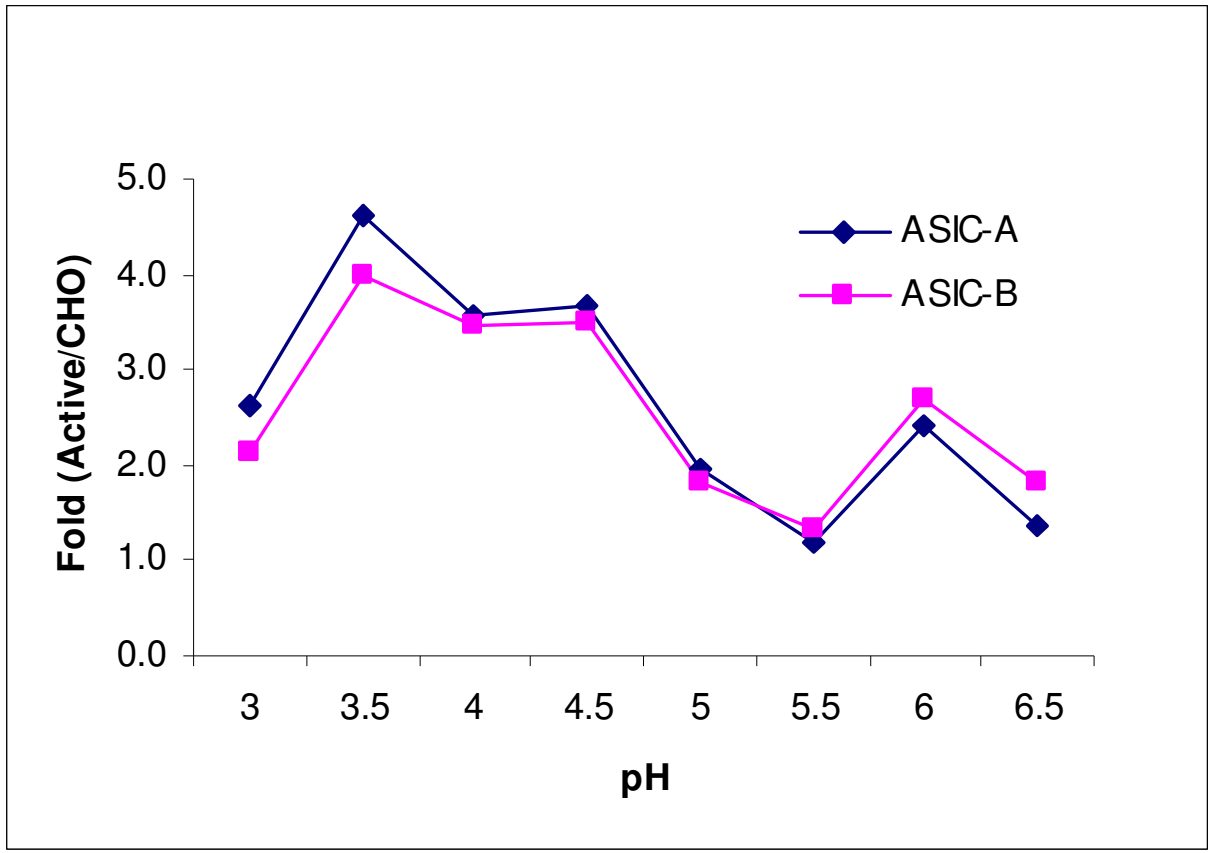
Fold - Activation of ASIC2a+3 & 3



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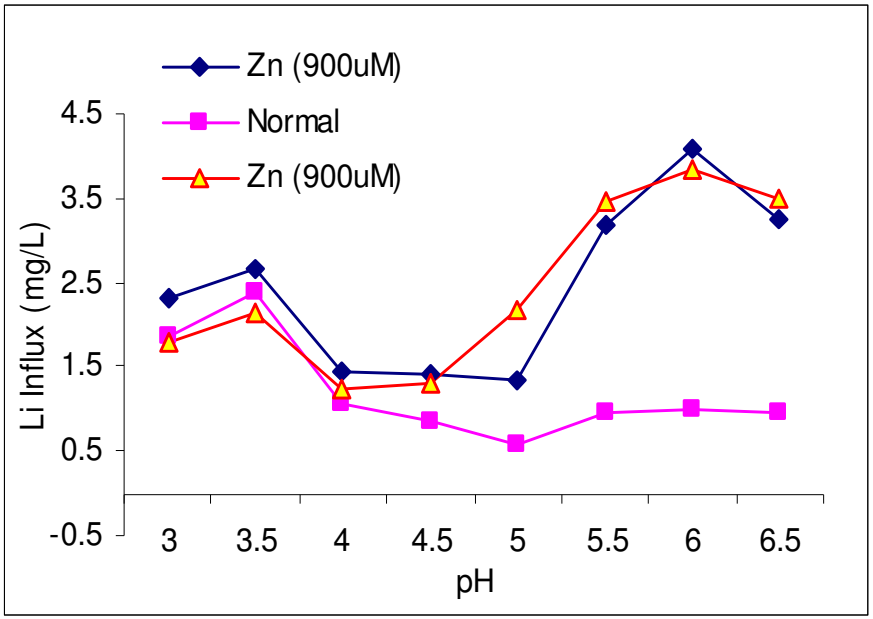
Effect of Zn^{++} on ASIC 2a+3

- Effect of Zn^{++} on ASIC 2a+3
- n=4 carried twice in the same plate to look at the reproducibility
- Thus showing a significant effect on ASIC 2a+3



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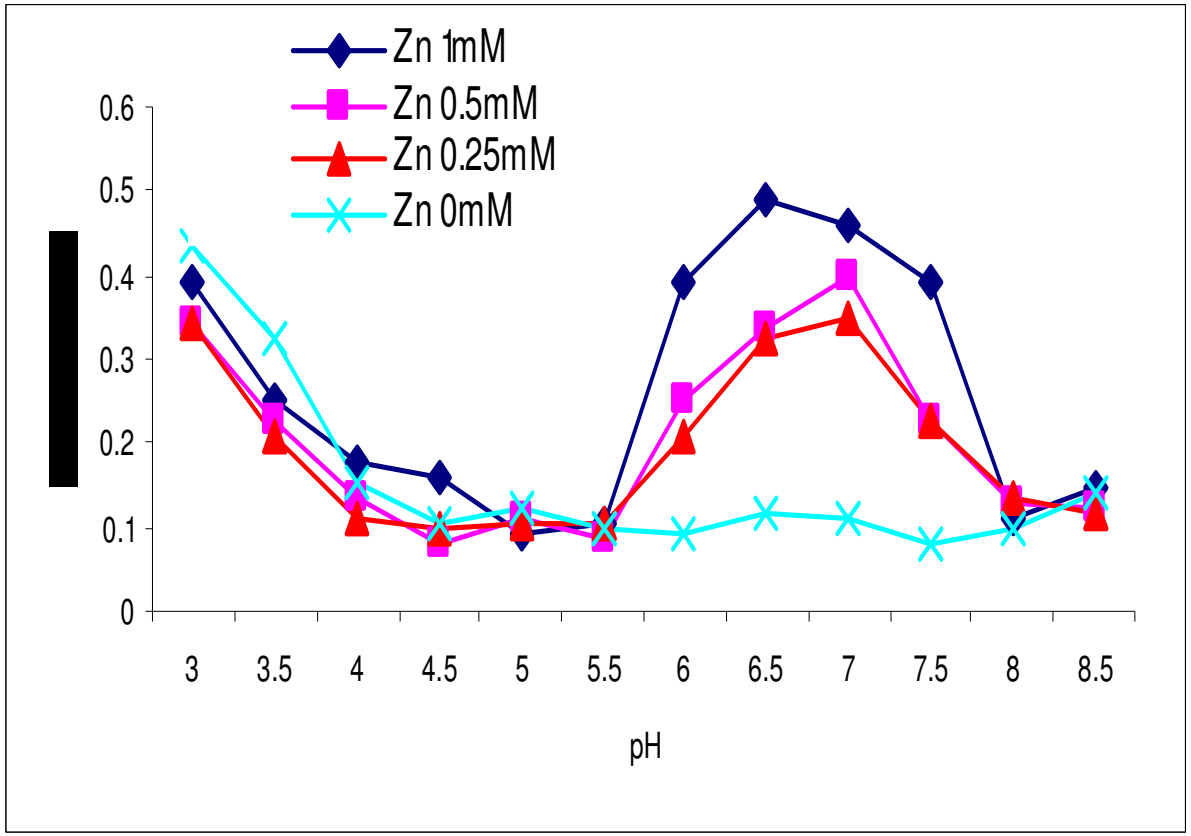
Optimized Zn^{++} on ASIC 2a+3

- Effect of Zn^{++} on ASIC 2a+3



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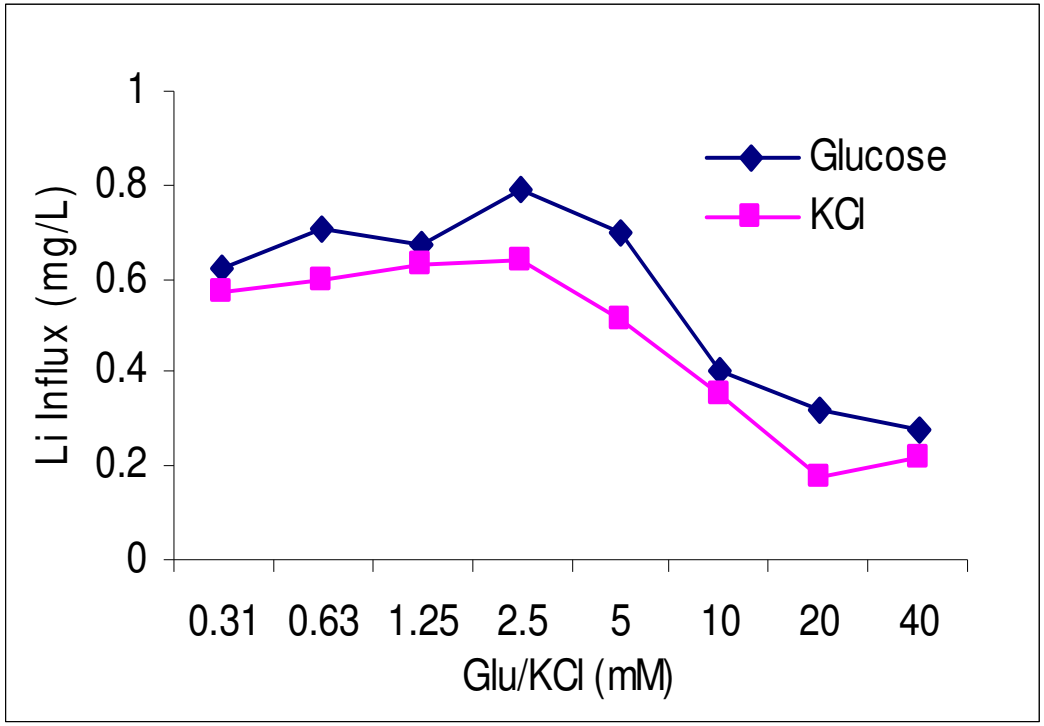
Effect of Glucose & KCl

- Effect of Glucose and KCl on ASIC 2a+3
- Thus showing effect on ASIC2a+3 beyond 5mM concentration



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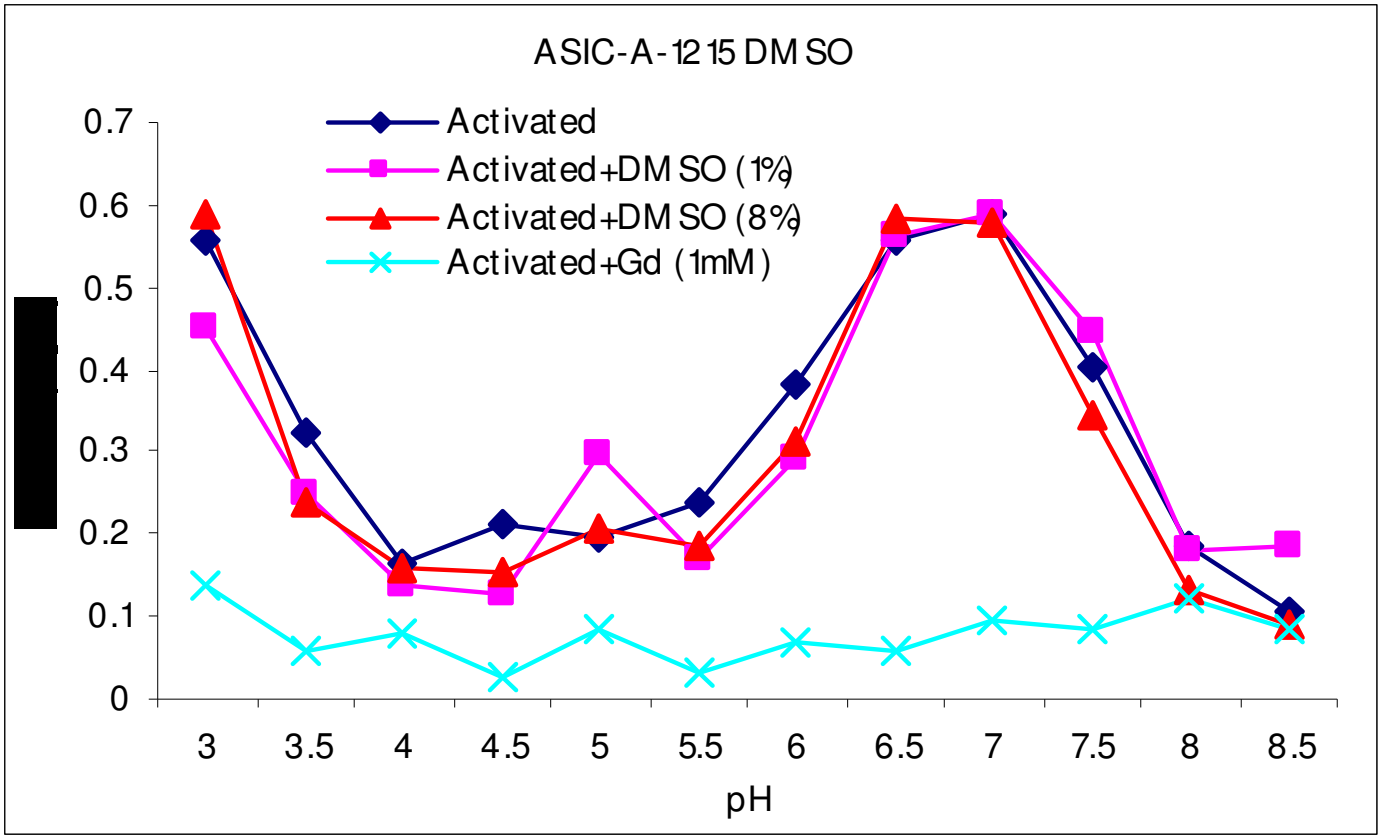
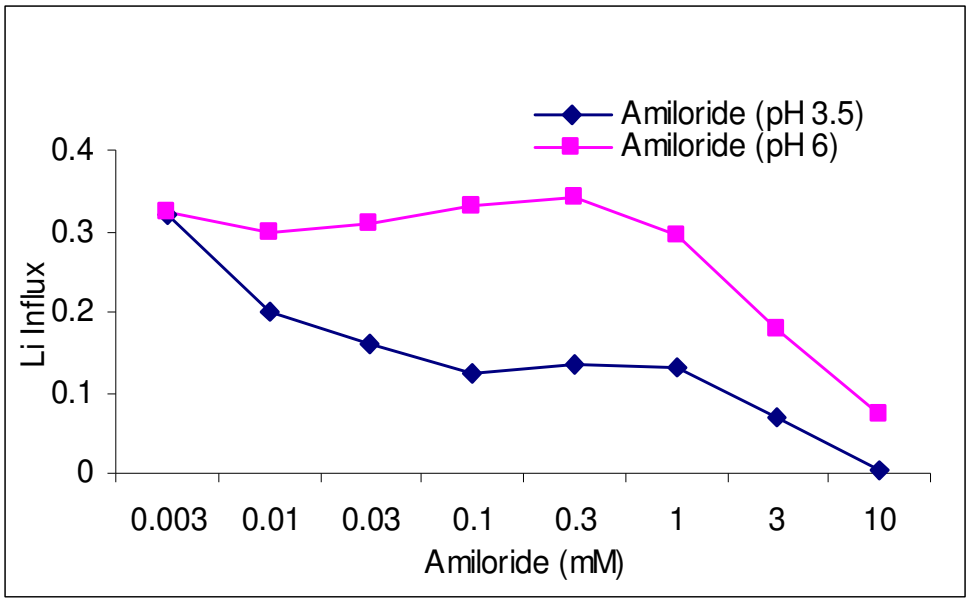
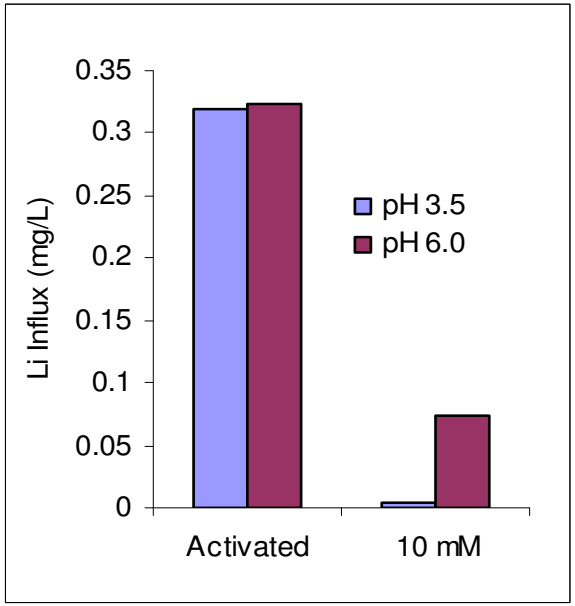
Effect of DMSO on ASIC2a+3



Amiloride @ pH 6, and 3.5

- Comparison of the effect of amiloride
  - pH 6.0 & pH 3.5 on ASIC2a+3





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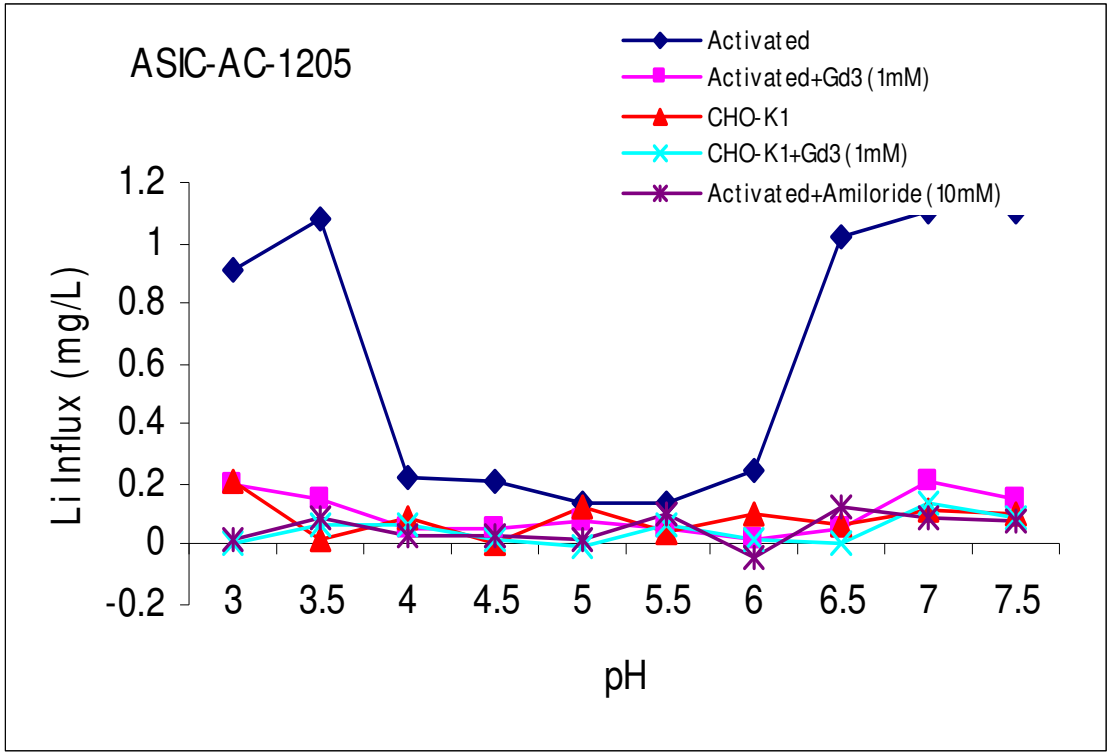
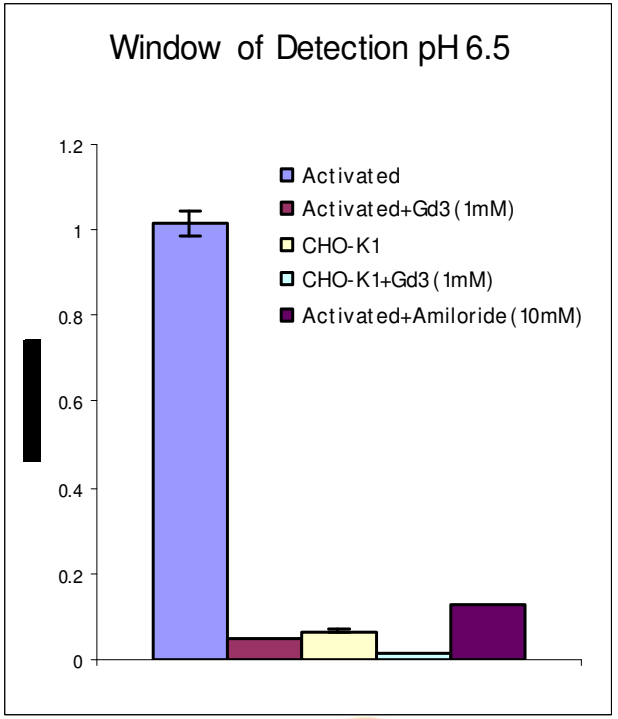
Gadolinium @ pH 6.5, and 3.5

- Comparison of the effect of Gadolinium
  - pH 7.5, 6.5 & 3.5 on ASIC2a+3





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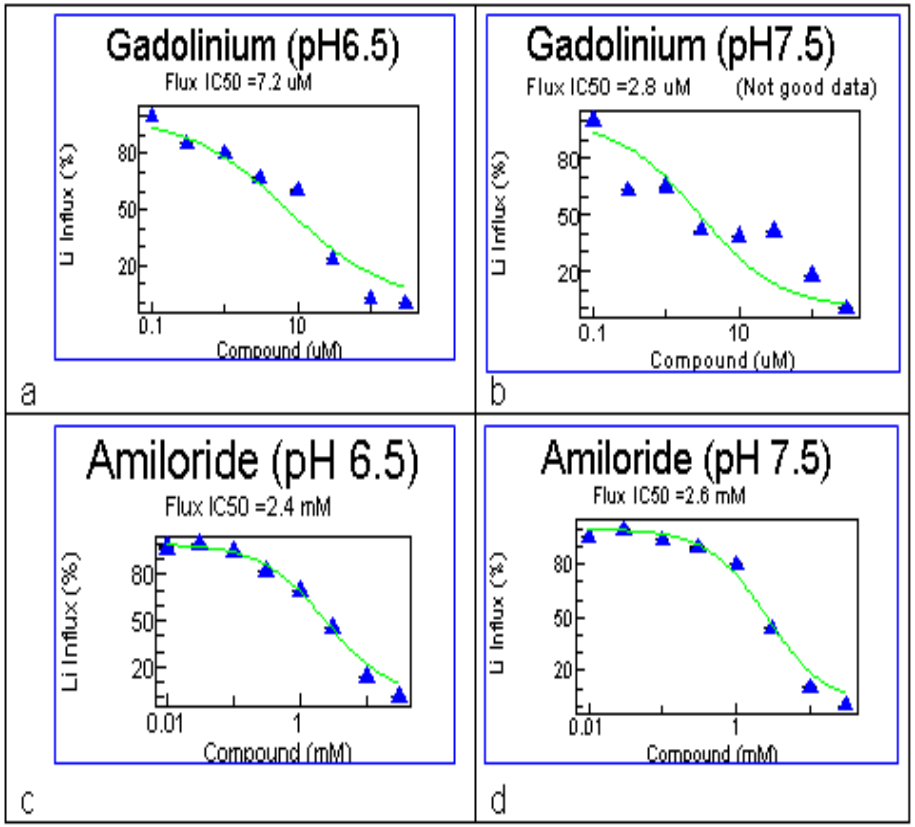
Gadolinium & Amiloride- pH range

- Comparison of the effect of Gadolinium & Amiloride
  - pH 7.5, 6.5 & 3.5 on ASIC 2a+3



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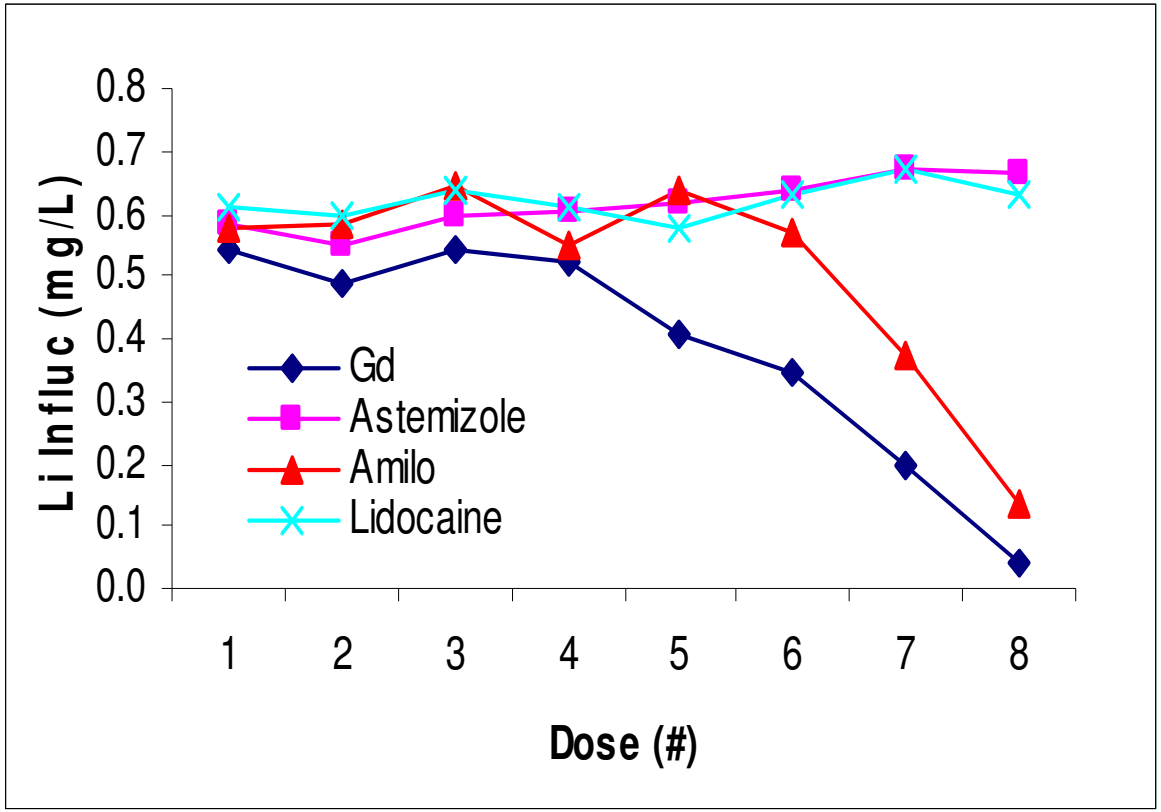
Other Compounds & ASIC2a+3

- Effect of Gadolinium & other compounds
  - pH 7.5, 6.5 & 3.5 on ASIC2a+3 subunits



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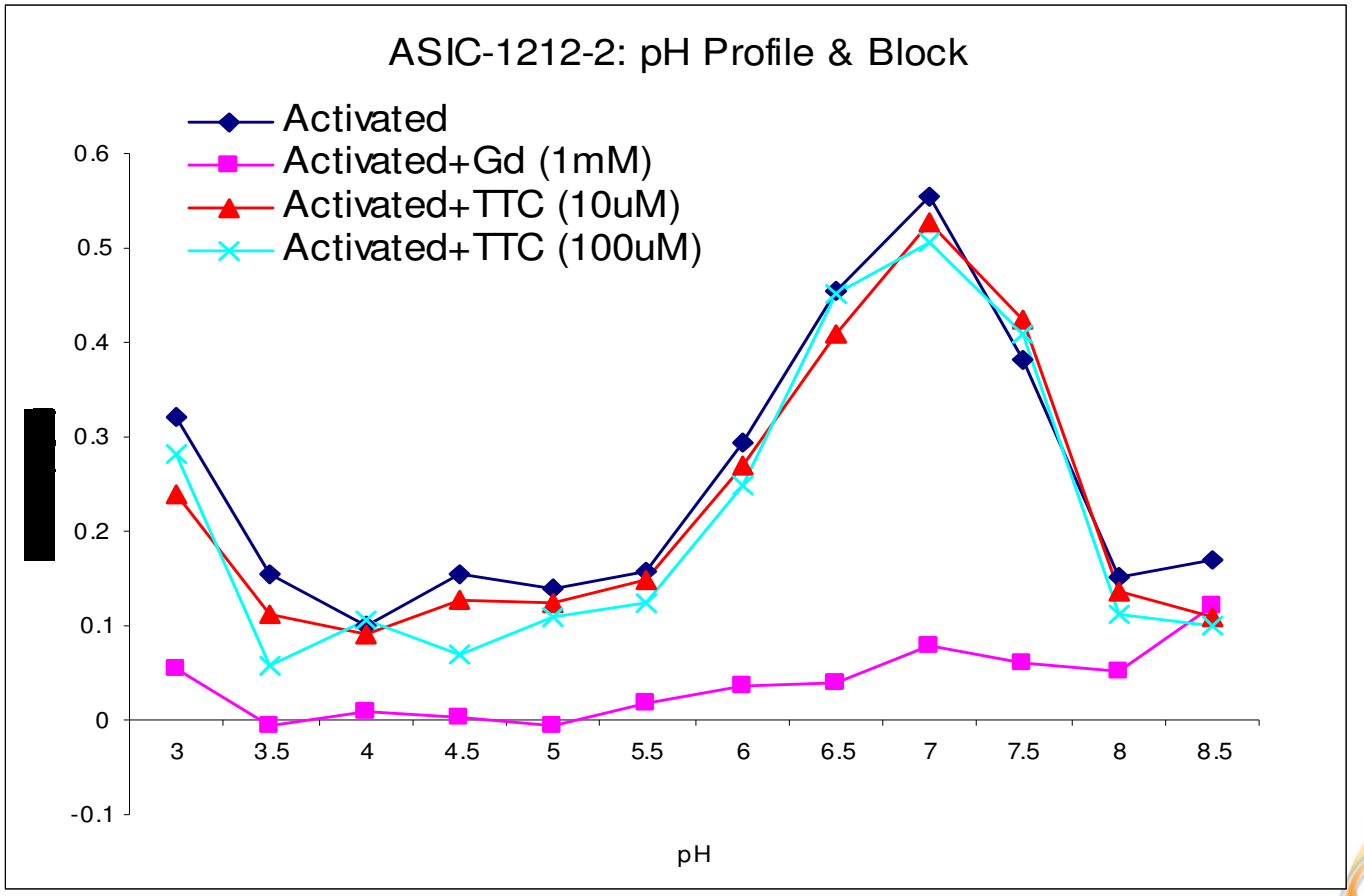
Gadolinium & TTC

- Effect of Gadolinium & Tetracaine (general anaesthetic)



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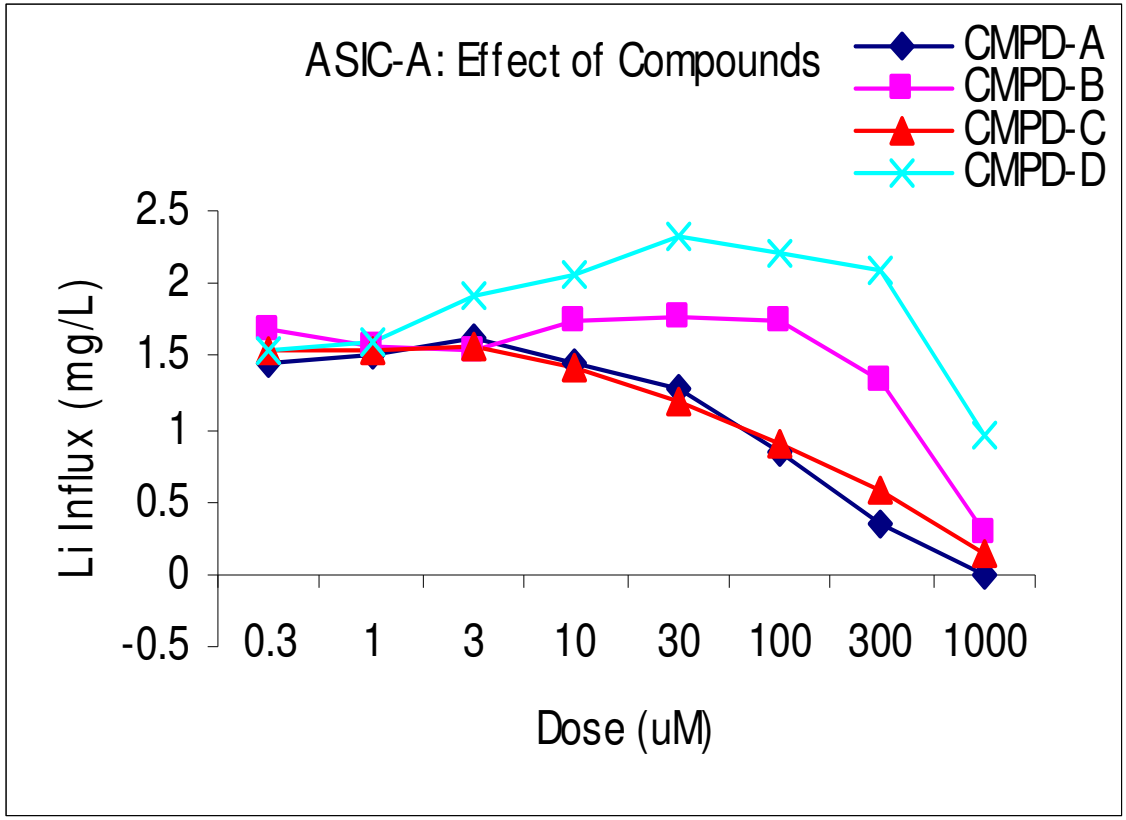
Test Compounds: ASIC2a+3

- Effect of 4 blinded compounds



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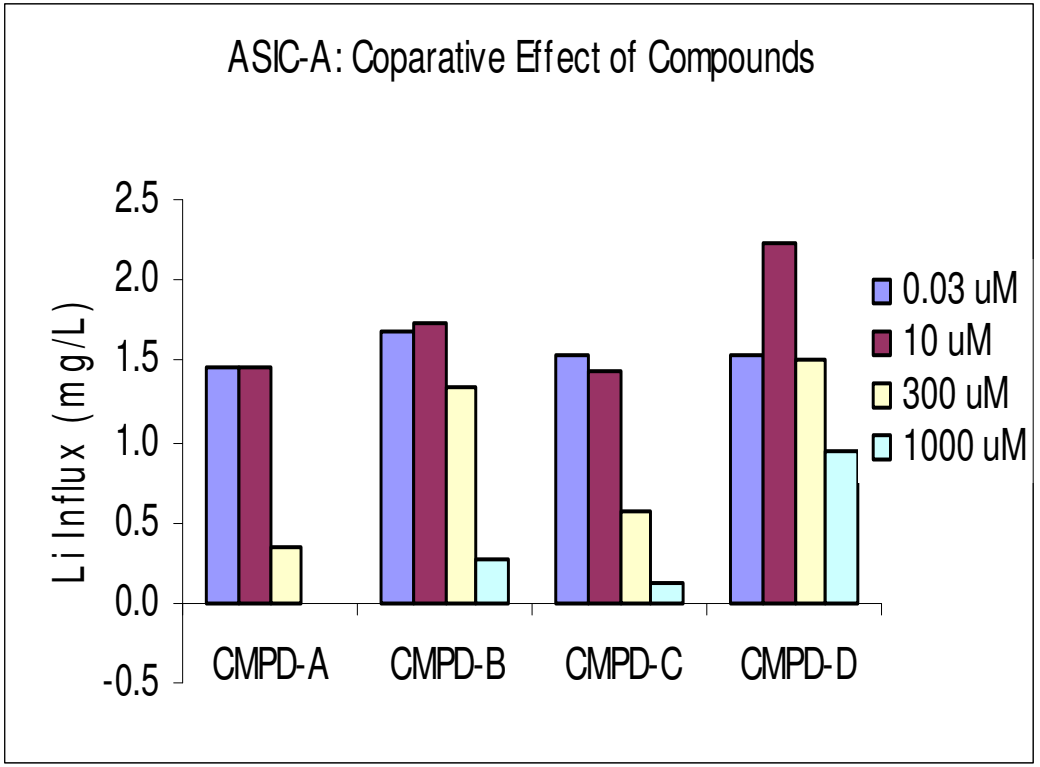
Test Compounds: ASIC2a+3

- Effect of 4 blinded compounds



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Test Compounds: ASIC12a+3

	Clone A(Presence of Zn)			Clone A (Absence of Zn)		
Compound	**IC50 (Upto 1000uM dose)**	**IC50 (Upto 100uM dose)**	**Rank Order**	**IC50 (Upto 1000uM dose)**	**IC50 (Upto 100uM dose)**	**Rank Order**
B	NA	2.4	1	NA	2.25	1
D	17.21	NA	2	19.47	NA	2
A	22.29	22.83	3	24.26	NA	3
C	32.52	NA	4	55.49	NA	4

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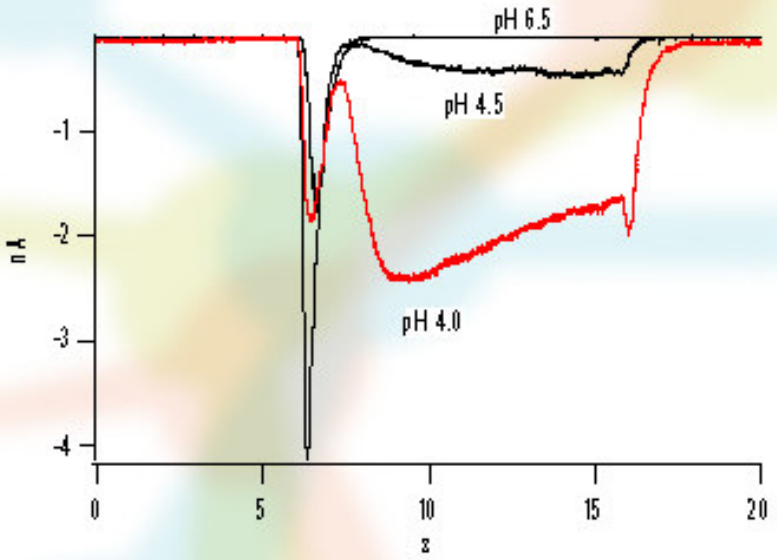
EP Profile of ASIC3

The effect of pH on CHO clone B



pH stimulation from 7.0 to 4.5 gives maximum peak amplitude

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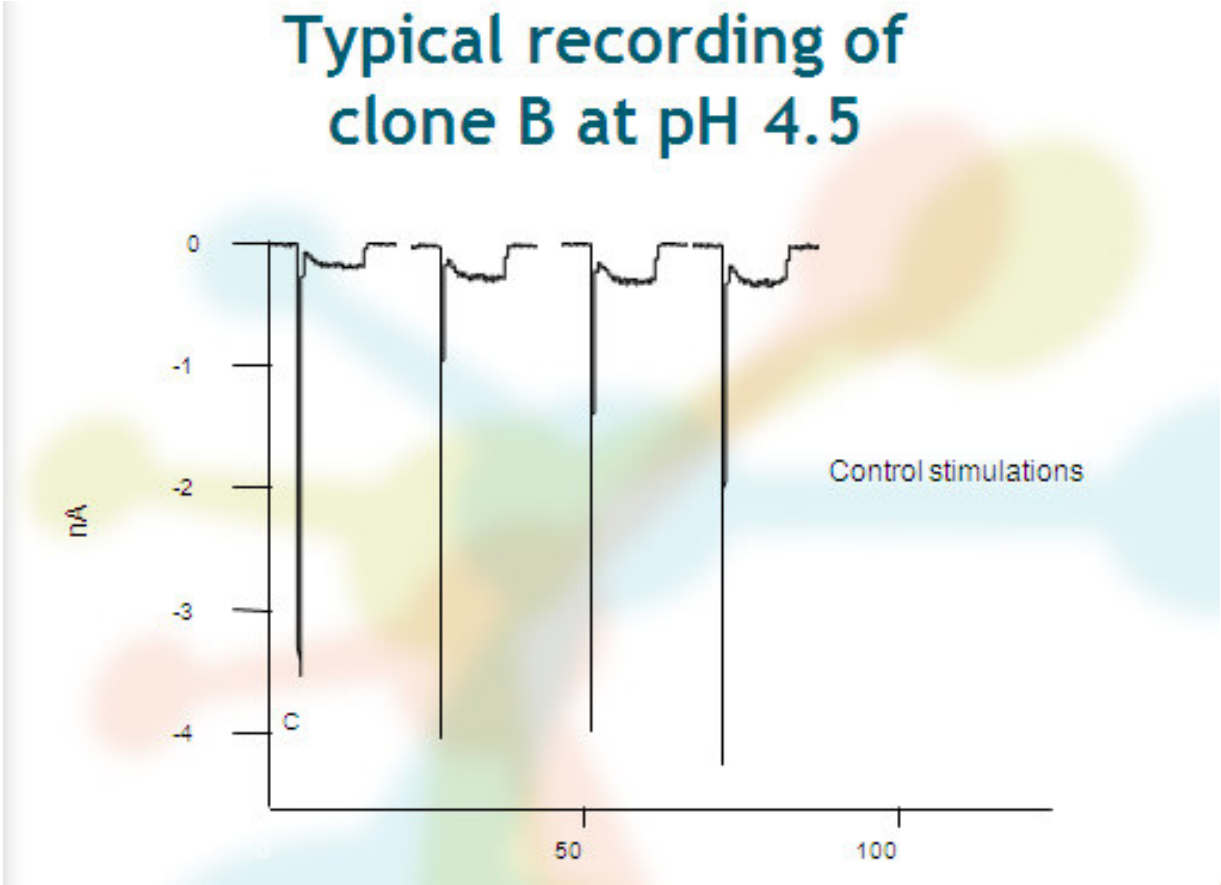
EP Profile of ASIC3



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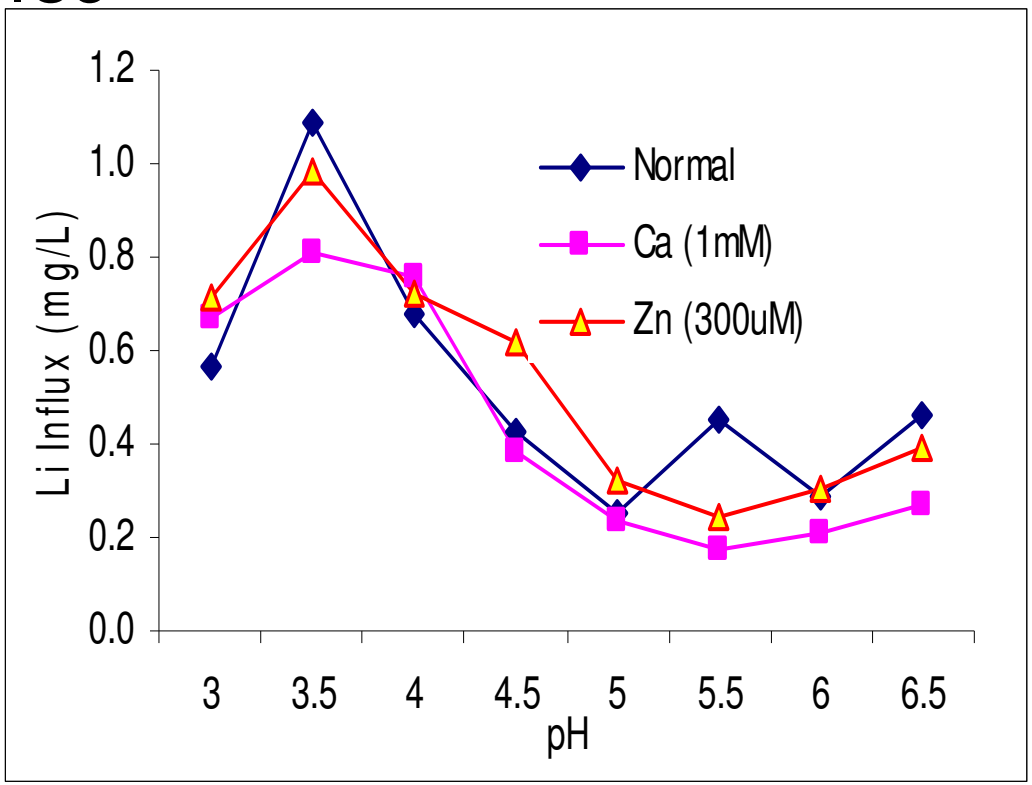
Effect of Zn^{++} on ASIC3

- pH range of ASIC3 activation
- Effect of Zn^{++} and Ca^{++} - No significant effect on ASIC3



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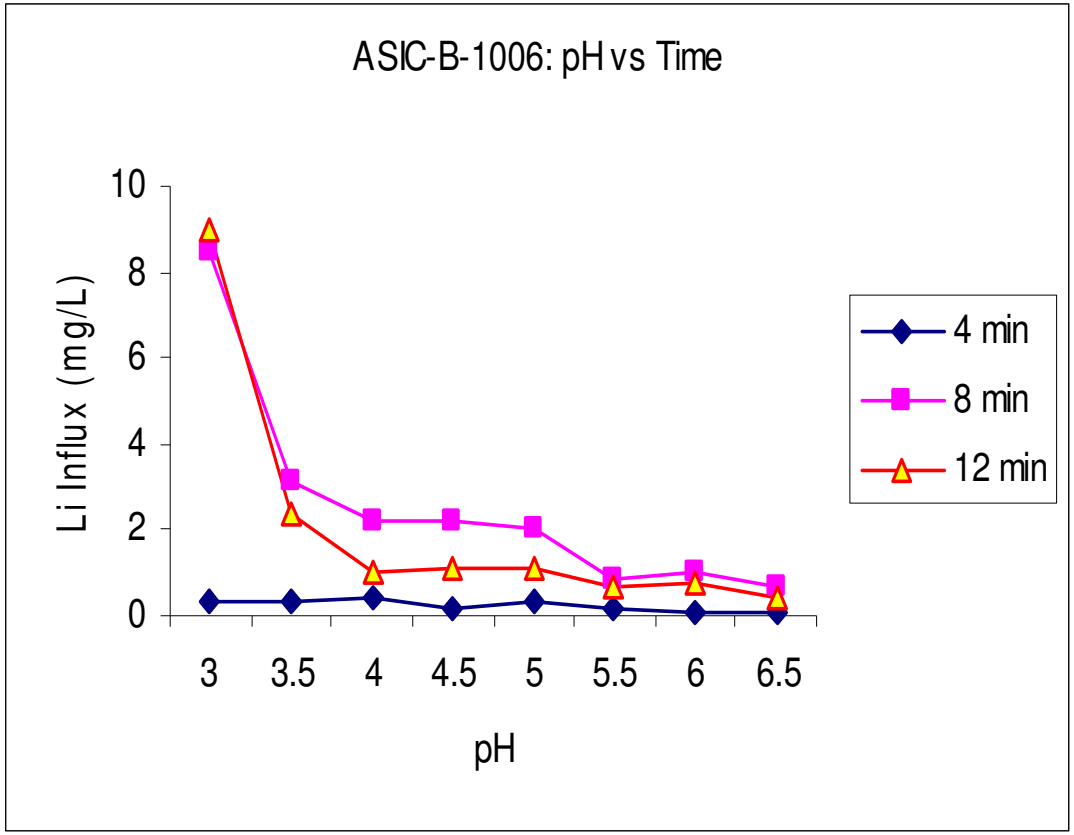
ASIC 3 activation-pH & duration

- Effect of pH & duration on ASIC 3



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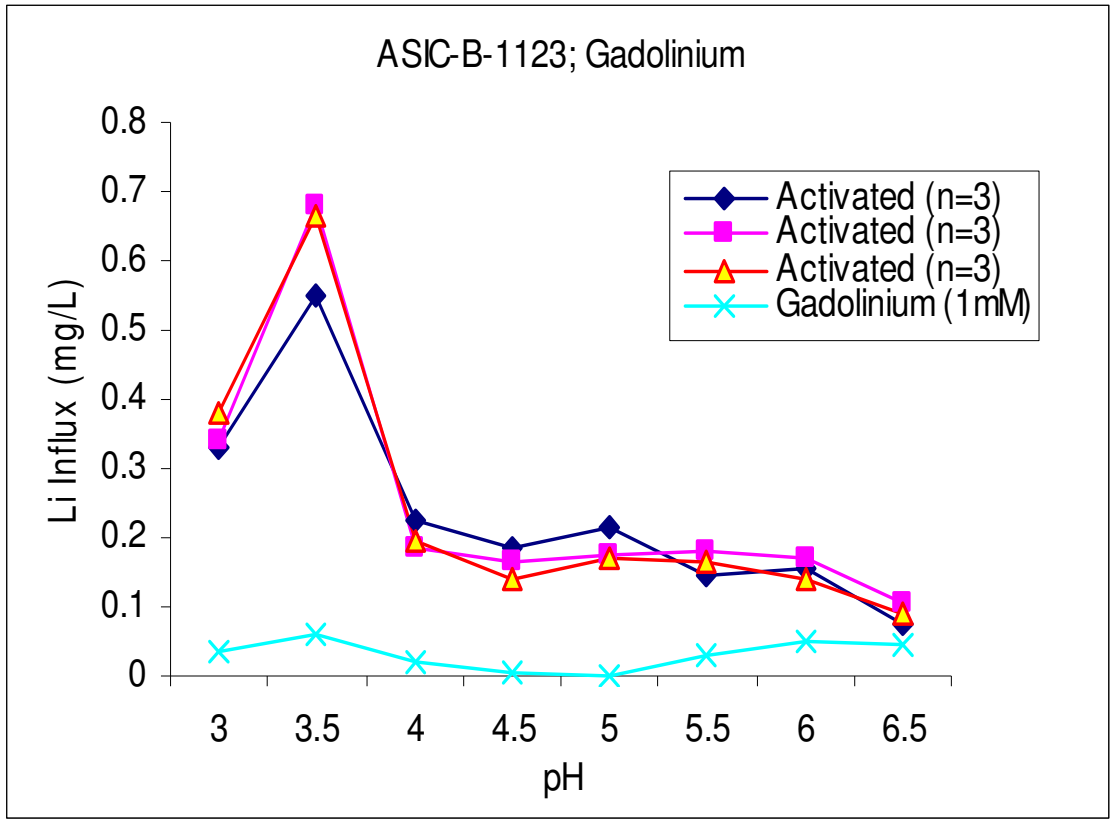
Gadolinium & ASIC 3

- Effect of Gadolinium on ASIC 3



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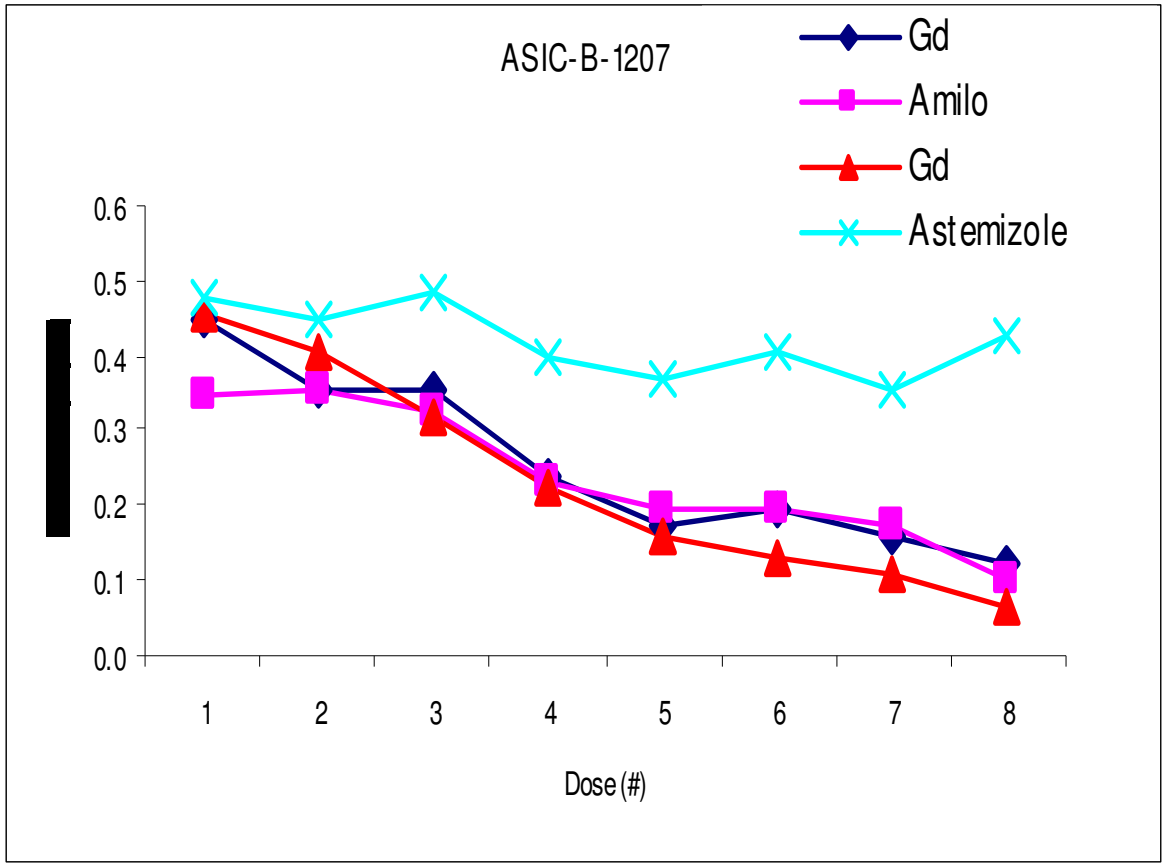
Effect on ASIC 3

- Comparison of the effect of some compounds on ASIC 3 at pH 3.5



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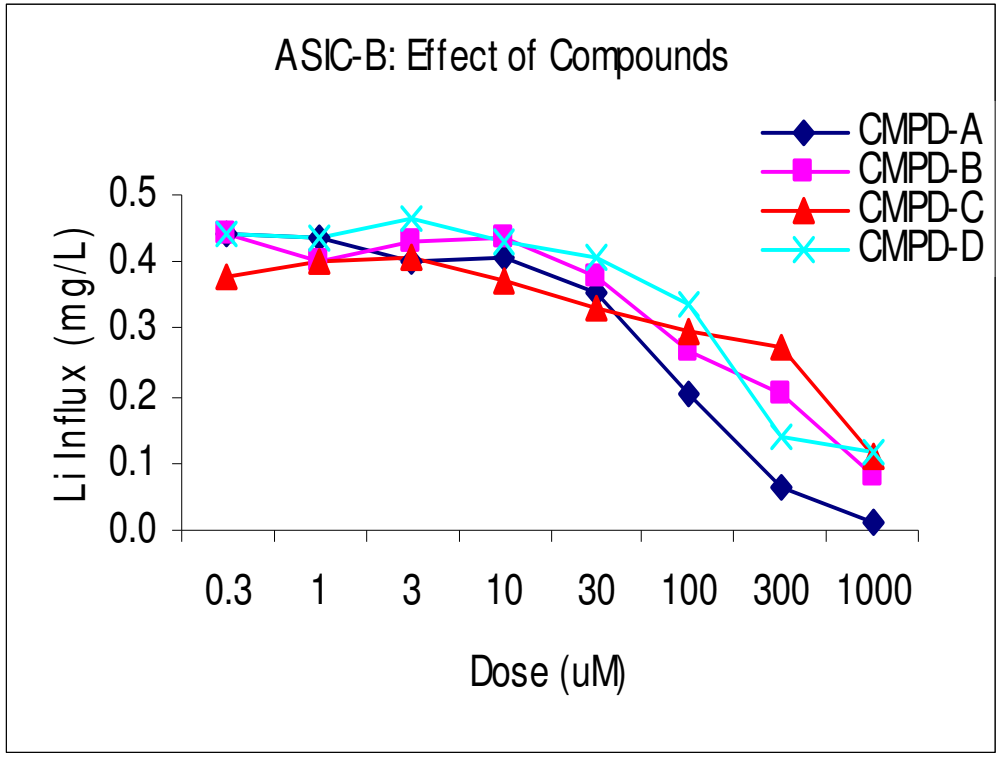
Test Compounds: ASIC3

- Comparison of the effect of some compounds on ASIC 3 at pH 3.5



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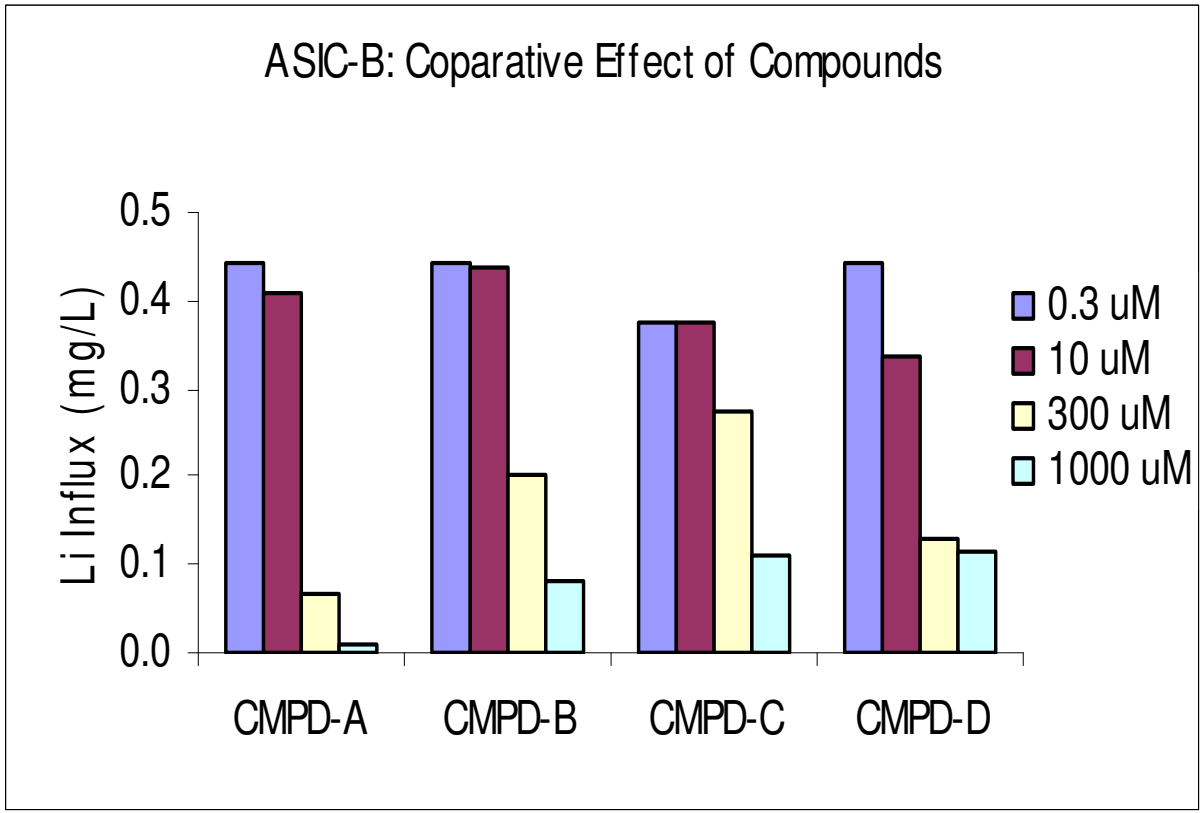
Test Compounds: ASIC3

- Comparison of the effect of some compounds on ASIC 3 at pH 3.5



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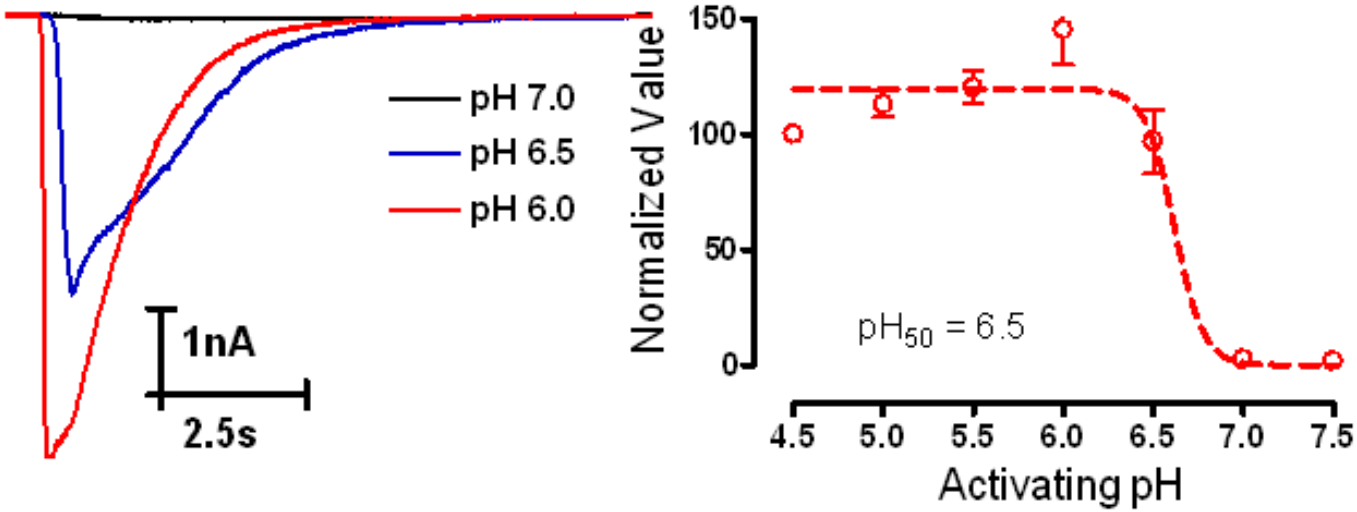
EP Profile of ASIC1a



- Zinc inhibits ASIC1a specific currents
- Calcium is important as the receptor is also calcium permeable

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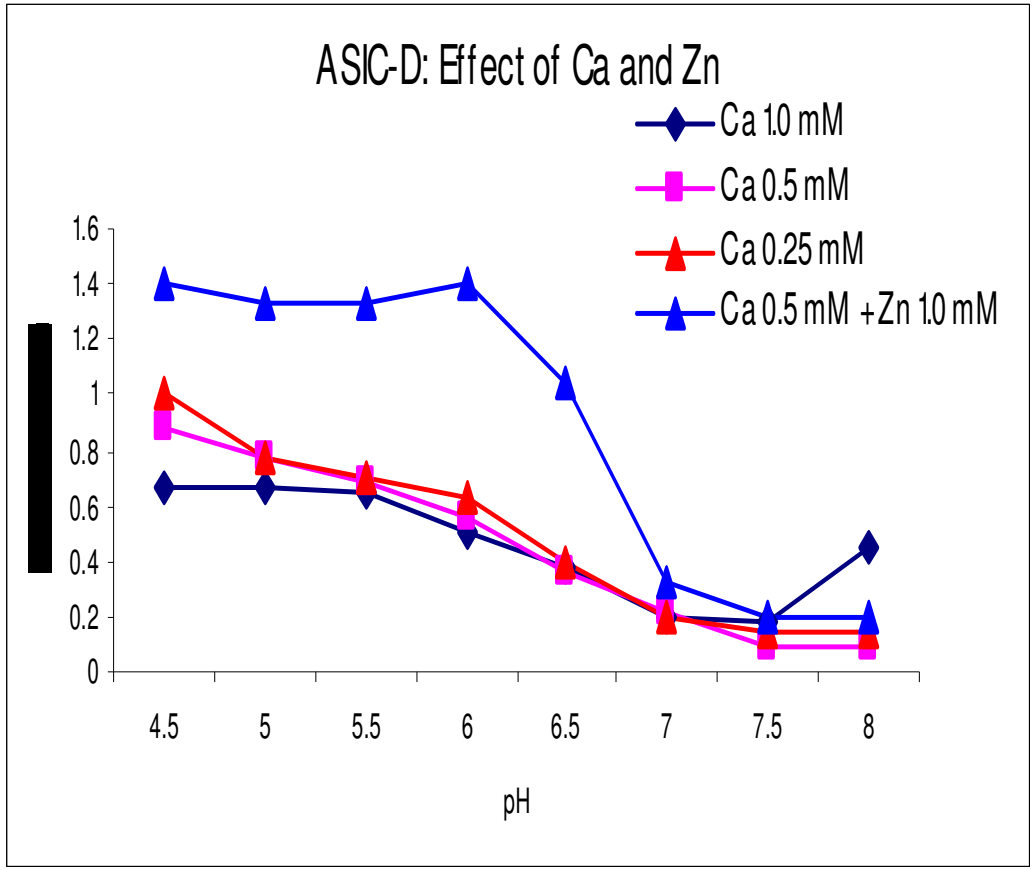
Activation of ASIC1a

- Effect of Ca^{++} Zn^{++} on ASIC 1a



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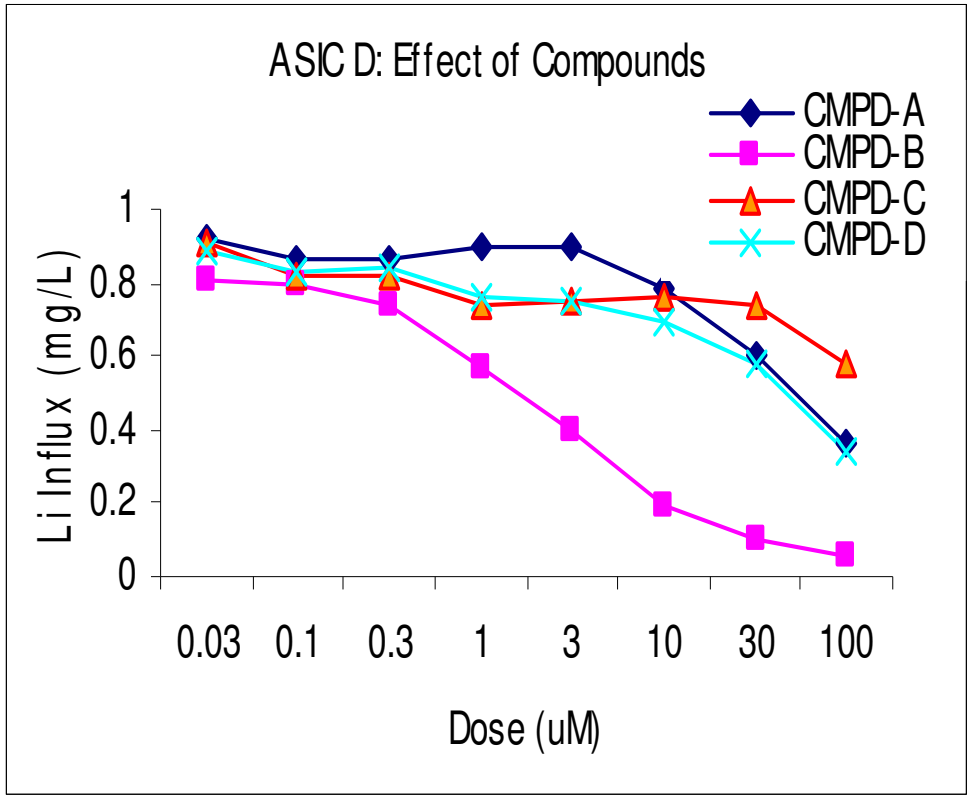
Test Compounds: ASIC1a+2a

- Effect of Gadolinium on clone D: ASIC 1a



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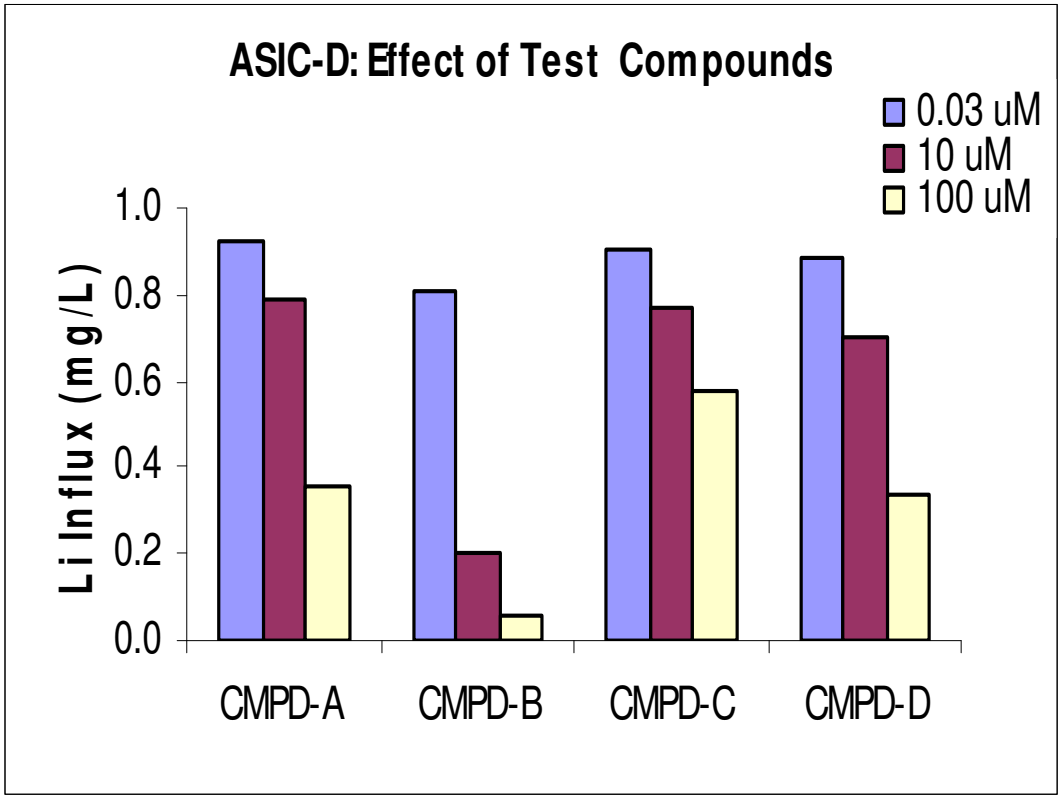
Test Compounds: ASIC1a

- Effect of Gadolinium on clone D: ASIC 1a



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Stretch Activated Ion Channels

Therapeutic Importance

- Stretching the myocardium can have profound effects
  - Electrical activity called mechanoelectric feedback
    - Can be powerful enough to trigger arrhythmias
  - Likely the underlying trigger for some clinically intraceable arrhythmias
- Involves two types of mechano-sensitive ion channels
  - A nonspecific cation channel & potasium channel(s)
    - Former channel currents well characterized
    - Blocked by a pep-toxin (*Grammastola spatulata)*
      - An effective antiarrhythmic agent against stretch-induced atrial arrhythmias

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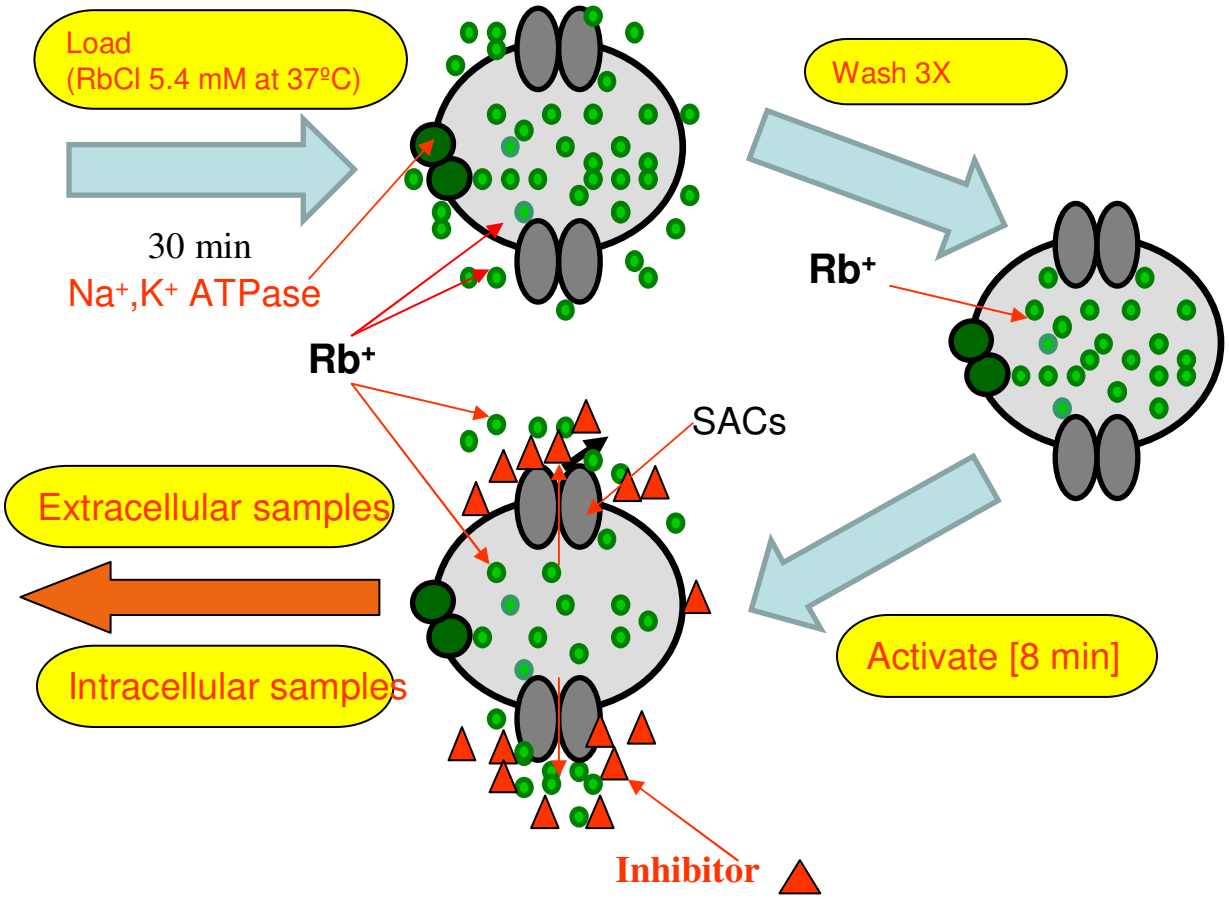
Therapeutic Importance (contd)

- Although some nonspecific activators and blockers of these channels exist
  - No specific blocker is available
    - Limits investigation of these channels in arrhythmogenesis
- The potential for the development of blockers of both types of stretch-activated channels as therapeutic agents needs to be carried

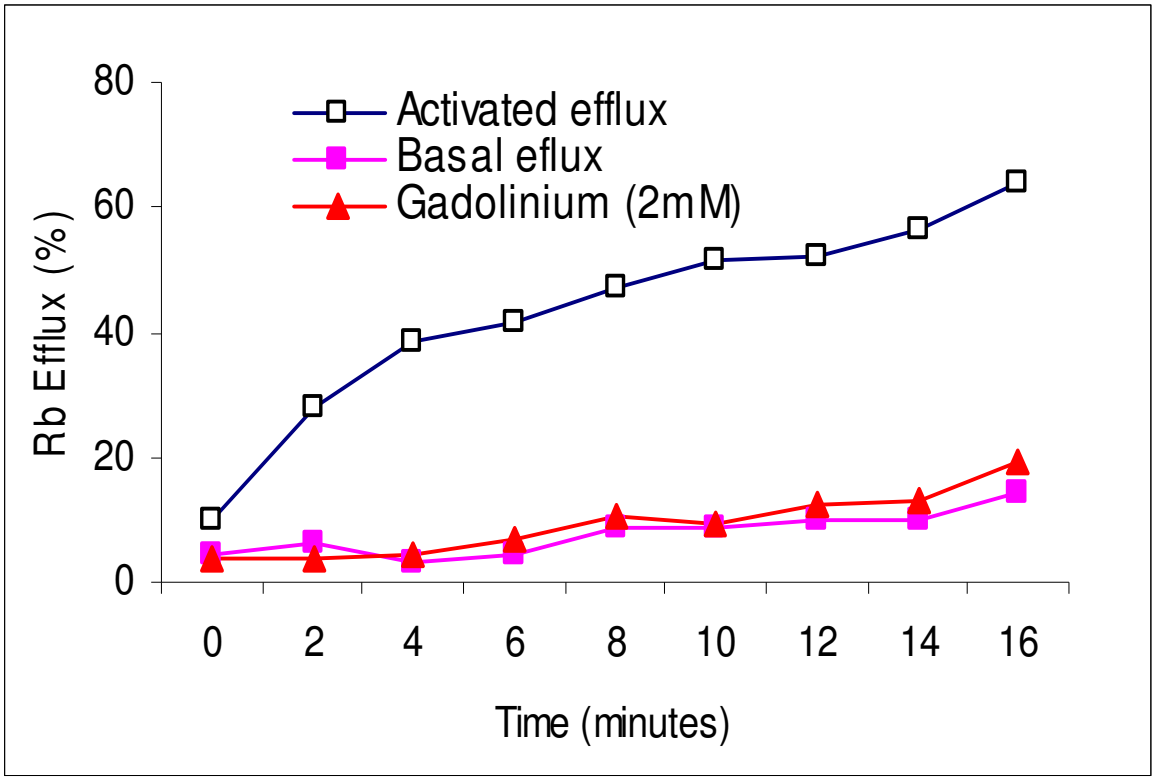
Protocol: SACs Rb^+ Efflux

CHO-K1
50,000 Cells/Well
[Incubate 5% CO2 at 37ºC, 24h]

ICR 8000



Period of Activation



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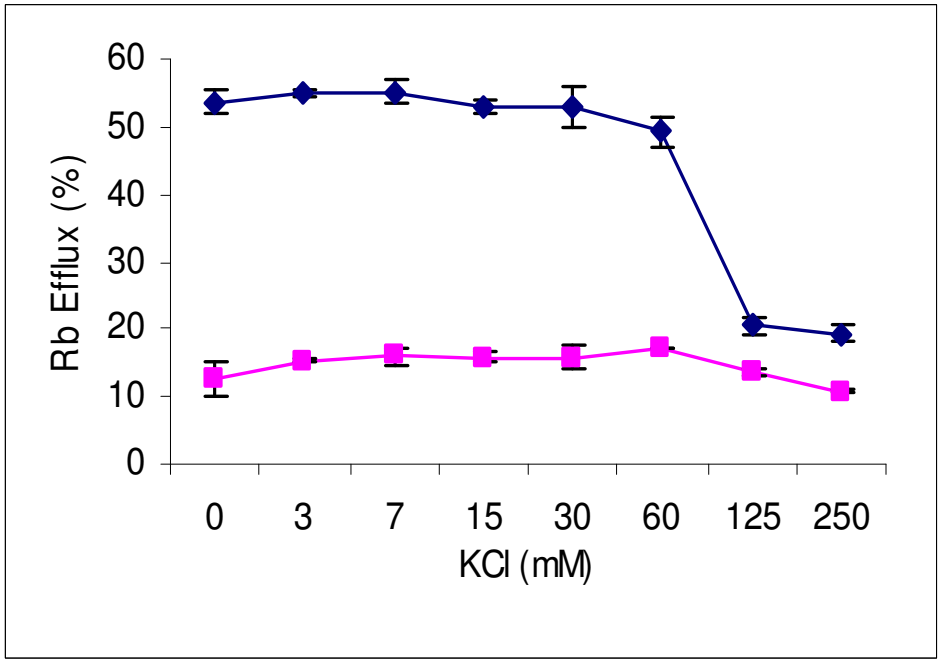
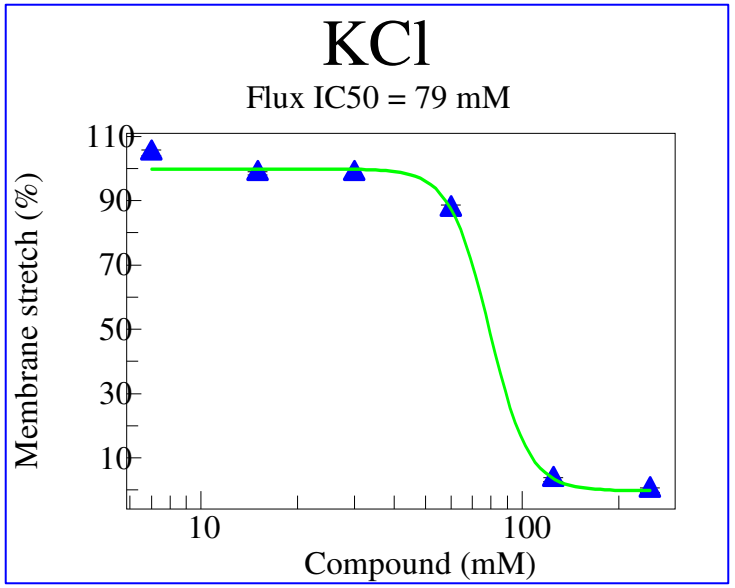
Effect of Depolarization

- CHO-hERG Cell line
  - 60mM of KCl does not effect the efflux of SAC





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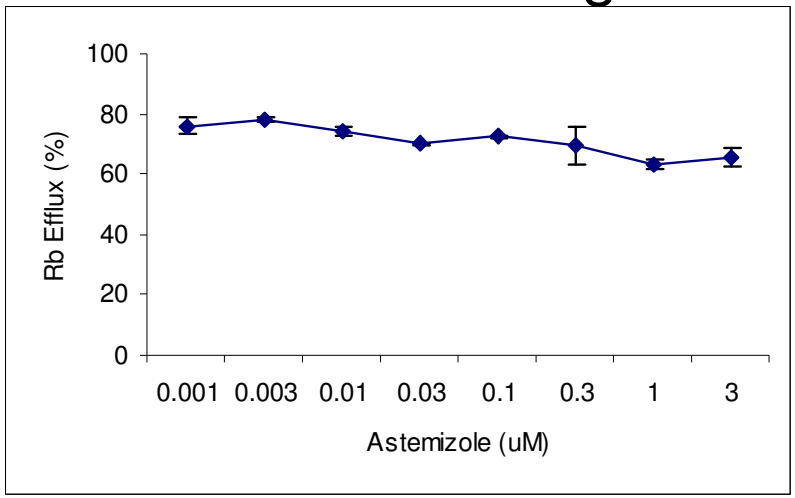
Effect of Depolarization

- CHO-hERG Cell line
  - hERG does not get activated under these conditions
  - 60mM of KCl does not effect the efflux of SAC
- Suggested that the SACs are not voltage gated
  - Presence of inward rectifier hERG
  - Astemizole had no significant effect on Rb+ efflux.



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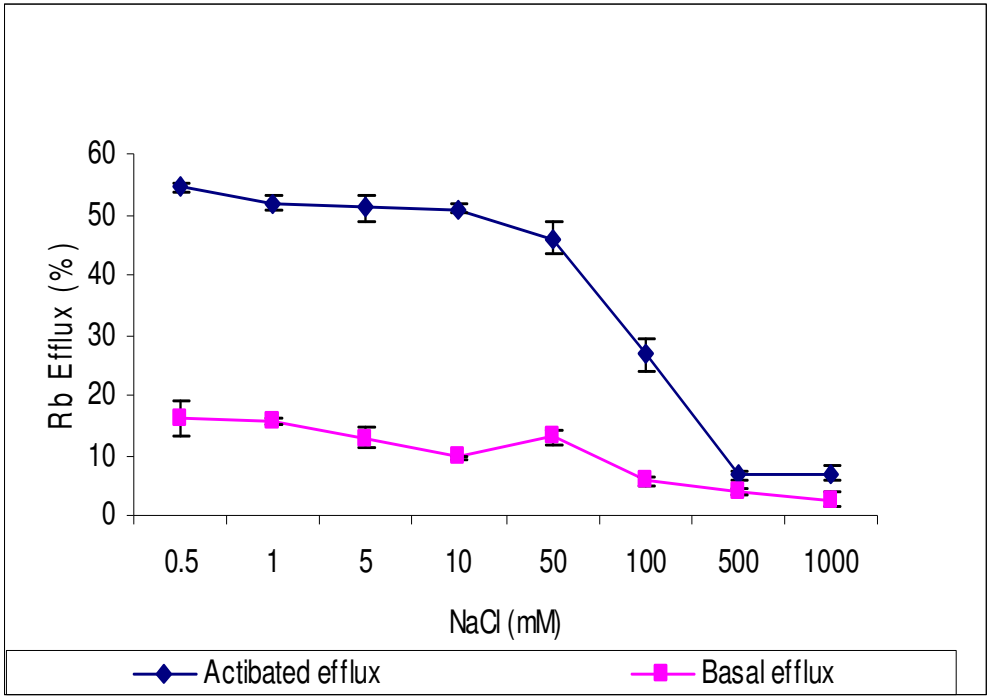
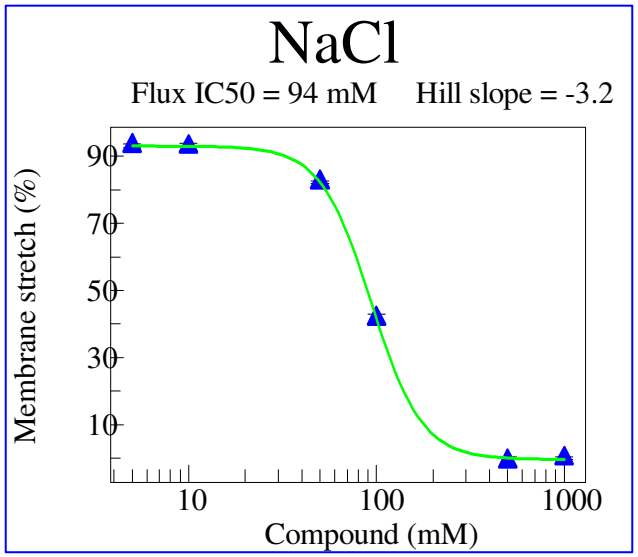
Effect of NaCl





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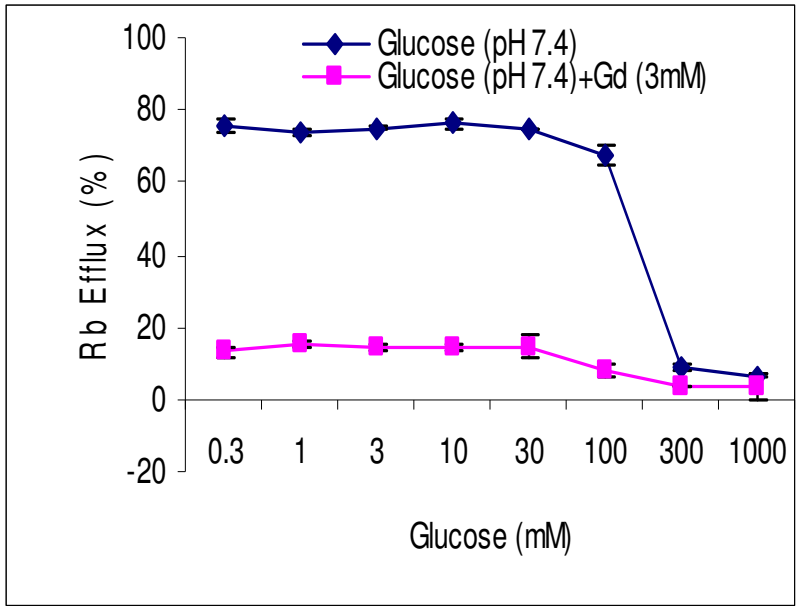
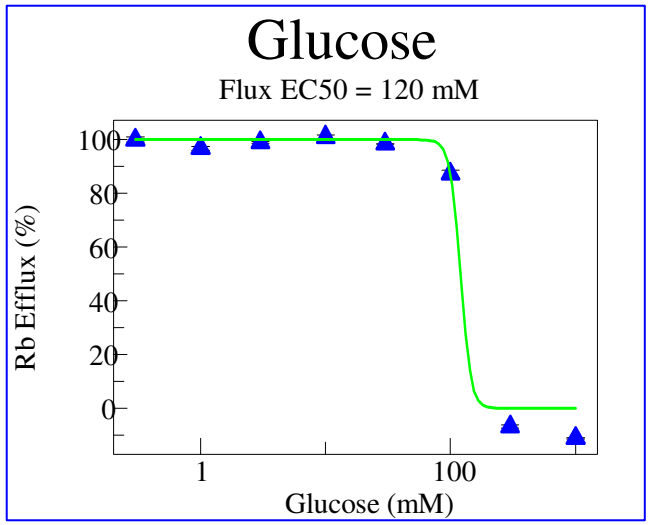
Effect of Glucose





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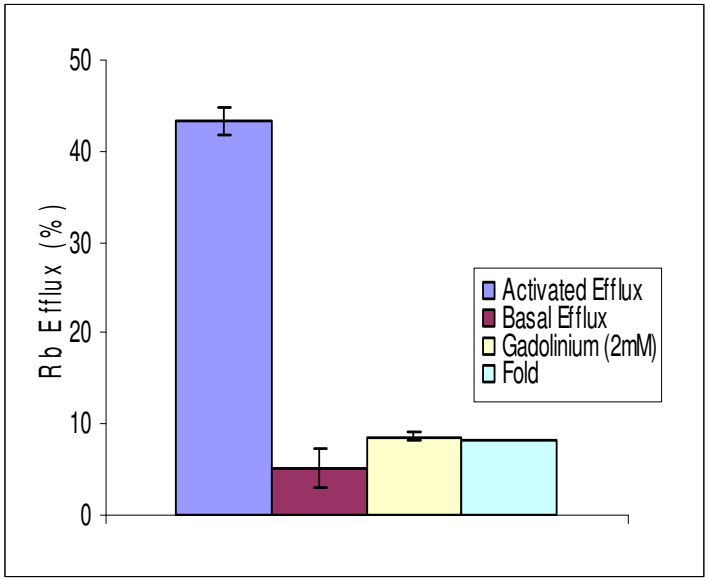
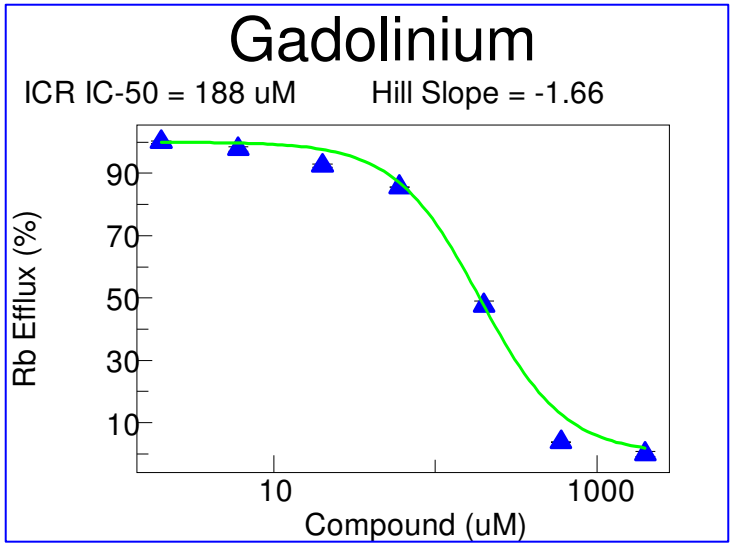
SA K^+ Channels





EP IC_{50}= 46.2 uM Zhang et al, 2000

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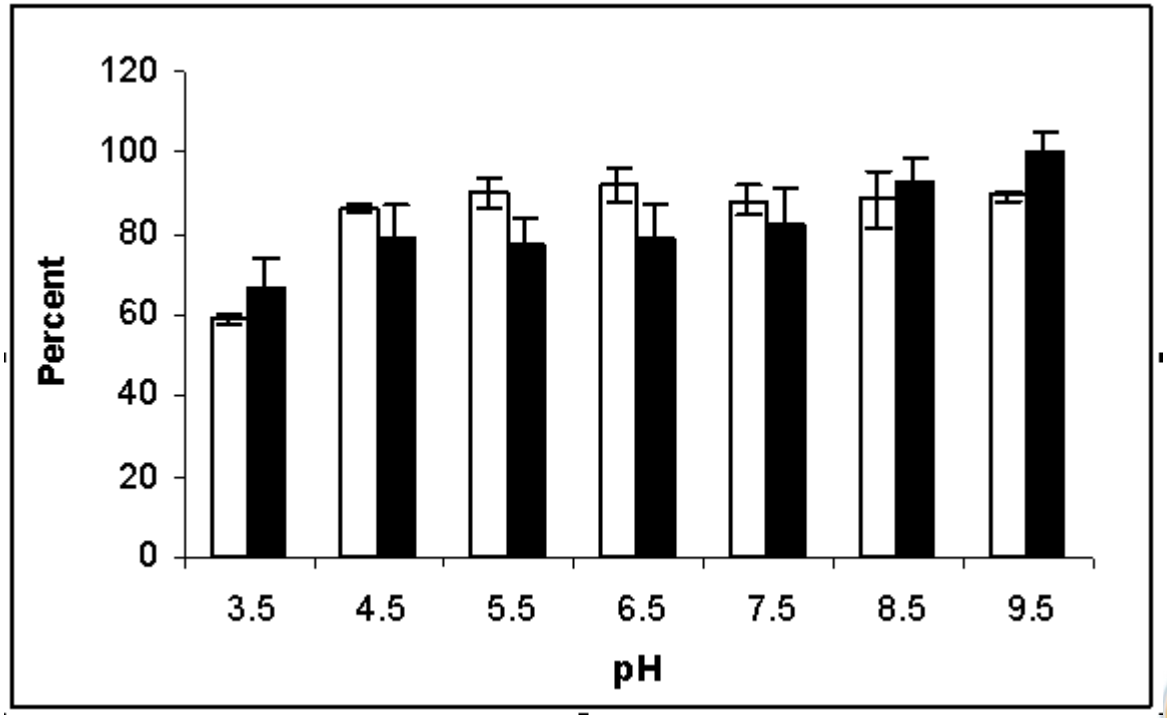
Effect of pH

- pH showing no significant effect on the Rb+ efflux except pH 3.5 where total Rb uptake (open bars) of the cells was also affected



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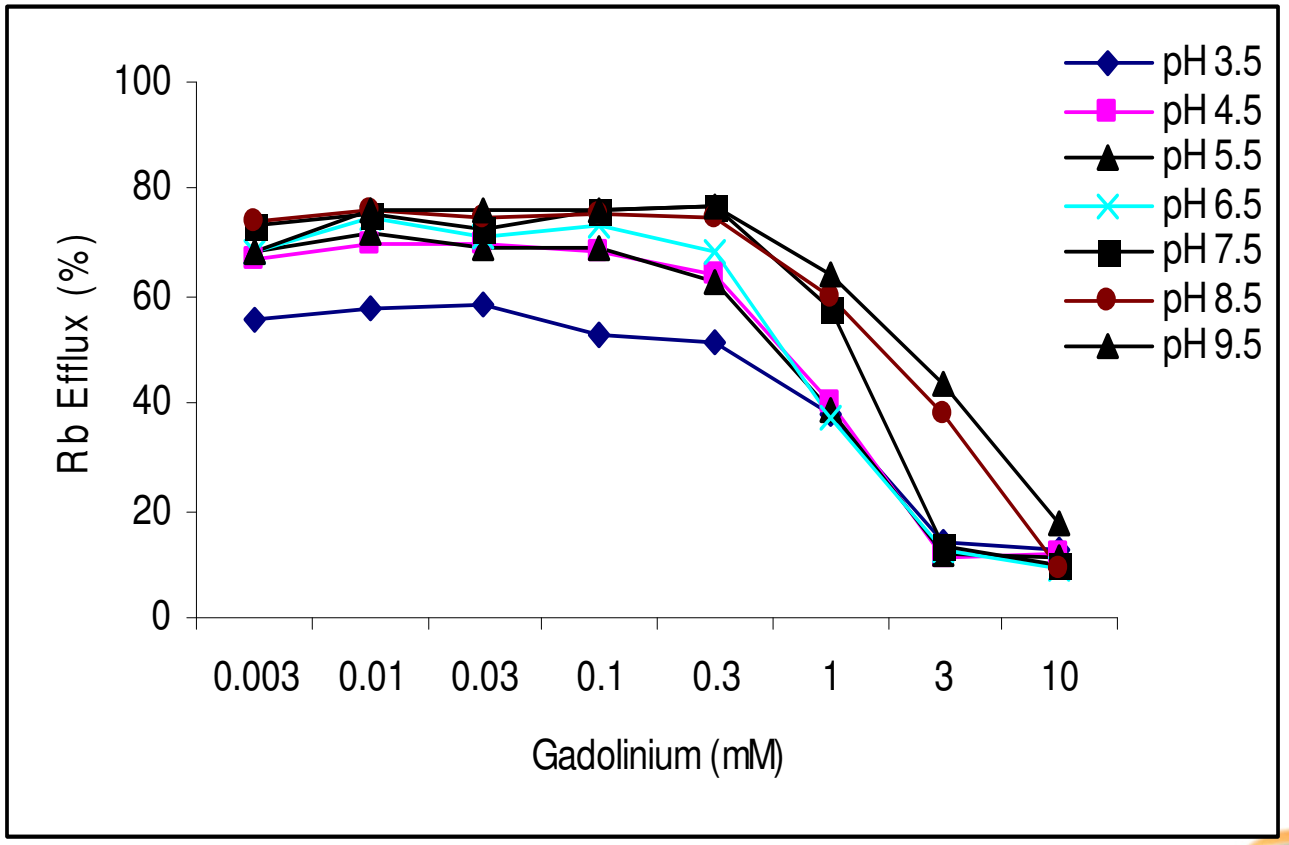
Effect of pH on Gadolinium



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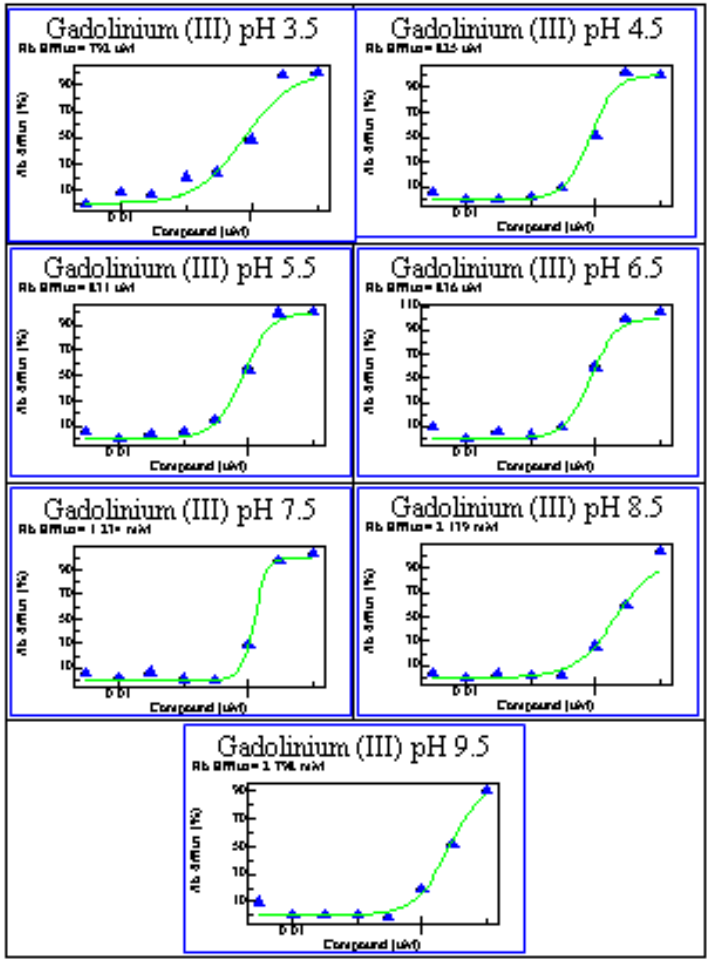
Effect of pH

pH	IC_{50} (uM)	
	CHO cell line transfected with hERG	CHO cell line
3.5	792	92
4.5	825	111
5.5	831	127
6.5	836	167
7.5	1234	188
8.5	2139	354
9.5	2798	402



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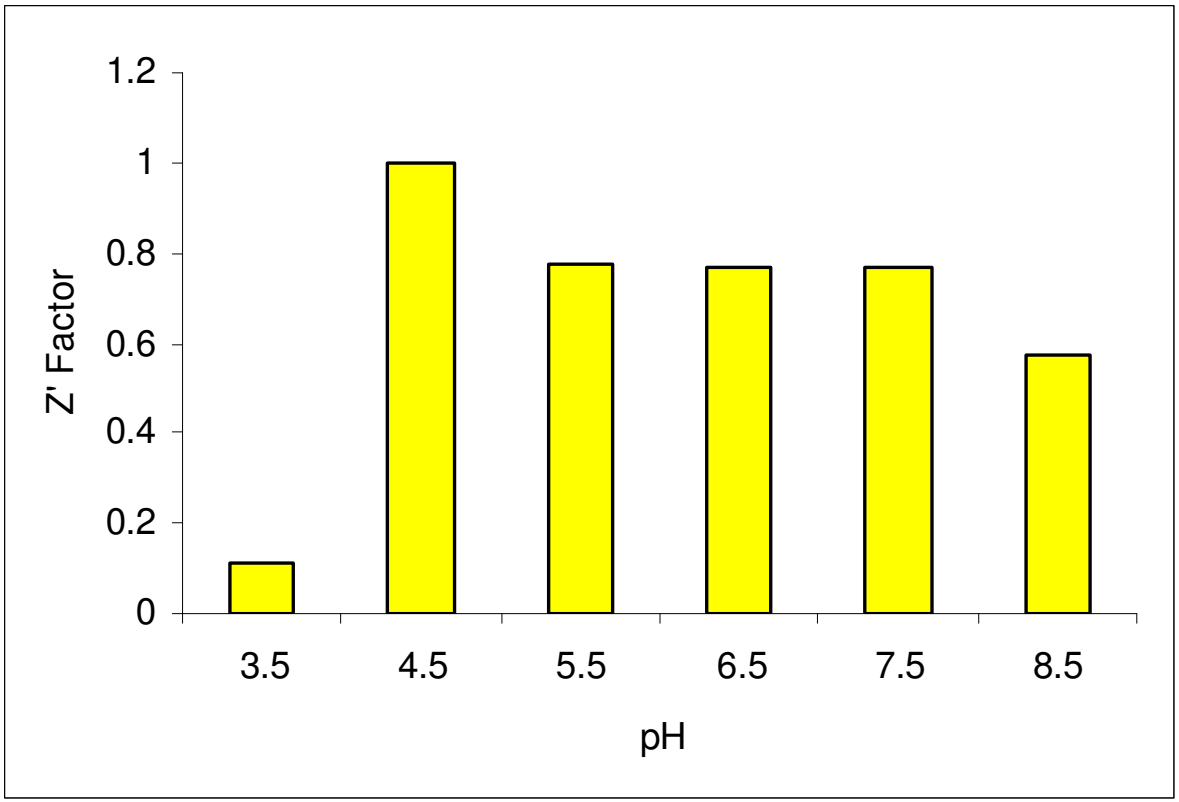
Effect of pH on Gadolinium

- Effect of pH on Gd^{3+} interaction of with SACs showing the pH range for Gd3+
  - Most optimal pH4.5 to 6.5
  - Least effect 8.5 to 9.5 had on the Rb efflux inhibition
  - Lowest IC_{50} pH 3.5
    - May be due to the cell health at this drastic pH
    - Aa the total Rb uptake carried by Na+,K+ ATPase was also low in comparison to rest of the pH range.

Z-Factor Inter Assay Variability



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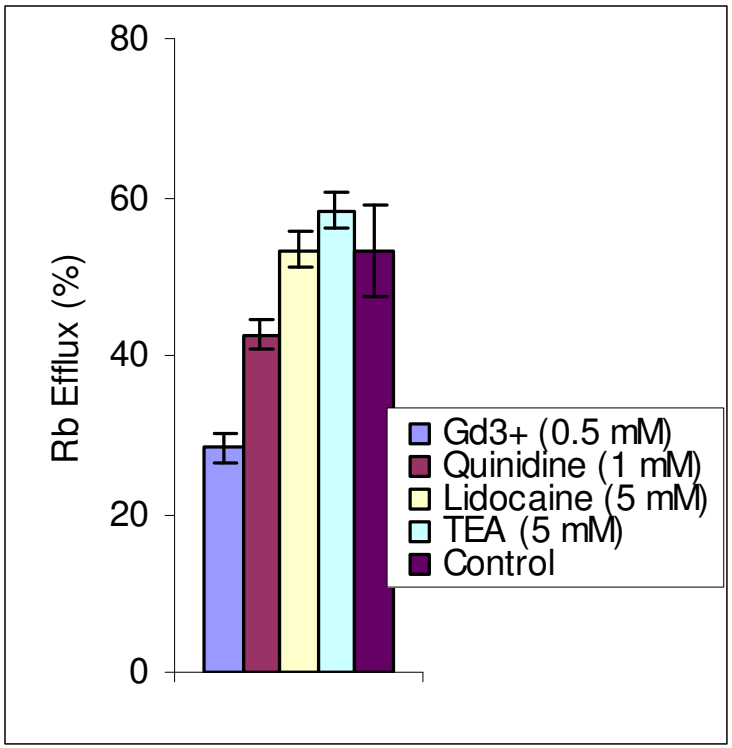
Effect of Drugs



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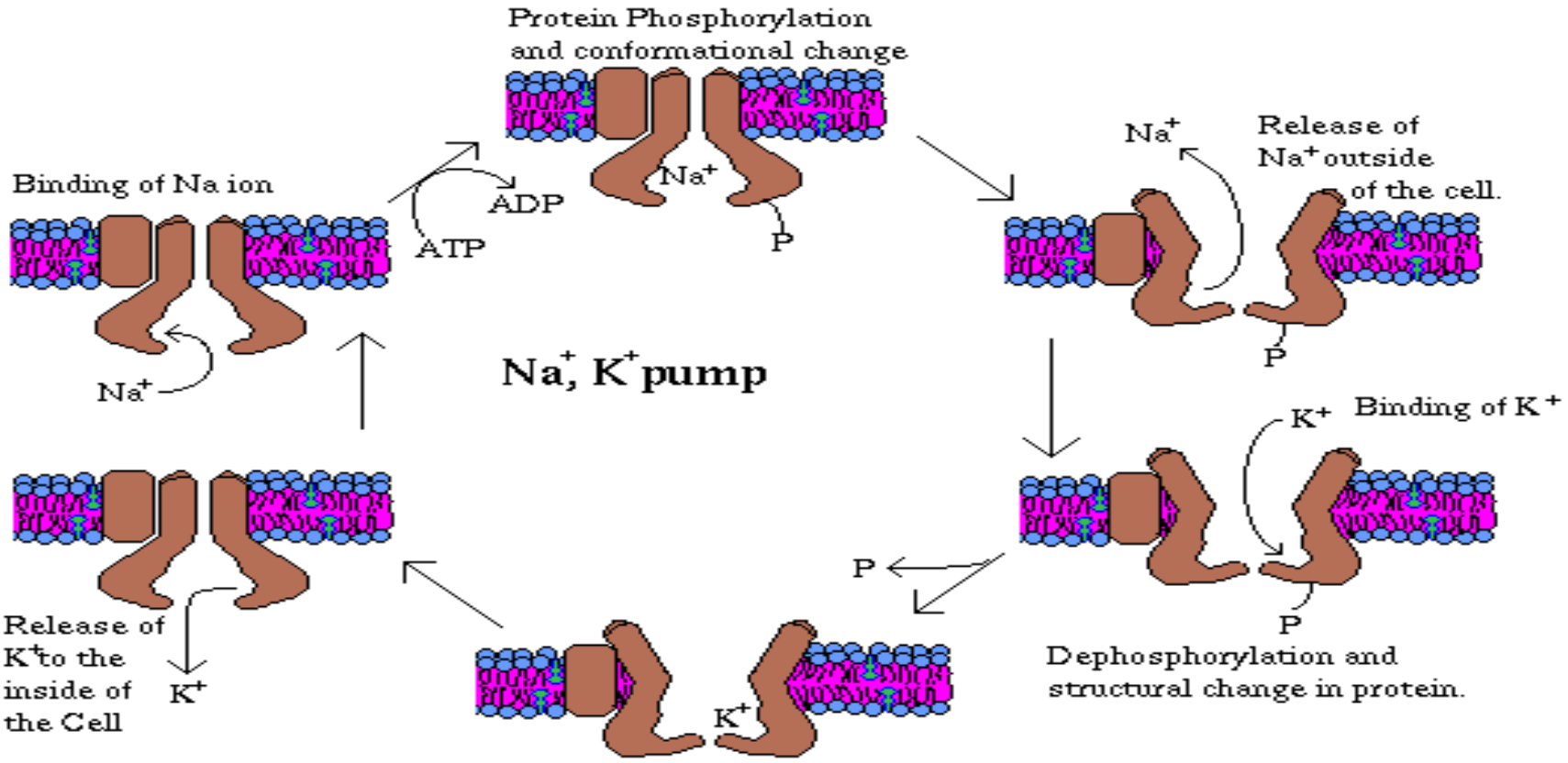
Na^+, K^+ ATPase

Na +, K+ ATPase (PUMP)



- The Top is the Outter membrane.
- The Bottom is the inner membrane (inside of the Cell)

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Tracer for Na^+, K^+ ATPase

- Human Na^+,K^+ ATPase is emerging as an important drug target
  - Cardioglycosides- digoxin and digitoxin
    - The treatment of congestive heart failure and related conditions

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Tracer for Na^+, K^+ ATPase

- In non-cell-based assays, the activity has been determined by using purified enzyme preparations to hydrolyze ATP
- In cell-based assays, the techniques such as
  - patch clamping, fluorescence,
  - H^3-Ouabain binding, and
  - Radio-tracer (Rubidium^{86}), and
  - Cold Rubidium in either recombinant cell lines or in cells other than primary cardiomyocytes

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Tracer for Na^+, K^+ ATPase

- However, the primary cardiomyocytes can not be used due to:
  - Heterogeneity
  - Sensitivity, and
  - Surface binding properties in developing cell based assays in an HTS format
- Alternative-Cultured mouse atrial cardiomyocytes derived from mouse embryonic stem cells as a model
  - Derived from transgenic mouse embryonic stem cells
    - Puromycin resistant.

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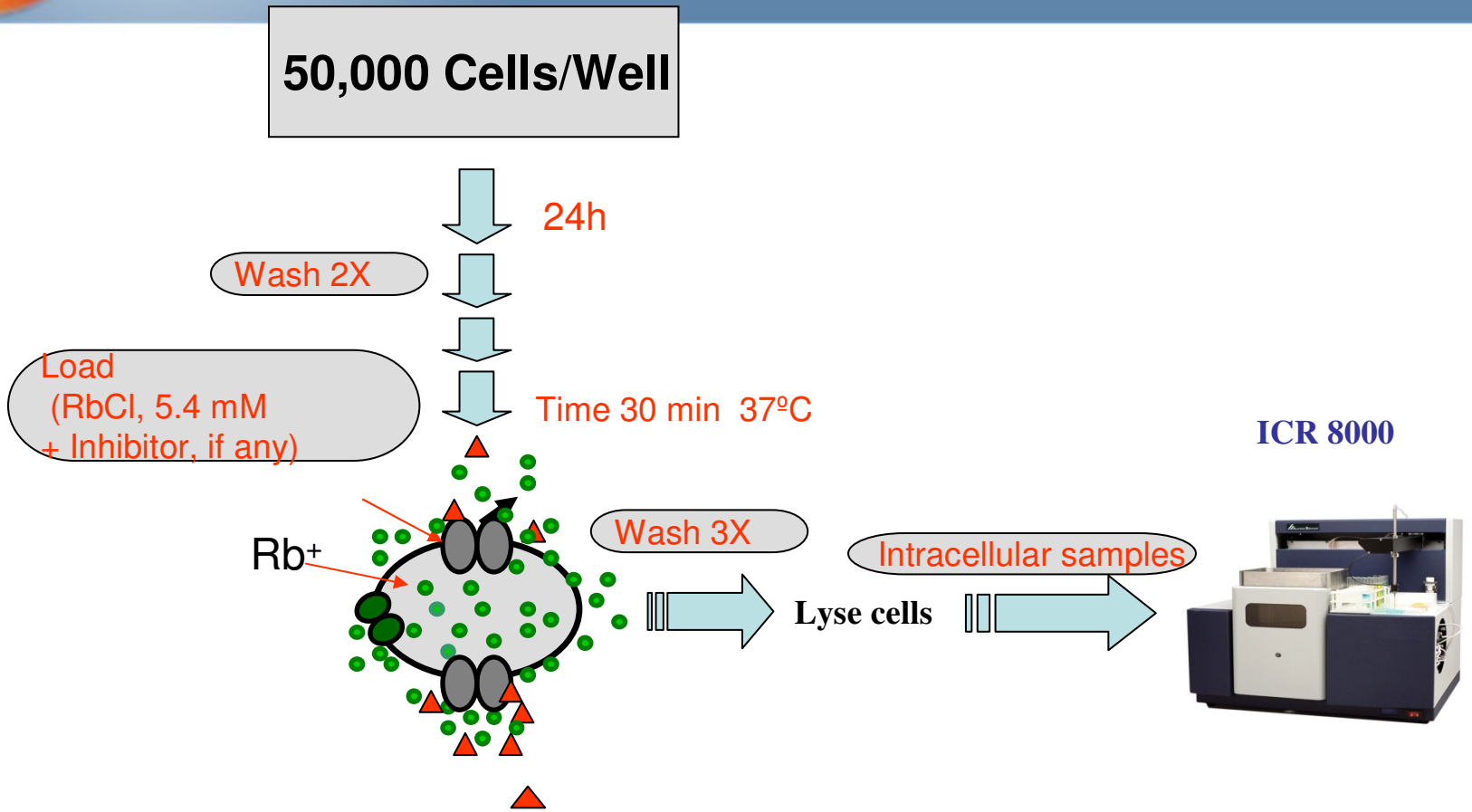
Na +, K+ ATPase Assay Steps



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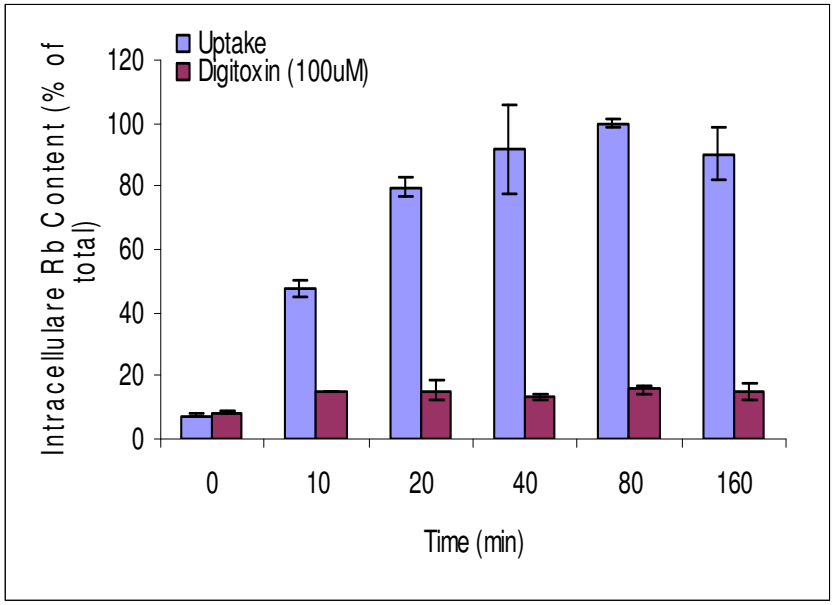
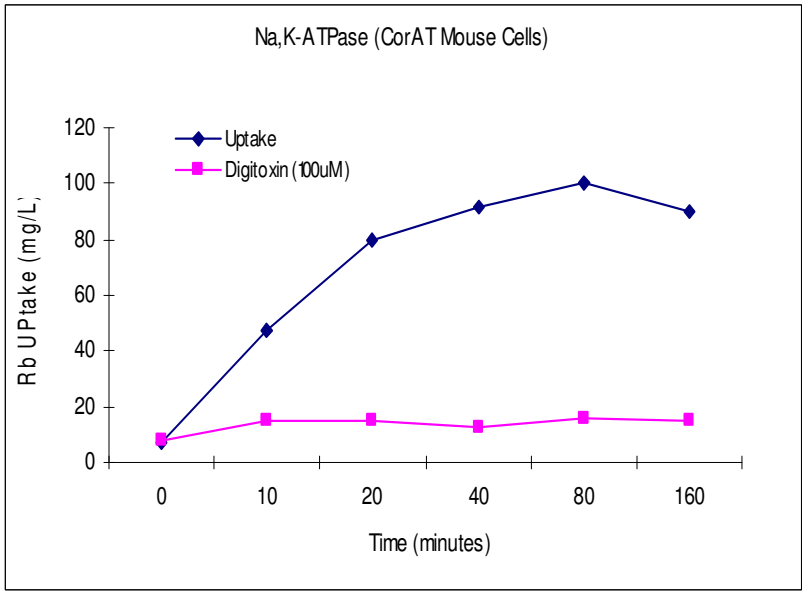
ES-Cardiomyocytes





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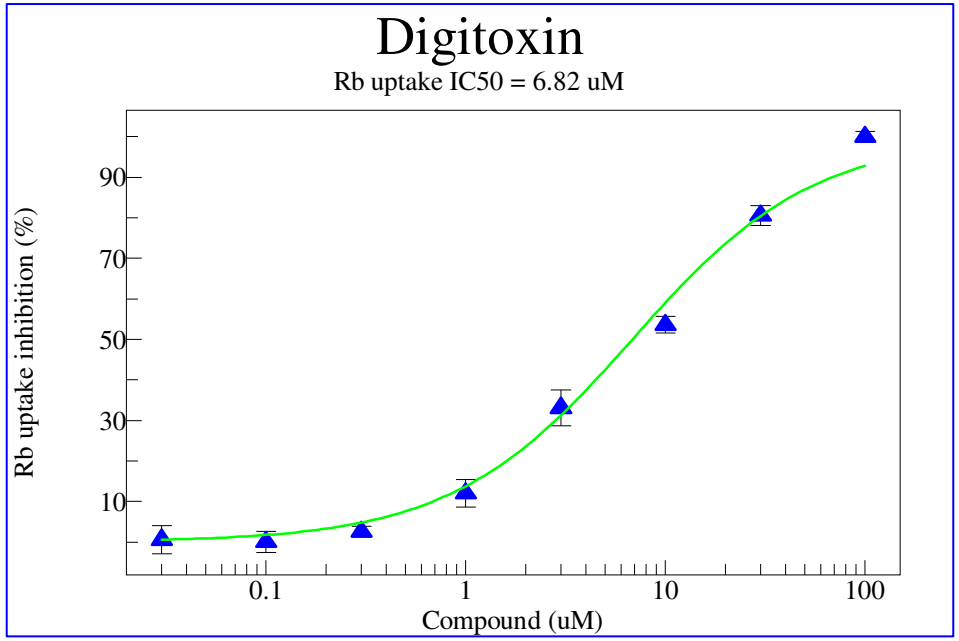
ES-Cardiomyocytes



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CONCLUSION

- ICR technology is applicable
  - Diverse channels
    - K^+, Na^+, Ca^{2+}, Cl^- and Pore forming proteins
- Continue to develop new applications for ICR
- ES-cardiomyocytes may emerge as good models for Na^+, K^+ ATPase target

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