Title: Calcium-Dependent Phosphorylation Regulates Neuronal Stability and Plasticity in a Highly Precise Pacemaker Nucleus

Running Title: Calcium-Dependent Mechanisms of Neuronal Stability and Plasticity

Authors and author addresses:
Andrew A. George
Section of Neurobiology &
Institute for Neuroscience
Patterson Laboratory, Rm 319
The University of Texas at Austin
Austin, TX 78712

Gregory T. Macleod
Department of Physiology-MC 7756
The University of Texas Health Science Center at San Antonio
7703 Floyd Curl Drive
San Antonio, TX 78229-3900

Harold H. Zakon
Section of Neurobiology &
Institute for Neuroscience
Patterson Laboratory, Rm 319
The University of Texas at Austin
Austin, TX 78712

Corresponding author:
Andrew A. George
Department of Biology
Washington University in St. Louis
St. Louis, MO 63130-4899
Email address: ageorge@biology2.wustl.edu
Phone #: 512-709-7352
ABSTRACT

Specific types of neurons show stable, predictable excitability properties while other neurons show transient adaptive plasticity of their excitability. However, little attention has been paid to how the cellular pathways underlying adaptive plasticity interact with those that maintain neuronal stability. We addressed this question in the pacemaker neurons from a weakly electric fish because these neurons show a highly stable spontaneous firing rate as well as an NMDA receptor-dependent form of plasticity. We found that basal firing rates were regulated by a serial interaction of conventional and atypical PKC isoforms and that this interaction establishes individual differences within the species. We observed that NMDA receptor-dependent plasticity is achieved by further activation of these kinases. Importantly, the PKC pathway is maintained in an unsaturated baseline state to allow further Ca^{2+}-dependent activation during plasticity. On the other hand, the Ca^{2+}/calmodulin-dependent phosphatase calcineurin does not regulate baseline firing but is recruited to control the duration of the NMDA receptor-dependent plasticity and return the pacemaker firing rate back to baseline. This work illustrates how neuronal plasticity can be realized by biasing ongoing mechanisms of stability (e.g. PKC) and terminated by recruiting alternative mechanisms (e.g. calcineurin) that constrain excitability. We propose this as a general model for regulating activity dependent change in neuronal excitability.

Keywords: Sensorimotor Adaptation, Intrinsic Excitability, PKC, Calcineurin and Neuronal Stability
INTRODUCTION

The electrical properties of neurons are tuned to maintain stable excitability, yet many neurons show significant plasticity in their excitability, often as a result of brief synaptic stimulation (Wierenga et al. 2005; Aizenman and Linden, 2000; O’leary et. al., 2010). Emerging themes from several studies indicate that protein kinases and phosphatases play a key role in neuronal plasticity (Winder et al., 1998; Malleret et. al., 2001; Nelson et al., 2005). However, do pathways that enable plasticity of electrical activity "tap into" those that control stability, or are they distinct so as not to disrupt the regulation of stability?

We addressed these questions in a weakly electric fish model that is particularly well suited for this study. The brown ghost (Apteronotus leptorhynchus) knifefish constantly emits electric organ discharges (EODs) for communication and the location of objects. The EOD is generated by brainstem pacemaker neurons that drive the electric organ in a 1:1 fashion (each spike in electrotonically coupled pacemaker neurons generates a pulse of the EOD, Fig. 1). The EOD is highly precise; it is, perhaps, the most stable biological oscillator known, with less than 1 microsecond of jitter (Bullock, 1969; Moortgat et al., 2000; Zakon et al., 2002). EOD frequency is individual- and, gender-specific in this species (Hopkins, 1988) and each fish discharges at a unique frequency that is stable over days. Thus, the pacemaker neurons of each fish have a tightly regulated and highly precise set point for spontaneous firing rate (Dye, 1987 and Dye, 1988).

However, this set point can be reset as a result of social interaction. If two fish with similar EOD frequencies meet, their electric fields jam each other. To avoid jamming, these fish have evolved a behavioral adaptation known as the jamming
avoidance response (JAR). The JAR is mediated by synaptic activation of NMDA receptors on pacemaker neurons (Harvey-Girard et al., 2007; Metzner, 1993) in which a fish's EOD frequency is transiently shifted to a higher frequency value. If the jamming signal persists, the firing rate of pacemaker neurons, and hence the EOD frequency, is reset to that new value. This long-term frequency elevation (LTFE) lasts tens of minutes to hours depending on the length of social interaction (Oestreich and Zakon, 2005).

LTFE is initiated by Ca\(^{2+}\) influx through the NMDA receptor (Oestreich et al., 2006; Dembrow et al., 2010). In this study we investigated the possible roles of the Ca\(^{2+}\) activated kinases Ca\(^{2+}\)/calmodulin dependant protein kinase II (CaMKII) and protein kinase C (PKC) and the calcium-sensitive serine/threoneine phosphatase calcineurin (protein phosphatases 2B or PP2B) in regulating basal firing rates and LTFE.

MATERIALS AND METHODS

Animals

In accordance with The University of Texas at Austin’s IACUC animal protocols, wild-caught individuals of the weakly electric fish *Apteronotus leptorhynchus* were obtained through Segrest Farms (Gibsonton, FL) and housed in community tanks in climate-controlled rooms (stable between 26°C and 28°C) and under a 12 hr dark/ light cycle. Fish were fed with frozen brine shrimp every 2 days. Individuals were allowed to acclimate for 1 month with water conductivity held at 800 uS/cm before being used in experiments.
PMn Slice Preparation

The *Apteronotus* PMn slice preparation has been previously described (Dye, 1987; Oestreich and Zakon, 2002). Briefly, fish were anesthetized in 0.1% 2-phenoxyethanol (Sigma, St. Louis, MO) and positioned on ice. To remove the brain, an incision was made through the skull and brains were irrigated with ice-cold artificial cerebral spinal fluid (ACSF; see below). Whole brains were transferred to Sylgard-coated Petri dishes containing oxygenated (95% O2–5% CO2) ice-cold artificial cerebral spinal fluid (ACSF: 124 mM NaCl, 2 mM KCl, 1.25 mM KH2PO4, 1.1 mM MgSO4, 1.1 mM CaCl2, 18 mM NaHCO3, and 10 mM glucose). Meninges were removed from the ventral surface of the brainstem, spanning from a point in close proximity of the caudal aspect of the pacemaker nucleus to the pituitary fossa. This region was dissected from the rest of the brain (thickness ~700 μm). Care was taken to incorporate the afferent fibers from the prepacemaker nuclei that run close to the ventral surface of the medulla (Dye, 1988; Maler et al., 1991; Zupanc and Horschke, 1997).

PMn Recordings

PMn slices were transferred to a Plexiglas recording chamber (designed by R. Turner and L. Maler; The University of Calgary, Calgary, Alberta, Canada and the University of Ottawa, Ottawa, Ontario, Canada) and oxygenated ACSF was bath perfused (flow rate = 12ml/min) continuously by means of a peristaltic pump (Dynamax RP-1; Rainin, Emeryville, CA). Bath temperature was tightly regulated (TH-10Km thermistor probe and TC2 temperature controller; Cell Micro-Controls, Norfolk, Virginia, USA) and maintained at 25°C.
Glutamatergic fiber tracts to PMn were activated with bipolar matrix microelectrodes (FHC, Bowdoin, ME, USA) under the following stimulus parameters: Train duration = 1 second, pulse width = 400 μs, rate = 500 Hz, and stimulus amplitudes ranging from 20 – 60 uA (Grass Stimulator, model S88; Grass Technologies, West Warwick, RI, USA). In order to avoid saturation by overstimulation and to allow for recovery of LTFE in enough time to carry out multiple trials, current injection of 40 uA was used to elicit LTFE. This produced an LTFE of ~50% of the maximum amplitude.

An extracellular recording electrode (filled with 1M NaCl) was positioned (Sutter MP-285 micromanipulator, Novato, CA) directly above the pacemaker nucleus until the pacemaker field potential was detected with an oscilloscope. The PMn field potential was amplified using an Axoclamp 2A amplifier (Molecular Devices, Sunnyvale, CA) and an A-M Systems model 1700 differential AC amplifier (Carlsborg, WA). Analog to digital conversion was accomplished using a Digidata 1320 series digitizer (Molecular Devices, Sunnyvale, CA) and field potentials were recorded with pClamp software (Molecular Devices, version 8).

**Operational Definitions**

Stimulation of the afferent fiber tract to PMn produces a fictive JAR during which occurs NMDAR activation and Ca\(^{2+}\) influx into relay neurons. We define the induction of LTFE arbitrarily at exactly 40 seconds after stimulation. This time point is an indication that the cellular mechanisms that regulate the JAR have occurred completely intact. LTFE maintenance is then defined as the duration of the frequency elevation after LTFE induction. For experiments that required stimulus modifications to match LTFE
induction, stimulus amplitudes were adjusted between 20 uA and 60 uA with all other stimulus parameters held constant.

Pharmacology

Calmidazolium chloride, cyclosporin A and KN-62 were obtained from Tocris Bioscience (Ellisvile, MO) and the cell-permeable PKC inhibitors (Myr-PKCα/β; myristoylated psuedosubstrate sequence from PKCα and PKCβ, N-Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln) and cell permeable PKCζ/ι inhibitor (Myr-PKCζ/ι; myristoylated pseudosubstrate sequence from PKCζ and PKCι, N-Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Trp-Arg-Lys-Leu) were purchased from BioMol International (Plymouth Meeting, PA). Given the uncertainty in the specificity of these peptide inhibitors we compared the amino acid sequences of these peptide inhibitors to homologous regions of teleostean PKCs. We found the sequences of the conventional peptide inhibitor to be identical to those of teleostean PKC isoforms alpha, beta and gamma and the atypical peptide inhibitor to be identical to the teleostean PKC isoforms zeta and iota. The two peptides had little similarity to the catalytic domains of each other or with those of other isoforms of PKC (Table I). From this, we infer that they are specific. Control experiments were performed with a myristoylated-scrambled peptide (PKCζ pseudosubstrate scrambled inhibitory peptide, Myr-Arg-Leu-Tyr-Arg-Lys-Arg-Ile-Trp-Arg-Ser-Ala-Gly-Arg; Tocris Bioscience). Bisindolylmaleimide I (Bis I) was purchased from EMD Biosciences, Inc. (San Diego, CA). These compounds were diluted in oxygenated ACSF and then bath applied to PMn slice preparations. FK506 was purchased from A.G. Scientific Inc. (San Diego, CA). Cyclosporin A, FK506, and
okadaic acid (EMD Biosciences, Inc. San Diego, CA) were dissolved in DMSO and bath applied to PMn preparations with a final DMSO concentration of 0.1%. Controls experiments were performed with 0.1% DMSO alone.

Western Blotting

Total Brain and PMn protein extracts were harvested using M-PER protein extraction buffer containing Halt Protease Inhibitor Single-Use Cocktail, dithiothreitol (DTT), and PMSF (phenylmethylsulfonyl fluoride; Pierce Biotechnologiy Inc., Rockford, IL). After extraction, samples were boiled in Lane Marker Reducing Sample Buffer (Pierce Biotechnology Inc., Rockford, IL) and cooled at room temperature for 5 minutes. Samples were loaded into 8-16% Precise Protein Gels (Pierce Biotechnology Inc., Rockford, IL) and run at 80 volts. Samples were transferred onto NitroBind nitrocellulose membranes (0.45 microns; Fisher Scientific, Pittsburg, PA). After transfer, membranes were blocked with blocking buffer (3% dried milk, 0.2% Tween-20 in 1X TBS) for 1 hr at 22ºC. An Antibody against calcineurin (1:1000; abcam Inc., Cambridge, MA) was added directly to the blocking buffer [1:200] and gently shaken overnight at 4ºC. Western analyses for conventional and atypical PKCs were excluded from this study due to the non-specific effects of commercially available antibodies. For loading controls, additional samples were incubated with a primary antibody against α-tubulin (1:200; abcam Inc., Cambridge, MA) and gently shaken overnight at 4ºC. After incubation, blots were washed in 1X TBS with 0.1% Tween-20 (3X, 5 minutes each at 22ºC). Blots were incubated in blocking buffer with HRP-conjugated 2º antibody (goat anti-rabbit [1:100,000]; 1 hr at 22ºC). Blots were washed in 1X TBS with 0.1% Tween-
200 20 (3X, 5 minutes each at 22°C) and in 1X TBS (2X at 22°C). Bands were visualized using Pierce Fempto Detection kit (Fempto HRPL substrates, Pierce Biotechnology Inc., Rockford, IL), apposed to film (exposure time = 20 seconds) and developed using standard techniques.

205 PMn Calcium Imaging

206 Apteronotus PMn slices were prepared (described above) and individual pacemaker and relay neurons were iontophoretically microinjected (injection current = 1 nA) with Fura-Dextran (dissolved in 200 mM KCl). PMn recordings were obtained (described above) and cytosolic Ca\(^{2+}\) transients were captured simultaneously using an iXon DV887 EMCCCD camera and controlled by iQ Imaging Software (Andor Technologies, Belfast, Ireland). Ca\(^{2+}\) transients were captured every 2 seconds through a 510/84 nm bandpass filter as a mercury arc lamp illuminated slice preparations alternately through 340/26 nm and 387/11 nm bandpass filters (Semrock, Rochester, NY). Ratiometric values indicating relative cytosolic Ca\(^{2+}\) levels were calculated by dividing the emission intensity at 510/84 nm when excited at 340/26 nm by the emission intensity at 510/84 nm when excited at 387/11 nm.

218 Statistical Analysis

219 All statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software Inc., San Diego CA). Means and standard error of the mean were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Groups were prepared using unpaired Student’s t test and repeated measures analysis of variance with significance
determined by \( p < 0.05 \). For linear regression, goodness-of-fit (\( R^2 \) and F values) and significance were determined using Graphpad's built-in linear regression functions. Multiple regression analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL).

**RESULTS**

**CaMKII and Calcineurin Do Not Influence Pacemaker Spontaneous Activity**

We first tested whether specific antagonists of calcium-dependent enzymes altered baseline firing rates. The CaMKII inhibitor KN-62 (10 \( \mu \text{M} \)) did not affect PMn basal firing rate (Fig. 2). The calmodulin antagonist, calmidazolium chloride (30\( \mu \text{M} \)) slightly decreased PMn basal firing rate by 10.2 Hz \( \pm \) 3.3 Hz, representing an average reduction of 1.7\% (Fig. 2). The calcineurin antagonists cyclosporin A (cyclo A; 10 \( \mu \text{M} \)) and FK506 (3\( \mu \text{M} \)) had no effect on PMn basal firing (Fig 2). Vehicle alone (DMSO 0.1\%) had no affect on basal firing rate (data not shown).

**Pacemaker Spontaneous Activity is Regulated By Conventional and Atypical PKCs**

We next tested for the involvement of conventional PKCs with the specific myristoylated protein kinase C inhibitor (Myr-PKC\( \alpha/\beta \); psuedosubstrate sequence from PKC\( \alpha \) and PKC\( \beta \); 10nM). Because we are unable to differentiate between the enzymatic activity of PKC\( \alpha \) and PKC\( \beta \) in this study, we use the term PKC\( \alpha/\beta \) to refer to the possibility that either or both enzymes may be involved. The PKC\( \alpha/\beta \) inhibitor decreased PMn basal firing rate by an average of 48.2 Hz or 8.8\% \( \pm \) 1.5\% (Fig. 2). To corroborate these findings, the application of Bis I also reduced PMn spontaneous activity by an average of 32 Hz, representing a 6.8\% \( \pm \) 2.5\% change in basal firing rate. At higher
concentrations (1 uM), the application of Bis I had no further effect on PMn firing rate. (Fig. 12). Application of an atypical protein kinase C inhibitor (Myr-PKCζ/ι; psuedosubstrate sequence from PKCζ and PKCι; 10nM) also reduced PMn basal firing rate by an average of 44.6 Hz or 6.7% ± 0.75% (Fig. 2). These results demonstrate that conventional and atypical PKCs regulate PMn basal firing rate and not the calcium-calmodulin dependant protein kinase CaMKII.

Conventional and Atypical PKCs Interact to Regulate Spontaneous Activity

Since inhibition of both conventional and an atypical form of PKC reduced PMn basal firing rate we tested whether the effects of blocking these two kinases were additive. We initially treated pacemaker nuclei with increasing concentrations of the conventional PKC antagonist to identify a saturating concentration (Fig. 3A; Myr-PKCα/β; 40 nM). PMn firing frequency decreased 72.4 Hz ± 4.5 Hz and no further decrease in firing frequency was observed with higher concentrations (Fig. 3A; time point: 55 minutes). After ~15 minutes of stable activity, we added the atypical antagonist (Myr-PKCζ/ι) to the bath in the continued presence of the PKCα/β antagonist (Fig. 3A; time point: 70 minutes), increasing the concentration until saturation (Myr-PKCζ/ι; 40 nM) and a final decrement in firing rate of 147.5 Hz ± 3.3 Hz (Fig. 3A; time point: 158 minutes).

Surprisingly, reversing the order of application produced a different result. Superfusing PMn slices with the PKCζ/ι antagonist at a saturating dose (Fig. 3B; Myr-PKCζ/ι; 40 nM) decreased PMn firing rate 143.7 Hz ± 19.5 Hz. Subsequent application of the PKCα/β antagonist (Fig. 3B; time point: 120 minutes) had no further effect on
firing rate. To ensure that the inability of PKCα/β blockers to further lower firing rate was not an artifact of reaching some intrinsic lowest limit of PMn firing rate, we reduced the external KCl concentration (Fig. 3B, 2mM to 1mM; time point: 140 minutes). This caused a further reduction of PMn basal firing rate by 23.8 Hz ± 8.5Hz. These results show that the interaction of the two kinases is non-additive and that blocking an atypical kinase completely occludes the effects of blocking conventional PKC isoforms. As a control, we bathed applied a myristoylated-scrambled peptide to PMn slice preparations to test whether myristoylated peptides modulate neuronal excitability in a non-specific manner. This had no effect on PMn spontaneous firing rate (Fig. 3C).

We further analyzed the effect of PKC blockade on basal firing rate. Regression analysis showed a significant correlation between the total magnitude of reduction in spontaneous firing and the original PMn firing frequency (i.e. the higher the initial frequency, the greater the decrease in firing rate) for experiments where inhibition of PKCα/β occurred before the inhibition of PKCζ/ι (Fig. 3D, p < 0.05, \( r^2 = 0.8068, F = 12.53, \) slope = 0.5198 ± 0.1469) and those where inhibition of PKCζ/ι occurred before the inhibition of PKCα/β (Fig. 3D, p < 0.05, \( r^2 = 0.8601, F = 18.45, \) slope = 0.4382 ± 0.1020). Regression analysis also showed a significant correlation when combining both data sets (Fig. 3E, p < 0.05, \( r^2 = 0.6663, F = 15.97, \) slope = 0.4203 ± 0.1052). If individual differences in pacemaker firing frequency were completely accounted for by PKC, then the final firing rate after PKC blockade ought be the same for each pacemaker. However, a positive correlation still existed when determining the final frequency as a function of original frequency (Fig. 3F, p < 0.05, \( r^2 = 0.9101, F = 30.36, \) slope = 0.5608 ± 0.1018). Controlling for the change in PMn firing rate and holding the final frequency as
a constant, multiple regression analysis demonstrated that PKCs contribute to ~ 33% of the total variation.

CaMKII Does Not Contribute to LTFE

We then tested whether blockade of any of the enzymes influences LTFE. Not only did KN-62 have no affect on baseline firing rate (see Fig. 2), pre-treatment of PMn slices with KN-62 (10 μM) did not affect maintenance or induction of LTFE (Figs. 4A and B’ respectively, p > 0.05). These results are in accordance with the lack of expression of CaMKII in the PMn (Maler and Hincke, 1999). On the other hand, KN-62 at a lower concentration (3.5 μM) blocks post-tetanic potentiation in brain regions of this species in which CaMKII is highly expressed (Wang and Maler, 1998).

Calmodulin Blockade Paradoxically Enhances LTFE

Since CaMKII is activated by calmodulin (CaM), the simple prediction is that blocking CaM should affect LTFE in the same manner as CaMKII. To test this we pretreated PMn slices with the calmodulin antagonist, calmidazolium chloride (30uM). Paradoxically, inhibition of CaM enhanced LTFE maintenance (Fig. 4C, p < 0.001) with no change in LTFE induction (Fig. 4D, p > 0.05). We believe that this is due to CaM activating the protein phosphatase calcineurin (see below).

LTFE Requires Conventional Isoforms of PKC

Because PKCs exert a powerful regulatory effect on spontaneous firing rate, we reasoned that they might be influential in regulating LTFE as well. We tested whether
conventional PKCs are involved in LTFE by pretreating slices with the myristoylated protein kinase C inhibitor (Myr-PKC\(\alpha/\beta\); 10nM). Inhibition of the conventional PKC isoforms \(\alpha\) and \(\beta\) resulted in a decrease in the maintenance and induction of LTFE (Figs. 5A and 5B, respectively, \(p < 0.001\))

Because bath-applied agents might act presynaptically and/or postsynaptically, the attenuation in maintenance of LTFE could be explained by reduced induction of LTFE through decreased presynaptic release or postsynaptic response to glutamate. Therefore, we increased the stimulus intensity to the glutamatergic afferents that synapse within the PMn (see methods) to match LTFE induction values (Fig. 5C; \(p > 0.05\)). The conventional PKC antagonist still resulted in a significant attenuation of LTFE (Fig. 5D, \(p < 0.001\)).

The previous experiment showed that reduction of LTFE by PKC inhibitors is not due to a decrease in transmitter release or amount of postsynaptic depolarization by the transmitter. However, since Ca\(^{2+}\) is not a major charge carrier through the NMDA receptor (Tsien and Tsien, 1990), if PKC antagonists decrease the Ca\(^{2+}\) permeability of the NMDA receptor (Lan et. al., 2001) we would be unable to detect it by monitoring firing rate alone. Furthermore, PKC blockers might also influence the regulation of intracellular stores of Ca\(^{2+}\). To test for these effects, relay and pacemaker neurons were loaded with fura-dextran (Fig. 6A) and relative cytosolic Ca\(^{2+}\)transients were imaged while simultaneously recording PMn activity.

Superfusion of the conventional PKC inhibitor reduced the relative magnitude of Ca\(^{2+}\) after stimulation by 46\% (Fig. 6B; \(p < 0.001\)) and the total Ca\(^{2+}\) (measured by the integral after induction) by 66\% (Fig. 6C, \(p < 0.001\)). To test whether this was solely due
to the reduction in synaptic transmission caused by PKC inhibition, we increased the stimulation intensity as above. Increasing the strength of stimulation matched LTFE induction values (Fig. 6D; p > 0.05), restored the Ca\(^{2+}\) signal to baseline levels (Fig. 6E; p > 0.05) and still resulted in a significant reduction in LTFE maintenance (Fig. 6F, p < 0.001). These experiments support our hypothesis that conventional PKC isoforms mediate LTFE by working downstream of NMDAR activation.

**LTFE Also Requires the Atypical Isoforms PKCζ and PKCι**

Inhibition of PKCζ/ι reduced LTFE maintenance (Figs. 7A, p < 0.001) and reduced the magnitude of PMn Ca\(^{2+}\) transients by 66% (Fig. 7B; p < 0.001). However, increasing the synaptic stimulus intensity to the PMn was unable to restore the induction values when compared to the initial stimulation suggesting that the PKCζ/ι antagonist strongly affects neurotransmitter release. To address whether PKCζ/ι truly contributes to the maintenance of LTFE, we reduced the stimulus intensity to the PMn for the initial stimulation (from 40 uA to 20 uA; see methods). A reduction in stimulus intensity restored the relative Ca\(^{2+}\) transients to baseline levels (Fig. 7C) and still resulted in a significant reduction in LTFE maintenance (Fig. 7D, p < 0.001).

**Calcineurin As a Negative Regulator of LTFE**

Given that LTFE is established by protein kinases it is logical to assume that protein phosphatases restabilize PMn activity and act as inhibitory constraints on LTFE. Despite the absence of effect of calcineurin inhibitors on spontaneous activity, we tested whether inhibition of calcineurin influences LTFE. LTFE was elicited in the presence of
the calcineurin antagonists cyclosporin A (cyclo A; 10 μM) or FK506 (3uM). Cyclosporin A enhanced LTFE maintenance (Fig. 8A, p < 0.001) with no effect on LTFE induction (Fig. 8B, p > 0.05). Although cyclosporin A is a potent calcineurin antagonist, it is known to influence other enzymes than calcineurin (Jacinto et al., 1998). Therefore, we treated pacemaker slices with another calcineurin antagonist, FK506 (3 uM). Under the same stimulation parameters, the maintenance phase of LTFE was enhanced (Fig. 8C, p < 0.001) with no observable change in induction (Fig. 8D, p > 0.05).

To support these findings, we harvested total brain and PMn protein and confirmed the expression of calcineurin using western analysis (Fig. 9A). To verify that the pharmacological inhibition of calcineurin was not altering calcium levels ectopically, we simultaneously recorded PMn firing rate while imaging PMn Ca\textsuperscript{2+} transients before and after the application of cyclosporin A. As previously described, the application of cyclosporin A enhanced the maintenance phase of LTFE (Fig. 9B, p < 0.001) and did not alter LTFE induction or PMn Ca\textsuperscript{2+} signaling (Figs 9C and 9D, p > 0.05).

**DISCUSSION**

This study demonstrates that in a highly precise pacemaker nucleus the ongoing spontaneous firing rate and LTFE, a form of synaptically-induced neural plasticity, are both regulated by conventional (PKC\textalpha and PKC\textbeta) and atypical (PKC\textzeta and PKC\textiota) PKCs and do not require Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII). Conversely, the calmodulin-activated phosphatase, calcineurin, regulates the duration of LTFE, but does not influence basal firing rate. We show that the PKC pathway is never maximally stimulated allowing synaptic stimulation to access the same pathway and induce LTFE.
Finally, we demonstrate that conventional and atypical PKCs interact non-additively to regulate pacemaker output and, ultimately, establish individual differences in the EOD.

CaMKII Does Not Contribute to Long Lasting Changes in PMn Excitability

CaMKII has long been described as a biochemical switch that converts increases in intracellular Ca\(^{2+}\) into long lasting changes in synaptic strength (Hudmon and Schulman, 2002; Lisman et al., 1997; Lisman et al., 2002) or intrinsic excitability (Nelson et al., 2005; Smith et al., 2002). Blocking CaMKII with KN-62 did not affect PMn basal firing rate or LTFE. On the other hand, KN-62 at a lower concentration (3.5 \(\mu\)M) blocks post-tetanic potentiation in other brain regions of this species (Wang and Maler, 1998). In accordance with this, CaMKII is absent in the PMn but is highly expressed in the areas of the Apteronotid brain that show post-tetanic potentiation (Maler and Hincke, 1999). Furthermore, since CaMKII is activated by calmodulin, inhibiting calmodulin should reduce LTFE if CaMKII initiates LTFE. Paradoxically, inhibiting calmodulin enhances LTFE maintenance, an effect we believe is due to CaM activating the phosphatase calcineurin (see below).

Conventional and Atypical PKCs Interact to Regulate PMn Spontaneous Activity

The application of PKCo/β and PKCζ/ι inhibitors independently reduced PMn spontaneous firing (Fig. 2). PKCs influence bursting and other prolonged changes in neuronal activity including spontaneous activity (Magoski, 2004; Wilson et al., 1998) by modulating the activity of many ion channels directly (Chen et al., 2006; Desai et al., 2008; Numann et al., 1991). The PMn of A. leptorhynchus is regulated by at least four
cationic currents (Oestreich et al., 2006; Smith, 2006) and it will be important to
determine in future experiments which channels are targeted by PKCα/β and PKCζ/ι.

Because both PKC inhibitors independently reduced firing rate, we asked whether
the two kinases worked in parallel or in series. We initially applied the PKCα/β
antagonist until saturating concentrations produced no further decrement in PMn firing
(Fig. 3A, ~75Hz). The subsequent application of the PKCζ/ι antagonist further reduced
PMn firing until saturating concentrations produced no further effect (average reduction
~150Hz) indicating that the effects of inhibiting PKCα/β and PKCζ/ι are additive. By
reversing the order, saturating doses of the PKCζ/ι antagonist alone reduced the average
firing frequency by ~150 Hz (Fig. 3B) and the application of the PKCα/β antagonist had
no further effect on reducing PMn firing rate. Because the inhibition of PKCζ/ι occluded
any further effects of PKCα/β inhibition it is likely that they work in series. One
interpretation of these results is that PKCα/β regulates PKCζ/ι. (see model below).

**Relationship of PKC to Sexual Dimorphism and Individual Differences in the EOD Frequency**

The EOD of *A. leptorhynchus* is sexually dimorphic. Females discharge at a rate
between 600-800 Hz and males between 800-1100 Hz (Hagedorn and Heiligenberg,
1985). Each fish has a unique and stable EOD frequency within the frequency range of
its gender (Schaefer and Zakon, 1996). The PMn controls EOD frequency in a 1:1
manner and the firing rate of the PMn in slice is approximately the same as the EOD
frequency of the fish from which it came (Meyer et al., 1987). Inhibition of PKC resulted
in a change in PMn firing rate that positively correlated with the initial firing frequency.
The maximum drop in firing rate following total blockade of PKC was \( \sim 250 \) Hz. This is comparable to the total range of either sex, or 50% of the total species range. We did not assess the gender of the fish in this study so we cannot directly address the extent to which PKC plays a role in determining EOD frequency within each sex. But since the PMn firing rate of every fish that we tested was affected by PKC block, we believe that PKC exerts a powerful effect on setting baseline EOD frequency in this species.

Further, no matter what the fish’s pacemaker firing frequency, it was capable of showing LTFE. In behaving fish, the magnitude of LTFE shows no sex difference and its maximum magnitude is about 15 Hz (Oestreich and Zakon, 2002). Thus, an important conclusion of this study is that the basal firing rate of the PMn is closely maintained below its maximum to accommodate LTFE.

A comparison of PMn firing rate after PKC inhibition with initial firing frequency, also yielded a strong positive correlation suggesting that PMn firing rate is governed jointly by PKC and additional regulatory mechanisms. EOD frequency in this species is shifted over days to weeks by steroid hormones (Zakon and Dunlap, 1999), likely through genomic transcriptional regulation. An interesting possibility is that basal pacemaker frequency is co-determined by steroid hormones and protein phosphorylation.

Conventional and Atypical PKCs Contribute to LTFE Induction and Maintenance

Blocking conventional PKC isoforms significantly attenuates the induction of LTFE, the magnitude of the PMn \( \text{Ca}^{2+} \) transients as well as the maintenance of LTFE. LTFE induction and \( \text{Ca}^{2+} \) signaling can be restored to pre-drug levels by increasing the stimulus intensity to the presynaptic fibers. The restoration of induction is consistent
with previous reports that blocking conventional PKCs reduces the probability of neurotransmitter release (Herlitze et al., 2001; Malenka et al., 1986; Suh et al., 2008) and has been demonstrated in many systems (Blackwell, 2006; Yawo, 1999; Zamponi et al., 1997).

Ca\(^{2+}\) transients in the pacemaker nucleus were no different from control after matching the induction demonstrating that inhibition of PKC does not alter NMDAR Ca\(^{2+}\) permeability, aspects of the Ca\(^{2+}\) regulatory process (e.g. Ca\(^{2+}\) pumps) that are not involved in LTFE, nor act ectopically on intracellular sources of Ca\(^{2+}\) (Banke et al., 2000; Chen et al., 2004; Tong et al., 1995; Yakel, 1997). Nevertheless, after matching LTFE induction and magnitude of the Ca\(^{2+}\) signaling, we still observed a significant attenuation in LTFE maintenance.

We found that the application of a specific PKC\(\zeta/\iota\) antagonist also significantly reduced LTFE induction, maintenance, and magnitude of the Ca\(^{2+}\) signal. Increasing the stimulus intensity to the PMn could not overcome the effect of PKC\(\zeta/\iota\) inhibition. Blocking PKC\(\zeta\) in the photoreceptors of mice decreases neurotransmitter release by inhibiting L-type presynaptic Ca\(^{2+}\) channels (Lee et al., 2007). Our data suggest a similar effect in the presynaptic terminals of afferents to the PMn, with the exception that prepacemaker afferents express N-type Ca\(^{2+}\) channels (Oestreich et al., 2006). Our study and Lee et al. (2007) emphasize that PKC\(\zeta\) and/or PKC\(\iota\) may be a general regulator of neurotransmitter release in different cells types.

To test whether PKC\(\zeta/\iota\) also plays a role in LTFE, we decreased the intensity of the initial stimulation (see methods) to allow us to match LTFE induction before and after drug application and observed that the PMn Ca\(^{2+}\) transients were not significantly
different but that the significant decrease in LTFE maintenance remained. The ability to match PMn Ca\(^{2+}\) transients while still attenuating LTFE maintenance suggest that the increase in firing rate is not due to an increase in Ca\(^{2+}\) currents but rather the regulation of other cationic currents.

At this time it is unclear whether an interaction between conventional and atypical PKCs plays a role in LTFE. It would be nearly impossible to determine an interaction because the stimulus paradigm would require matching LTFE induction values by increasing the stimulus presented in the presence of the PKC\(\alpha/\beta\) antagonist and subsequently trying to match LTFE induction by decreasing the stimulus amplitude in the presence of the PKC\(\zeta/\iota\) antagonist. Because the inhibition of PKC\(\zeta/\iota\) cannot be overcome by increasing the stimulus intensity (see results) we would not know whether the effect was synergistic or simply due to the effect of PKC\(\zeta/\iota\) inhibition alone. However, we assume they interact during LTFE in the same way that they do in regulating basal firing rate (see below).

**Calcineurin is Necessary for Regulating the Duration of LTFE**

Calcineurin inhibition significantly enhances LTFE maintenance with no effect on PMn spontaneous activity (Figs. 2 and 8). Inhibition of calcineurin signaling leads to enhanced LTP and/or a lower threshold for eliciting potentiation (Ikegami et al., 1996; Wang and Kelly, 1996), whereas increases in calcineurin activity attenuate LTP (Winder et al., 1998). In the present study, calcineurin inhibition did not alter LTFE induction or PMn Ca\(^{2+}\) transients (Fig. 9). These data suggests that the inhibition of calcineurin does not alter neurotransmitter release, NMDAR function, or any other postsynaptic source of
Ca\textsuperscript{2+}. Inhibition of calmodulin also enhances the maintenance of LTFE with no effect on LTFE induction. This is not surprising because the activation of calcineurin is dependent on calmodulin and these data support the findings that calcineurin plays an influential role in signaling pathways that are recruited for synaptically induced plasticity (Malleret et al., 2001). Calcineurin’s effects could be mediated through the disinhibition of other protein phosphatases (Halpain et al., 1990). The application of okadaic acid (100 nM; inhibitor of protein phosphatase 1 and protein phosphatase 2A) had similar effects to the direct inhibition of calcineurin (Fig. 11). Together, these data support our hypotheses that NMDAR activation also leads to the activation of neuronal protein phosphatases involved in regulating the duration of LTFE.

A Model for Phosphorylation-Dependent Regulation of Firing Rate and LTFE

We propose a model for regulation of spontaneous firing and LTFE in the pacemaker nucleus (Fig. 10). PMn spontaneous activity is regulated by conventional and atypical PKCs in the absence of synaptic stimulation. Conventional PKCs are activated by diacylglycerol (DAG) and/or Ca\textsuperscript{2+}. It is likely that DAG constitutively activates conventional PKCs in the PMn because blockade of phospholipase C markedly lowers PMn spontaneous firing rate (A. George, unpubl.). However, PKC cannot be maximally stimulated or it would be unaffected by Ca\textsuperscript{2+} influx following NMDAR activation.

In other systems, atypical PKCs (e.g. PKCζ) is activated by other kinases, such as PDK1 kinase (Kelly et al., 2007). We do not know which kinases regulate atypical PKCs in the PMn although our results suggest a serial interaction between conventional and atypical PKCs where PKCα/β are upstream of PKCζ/ι. However, because blockade of
PKCα/β does not occlude the effects of PKCζ/ι inhibitors, atypical PKCs must be activated by other kinases as well. While we present this model as a simple interpretation of the relationship between PKC activity and PMn stability, we do not exclude the possibility that conventional and atypical PKCs act independently, exclusively targeting specific types of phosphoproteins. For example, PKCα/β may regulate a subset of ion channels regulated by PKCζ/ι and, in turn, set pacemaker firing rate. Finally, because the inhibition of calcineurin, PP1, and PP2A does not affect PMn basal firing rate (A. George, unpubl.), another constitutively active phosphatase must be involved in regulating PMn basal activity. We suppose that this phosphatase is not in high concentration or very efficient as it takes an hour for basal firing rate to decrease to a minimum after total blockade of both PKC isoforms (Fig. 3).

With activation of the NMDA receptor and the subsequent Ca\textsuperscript{2+} influx that induces LTFE, we propose that Ca\textsuperscript{2+} boosts the activity of already active conventional PKCs as well as activating calcineurin. When calcineurin is pharmacologically blocked, PMn firing rate remains elevated for many minutes, again, indicating that the endogenous unidentified phosphatase does not act rapidly. Thus, calcineurin is recruited as a brake in the system and is pivotal in determining the duration of LTFE.

**Relationship to Other Systems**

Long-term changes in intrinsic excitability have been described in a number of neuron types. Remarkably, second messenger pathways have yet to be investigated in many of these (deep cerebellar nuclei: Aizenman and Linden, 2000; entorhinal cortex; Egorov et al., 2002; frog tectum: Pratt and Eizenman, 2007). In the handful of studies in which this was examined PKC appears not to regulate basal firing rate in the majority of
examples (pyramidal neurons in piriform cortex: Seroussi et al. 2002; hippocampal CA1 pyramidal neurons: Yuan et al., 2002, Hoffman and Johnston, 1998, Alroy et al. 1999; Aplysia bag cells: Conn et al., 1989). However, as in the pacemaker nucleus, blockade of PKC in Aplysia sensory neurons decreased basal excitation as well as attenuating long-term enhancement of excitability (Manseau et al., 1998).

One important point is that in those systems where PKC regulates basal excitability as well as synaptically-activated changes in excitability, it is imperative that PKC not be saturated in the resting condition.

The majority of studies focus on conventional PKCs or use blockers that cannot distinguish between different classes of PKCs. A novel aspect of our work is that we have identified a putative non-linear interaction between conventional and atypical kinases suggesting that they are acting in series. It will be intriguing to know if such a relationship occurs in the other cases of intrinsic plasticity in which PKCs are involved.

Calcineurin's role in synaptic plasticity in most systems is to “tamp down” synaptic excitation (i.e. generate LTD or oppose LTP). Surprisingly little attention has been paid to the role of phosphatases or agents that constrain excitability in most studies of intrinsic plasticity. This is especially important in cases of facilitation in which membrane excitability could “run wild.” In hippocampal pyramidal neurons excessive synaptic input initiates a compensatory decrease in intrinsic excitability (Misonou et. al., 2004) via activation of calcineurin. As in pacemaker neurons, calcineurin is inactive prior to strong synaptic activation. Calcineurin is active in resting cortical neurons (Zhang et al., 2004) and the balance between calcineurin and CAMKII activity regulates neural
threshold. However, whether calcineurin also is involved in reestablishing threshold in these cells following enhanced excitability has not been determined.

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References


Figure 1: Organization of the Electromotor System of *A. leptorhynchus* and the principle of long-term frequency elevation (LTFE). (A) The pacemaker nucleus (PMn) can be visualized on the ventral surface of the caudal medulla (outlined in red). (B) Schematic representation of the pacemaker nucleus. Pacemaker and relay neurons (labeled P and R respectively) are electrotonically coupled to one another and autodepolarize *en masse*. Glutamatergic inputs from sublemniscal prepacemaker nuclei (SPPn) activate NMDA (N-methyl-D-aspartate) receptors expressed on relay neurons and mediate the upward jamming avoidance response (JAR). Relay neurons are electrotonically coupled to electromotor neurons (EMN) located within the spinal cord and convey information to the electric organ in the tail. (C) Transmitted light images of pacemaker and relay neurons of the PMn (somas outlined in red; Scale bar = 100 microns). (D) The electric organ discharge (EOD) of *A. leptorhynchus*. (E) Schematic representation of the JAR and LTFE. Stimulation (solid black line) of SPPn afferents results in a rapid acceleration in PMn frequency. After LTFE induction, the firing rate moves to a new stable frequency value (as indicated by asterisk) and is maintained for a period of time before gradually returning to baseline.

Figure 2: Effects of inhibiting calcium-mediated phosphorylation and dephosphorylation and PMn baseline activity. Changes in PMn spontaneous firing after blocking calmodulin (calmidazolium chloride [30uM]); N=7 fish; *p* < 0.05), CaMKII (KN-62 [10 uM]); N=6 fish; *p* > 0.05), calcineurin (cyclosporin A [10 uM]), N=6; FK506 [3 uM], N=6; *p* > 0.05), PKCα and PKCβ (Bisindolylmaleimide I [10 nM]); N= 5 fish *p* < 0.01)
PKCα and PKCβ (myr-PKCα/β [10 nM]); N= 5 fish p < 0.001) and PKCζ and PKCι (myr-PKCζ/ι; [10 nM]); N=5 fish; p < 0.001).

Figure 3: Interaction between conventional and atypical PKC isoforms in regulating PMn basal activity. (A) PMn slices were exposed to increasing concentrations of the conventional PKC antagonist (Myr-PKCα/β; each symbol representing an increase in concentration) until a saturating dose did not produce any further decrement in PMn basal firing (40nM). After 20 minutes of stable activity, bath application of the atypical PKC antagonist (Myr-PKCζ/ι; each ζ represents an increase in concentration) further reduced PMn basal firing rate (saturating dose of Myr-PKCζ/ι at 40 nM; N=5 fish; gray bars represent standard error). (B) Reversing the order of PKC inhibition resulted in the occlusion of the effects of PKCα/β by the effects of PKCζ/ι. Reducing the concentration of external potassium (from 2mM to 1mM KCl) further reduced PMn firing rate (time point 140 minutes; N=5 fish: gray bars represent standard error). (C) The application of a myristoylated-scrambled peptide (indicated by arrow) had no effect on PMn spontaneous activity (gray bars represent standard error). (D) For both sets of experiments, the change in PMn firing rate positively correlated with initial firing frequency when the α/β inhibitor was applied first (green line with triangles, p < 0.05) or when the PKCζ/ι inhibitor was applied first (red line with circles, p < 0.05). (E) Regression analysis also showed a significant correlation when combining the two data sets (p < 0.05; N=10 fish). (F) A significant correlation still existed when plotting the final frequency as a function of the initial PMn frequency (p < 0.05).
Figure 4: Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) is not necessary for LTFE. (A) Application of a specific CaMKII inhibitor, KN-62 had no effect on LTFE maintenance or LTFE induction (A and B respectively; black trace: pre-drug stimulation, red trace: KN-62 application and green trace: washout stimulation; p > 0.05; N=6 fish). (C) Calmidazolium chloride, a specific calmodulin antagonist, enhanced LTFE maintenance (C; p < 0.001) with no change in induction (D; black trace: pre-drug stimulation, red trace: calmidazolium application and green trace: washout stimulation; p > 0.05; N=7 fish). Error bars indicate SEM.

Figure 5: Inhibition of conventional PKCs attenuates LTFE. (A) Application of a specific PKC antagonist (myristoylated PKC oligopeptide generated against the catalytic domains of PKC\textalpha and PKC\textbeta) significantly attenuated LTFE maintenance and induction (A and B respectively; black trace: pre-drug stimulation, red trace: application of Myr-PKC\textalpha/\textbeta and green trace: washout stimulation; p < 0.001; N=5 fish). Upon increasing synaptic stimulation to the pacemaker (see methods), no differences in LTFE induction were observed (C; p > 0.05). However, LTFE maintenance was still attenuated (D; p < 0.001; N=5 fish). Error bars indicate SEM.

Figure 6: The effects of PKC inhibition on Ca\textsuperscript{2+} signaling in the PMn. (A) Pacemaker (top panels) and relay neurons (bottom panels) were loaded with fura-dextran to measure the effects of PKC inhibition on relative cytosolic Ca\textsuperscript{2+} transients. For both cell types, transmitted light images are shown on the left and their respective false colored images after fura loading are shown on the right; scale bar = 100 microns. (B) A significant
reduction in the relative amplitude of cytosolic Ca\(^{2+}\) (green trace: pre-drug stimulation and red trace: application of Myr-PKC\(\alpha/\beta;\ p < 0.05\)) as well as total Ca\(^{2+}\) (C; p < 0.05; measured as the integral, \(\Delta F/F; N=6\) fish) accompanied attenuation of LTFE (as in Figure 5). No differences were observed in LTFE induction or cytosolic Ca\(^{2+}\) (D and E, respectively) after increasing synaptic stimulation to the pacemaker (p > 0.05; from 40 uA to 60 uA). However, attenuation of LTFE maintenance was still observed (F; p < 0.001; N=5 fish). Error bars indicate SEM.

Figure 7: Inhibition of atypical PKC isoforms and LTFE. (A) The application of the atypical PKC antagonist (Myr-PKC\(\zeta/\iota\) attenuated LTFE induction and maintenance (A; green trace: pre-drug stimulation; red trace: application of Myr-PKC\(\zeta/\iota, p < 0.001; N=5\) fish). The attenuation was accompanied by a reduction in the relative amplitude of cytosolic Ca\(^{2+}\) (B; green trace: pre-drug stimulation; red trace: application of Myr-PKC\(\zeta/\iota, p < 0.001\). However, increasing the synaptic stimulation to the PMn could not reverse the effect of the antagonist on LTFE induction or maintenance. After reducing the stimulus intensity of the pre-drug stimulation (40 uA to 20 uA; see methods), matching the amount of induction in the presence of the inhibitor, no differences were observed in the relative cytosolic Ca\(^{2+}\) transients (C; p > 0.05) and LTFE maintenance was still attenuated (D; p < 0.001; N=5 fish). Error bars indicate SEM.

Figure 8: Inhibition of calmodulin dependent phosphatase, calcineurin, enhances LTFE. Bath perfusion of specific calcineurin inhibitors, cyclosporin A enhanced LTFE maintenance (A; black trace: pre-drug stimulation, red trace: application of cyclosporin A
(10 uM) and green trace: washout stimulation; p < 0.001; N=6 fish) as well as FK506 (C; black trace: pre-drug stimulation, red trace: application of FK506 (3 uM) and green trace: washout stimulation; p < 0.001; N=6 fish). Cyclosporin A and FK506 did not effect LTFE induction (B and D respectively; p > 0.05). Error bars indicate SEM.

Figure 9: Calcineurin inhibition does not alter PMn Ca\(^{2+}\) signaling. (A) Expression of calcineurin in total brain and pacemaker protein extracts from *Apteronotus*. Western analysis reveals a single band corresponding to the molecular weight of calcineurin (~61 kDa). Simultaneous PMn recordings and Ca\(^{2+}\) imaging demonstrate that inhibition of calcineurin resulted in an enhancement of LTFE maintenance (B) with no change in LTFE induction (C). No effect was observed on relative PMn Ca\(^{2+}\) transients (D; green trace: pre-drug stimulation; red trace: application of cyclosporin A, p > 0.05; N=5 fish). Error bars indicate SEM.

Figure 10: Model of PMn Mechanisms of Stability and Plasticity. PMn spontaneous activity is regulated by a serial interaction between conventional and atypical PKCs (represented by thin arrows). The activity of both PKCs is maintained in an unsaturated state. During the jamming avoidance response, NMDAR activation leads to an influx of Ca\(^{2+}\) biasing the activity of conventional PKC isoforms and ultimately leading to the phosphorylation of target proteins involved in LTFE. The same Ca\(^{2+}\) signal activates the calmodulin/calcineurin pathway gradually leading to the dephosphorylation of proteins targeted by PKCs and ultimately, reducing the PMn firing rate to basal levels.
Figure 11: Inhibition of protein phosphatases 1 and 2A has similar effects on LTFE. The application of okadaic acid (OA) does not affect LTFE induction (A; black trace: pre-drug stimulation, red trace: application of OA [100 nM] and green trace: washout stimulation; \(p > 0.05\); \(N=8\)). Conversely, PP1 and PP2A inhibition enhanced the maintenance of LTFE (B; black trace: pre-drug stimulation, red trace: application of OA [100 nM] and green trace: washout stimulation; \(p < 0.001\); \(N=8\)).

Figure 12: Inhibition of PKC with Bisindolylmaleimide I (Bis I) and pacemaker spontaneous firing rate. The application of Bis I (10 nM) reduced PMn firing rate by 32 Hz \(\pm\) 8.5 Hz (indicated by arrow). Application of Bis 1 at higher concentrations (indicated by arrowhead; 1\(\mu\)M) produced no further reduction in firing rate.

Table 1: Similarity of peptide inhibitors with teleostean sequences of homologous PKC isoforms. Sequences from zebrafish, stickleback, catfish, minnow and fugu PKC isoforms were identified and compared with the peptide inhibitor sequences used in this study. No sequence overlap was observed between the sequence of the conventional peptide inhibitor (shaded in gray) and novel PKC sequences. In addition, the sequence of the peptide inhibitor used against atypical PKC isoforms (shaded in red) showed no overlap with conventional and/or novel PKC sequences. Accession numbers are listed for each species used for the comparison.
Figure 1

A

Rostral

B

Caudal

C

D

E

*
Figure 2

% Change in Firing Rate

- Calmidazolium
- KN-62
- Cyclosporin A
- FK506
- Bis I
- Myr-PKC α/β
- Myr-PKC ζ/λ

* p < 0.05
** p < 0.01
Figure 4
Figure 5

A

B

C

D

- Pre-drug
- Myr-PKCα/β
- Washout

Frequency Change (Hz)

0 250 500 750 1000

Time (seconds)

Frequency Change (Hz)

0 10 20 30

Time (seconds)

Frequency Change (Hz)

0 10 20 30 40 50 60 70 80 90 100

Time (seconds)

Frequency Change (Hz)

0 5 10 15 20

Time (seconds)
Figure 6

A

PM-Trans  PM-Fura

Relay-Trans  Relay-Fura

B

C

D

E

F

Pre-drug      Myr-PKCα/β (40 uA)

Pre-drug

Myr-PKCα/β (60 uA)

Pre-drug

Myr-PKCα/β (60 uA)

Pre-drug

Myr-PKCα/β (60 uA)
Figure 7

A

B

C

D

**

*
Figure 8

(A) Pre-drug Cyclosporin A Washout

(B) Pre-drug FK506 Washout

(C) Pre-drug FK506 Washout

(D) Pre-drug FK506 Washout
Figure 9

A

Total Extract  PMn Extract

61 kDa

B

Frequency Change (Hz)

Time (seconds)

Pre-drug  Cyclosporin A

C

Frequency Change (Hz)

Time (seconds)

Pre-drug  Cyclosporin A

D

Ratiometric Units (RU)

Time (seconds)

Pre-drug  Cyclosporin A

**
NMDAR Activation

\[ \text{Ca}^{2+} \]

PKC $\alpha/\beta$ $\rightarrow$ PKC $\zeta/\iota$ $\rightarrow$ CaM/Calcineurin

CaM/Calcineurin $\rightarrow$ Target Phosphoproteins $\rightarrow$ Pacemaker Output

- Endogenous Phosphatase

\[ \text{DAG} \]

?
Figure 11

A

Frequency Change (Hz)

Time (seconds)

B

Frequency Change (Hz)

Time (seconds)
## Table 1

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