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Involvement of Protein Kinase C and Protein Kinase A in the Enhancement of L-type Calcium Current by $GABA_B$ Receptor Activation in Neonatal Hippocampus

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Involvement of PKC and PKA in the Enhancement of L-type Calcium Current by GABAB Receptor Activation in Neonatal Hippocampus

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Abstract:

In the early neonatal period activation of $GABA_B$ receptors attenuates calcium current through N-type calcium channels while enhancing current through L-type calcium channels in rat hippocampal neurons. The attenuation of N-type calcium current has been previously demonstrated to occur through direct interactions of the βγ subunits of $G_{i/o}$ G-proteins, but the signal transduction pathway for the enhancement of L-type calcium channels in mammalian neurons remains unknown. In the present study, calcium currents were elicited in acute cultures from postnatal day 6–8 rat hippocampi in the presence of various modulators of protein kinase A (PKA) and protein kinase C (PKC) pathways. Overnight treatment with an inhibitor of $G_{i/o}$ (pertussis

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toxin, 200 ng/ml) abolished the attenuation of calcium current by the $GABA_B$ agonist, baclofen (10 μM) with no effect on the enhancement of calcium current. These data indicate that while the attenuation of N-type calcium current is mediated by the $G_{i/o}$ subtype of G-protein, the enhancement of L-type calcium current requires activation of a different G-protein. The enhancement of the sustained component of calcium current by baclofen was blocked by PKC inhibitors, GF-109203X (500 nM), chelerythrine chloride (5 μM), and PKC fragment 19–36 (2 μM) and mimicked by the PKC activator phorbol-12-myristate-13-acetate (1 μM). The enhancement of the sustained component of calcium current was blocked by PKA inhibitors H-89 (1 μM) and PKA fragment 6–22 (500 nM) but not Rp-cAMPS (30 μM) and it was not mimicked by the PKA activator, 8-Br-cAMP (500 μM – 1 mM). The data suggest that activation of PKC alone is sufficient to enhance L-type calcium current but that PKA may also be involved in the $GABA_B$ receptor mediated effect.

Keywords: L-type calcium channel, hippocampus, $GABA_B$ receptor, protein kinase C, protein kinase A, G-protein.

L-type calcium channels play a large role in the function of both cardiac and skeletal muscle. Thus, many investigators have concentrated on studying the regulation of L-type calcium channels in muscle tissue, particularly cardiac muscle (for review see Catterall, 2000). Most of these physiological studies have categorized current as L-type without attributing it to one of the four specific isoforms of L-type calcium channels that have been sequenced (Catterall, et al., 2005). Since not all isoforms of L-type channels are expressed in all tissues, the tissue type often gives a good indication of the isoform responsible for the current. For example, $Ca_v1.1$ is only expressed in skeletal muscle and $Ca_v1.2$ is the primary type of L-type channel expressed in healthy, adult cardiac tissue. Thus, the studies on regulation of L-type calcium channels in skeletal and cardiac muscle provide evidence that $Ca_v1.1$ and $Ca_v1.2$ are regulated by phosphorylation. However, these studies on native tissue do not provide any information on molecular sequencing. Much less is known about the regulation of L-type channels in neurons, which mainly consist of $Ca_v1.2$ and $Ca_v1.3$. The protein sequences of these channels demonstrate consensus sequences for a variety of protein kinases (Obenauer et al., 2003), suggesting that phosphorylation may be involved in their regulation. Regulation of other voltage-dependent calcium channels such as N-type ($Ca_v2.2$) and P/Q-type ($Ca_v2.1$)

calcium channels by neurotransmitters and phosphorylation has been well studied in neurons (for review see Dolphin, 2003).

One common neurotransmitter regulating neuronal activity is γ-amino butyric acid (GABA), either directly through ionotropic $GABA_A$ receptors or indirectly through metabotropic $GABA_B$ receptor modulation of ion channels. Upon $GABA_B$ receptor activation, neuronal calcium channels can be directly regulated by either Gα or Gβγ subunits or they can be indirectly regulated by G-proteins through second messenger systems (for review see Catterall, 2000; Dolphin, 2003; Tedford and Zamponi, 2006). We have previously demonstrated that $GABA_B$ receptor activation can lead to an attenuation of N-type calcium current and the enhancement of L-type calcium current in hippocampal cultures obtained from neonatal rat pups (Carter and Mynlieff, 2004; Bray and Mynlieff, 2009). The variety of responses seen in primary hippocampal cultures is likely due to the heterogeneity of the neuron types present. It is possible that the enhancement of calcium current by $GABA_B$ receptors is only present in a particular subset of cells. Current through N-type channels is primarily involved in neurotransmitter release, and therefore modulation of this current by presynaptic receptors is likely to be involved in decreasing neurotransmitter release (Hirata et al., 1995; Doze et al., 1995; Takahashi et al., 1998). L-type calcium channels are located on the soma and dendrites of neurons and thus current through L-type channels regulates many processes including enzymatic activity, excitability, and gene expression (for review see Lipscombe, 2004; Pinato et al., 2009). For example, in neonatal hippocampus calcium influx through L-type calcium channels contributes to the regulation of expression of the K^+Cl^- co-transporter (Bray and Mynlieff, 2009). It is well known that the inhibition of N-type current by $GABA_B$ receptor activation in mammalian neurons is mediated by Gβγ subunits from the $G_{i/o}$ protein family, which are sensitive to pertussis toxin (PTX; Kleuss et al., 1991; Ikeda et al., 1996; Kajikawa et al., 2001; for review see Tedford and Zamponi, 2006). However, the signal transduction pathway involved in the enhancement of L-type current by $GABA_B$ receptors in mammalian neurons is unknown.

In addition to direct effects of Gβγ subunits on calcium channels, activation of G-proteins can lead to activation of kinases, which can modulate channel activity as well. L-type channels have

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several consensus sites for phosphorylation by protein kinase C (PKC) and protein kinase A (PKA; Obenauer et al., 2003). $GABA_B$ receptor activation of PKC and PKA has been shown to enhance L-type calcium current in a subset of salamander retinal neurons (Shen and Slaughter, 1999). It is not clear that the same signal pathway would be utilized in the rat hippocampus. Differences in amphibian versus mammalian receptors and channels as well as differences in the retina versus hippocampus may cause variation in the signaling pathway. For example, in mammalian retinal cells, $Ca_v1.3$ and $Ca_v1.4$ are the primary isoforms of L-type channels expressed (Xu et al., 2002; Ko et al., 2007). However, in mammalian hippocampus, $Ca_v1.2$ and $Ca_v1.3$ are the primary isoforms of L-type channels expressed and their individual expression patterns differ throughout development (Nuñez and McCarthy, 2007; Schlick et al., 2010; M. Mynlieff, unpublished observations). The different isoforms of L-type calcium channels have different phosphorylation consensus sites within their sequences (Obenauer et al., 2003; http://scansite.mit.edu) and are thus likely to be regulated by different kinases. It has also been demonstrated that activation of PKC can enhance L-type calcium current in rat hippocampal neurons (Doerner and Alger, 1992). However, this response was not directly linked to $GABA_B$ receptors.

The current study was designed to investigate the involvement of PKC and PKA in $GABA_B$ receptor modulation of L-type calcium channels within the neonatal rat hippocampus. Although activation of PKC has been shown to enhance L-type calcium current within the hippocampus, this is the first study to directly investigate the potential role of PKC in $GABA_B$ receptor mediated enhancement of L-type current in mammalian hippocampus. Electrophysiological experiments were performed on cultured hippocampal neurons in the presence and absence of several PKC and PKA antagonists, as well as agonists for both types of kinases to determine their involvement in the signaling pathway of $GABA_B$ receptor activation and the subsequent enhancement of L-type current.

Section 2 – EXPERIMENTAL PROCEDURES

2.1 Isolation of Hippocampal Neurons

All animal protocols were approved by the Marquette University Institutional Animal Care and Use Committee and followed the guidelines set forth by the U.S. Public Health Service. Hippocampal neurons were isolated from 6–8 day old Sprague-Dawley rat pups as published previously (Mynlieff, 1997). Briefly, the animals were anesthetized with CO_2 and sacrificed by decapitation. The head was immersed in 70% ethanol for several minutes for decontamination and rinsed in sterile rodent Ringer's solution with glucose (146 mM NaCl, 5 mM KCl, 2 mM$CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 11 mM D-glucose, pH 7.4 with NaOH). The hippocampi were dissected from the brain using sterile technique in oxygenated, cold (~5°C), rodent Ringer's solution with glucose. For all experiments dissections were restricted to the superior region of the hippocampus (excluding the dentate gyrus) to minimize the heterogeneity of cell types within the cultures. The tissue was placed in PIPES-buffered saline (120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM D-glucose, 20 mM piperazine-N, N'-bis[2-ethanesulfonic acid], pH 7.0 with NaOH) and sliced into ~1 mm^3 sections. The tissue was incubated in 1 ml of 0.5% Trypsin XI and 0.01% DNase I (Sigma-Aldrich, St. Louis, MO) in PIPES-buffered saline at room temperature for 30 minutes with 100% oxygen blowing over the solution. This incubation was followed by 60 minutes at 35°C under continuous oxygen. The tissue was rinsed with 1 ml trypsin inhibitor (1 mg/ml trypsin inhibitor, Type II-O: chicken egg white and 1 mg/ml bovine serum albumin; Sigma-Aldrich, St. Louis, MO) in rodent Ringer's solution. The tissue was rinsed again with 1 ml Neurobasal-A growth medium (Invitrogen, Carlsbad, CA) fortified with B27 supplement, 0.5 mM glutamine, and 0.02 mg/ml gentamicin and triturated with a fire-polished Pasteur pipette in fresh growth medium. The cell suspension was plated onto the center of poly-L-lysine coated dishes (1 mg/ml, MW 38,500 – 60,000; Sigma-Aldrich, St. Louis, MO). The cultures were maintained at 37°C in a 5% CO_2 water-jacketed incubator.

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2.2 Electrophysiology

Calcium currents were measured using whole-cell patch clamp recording in voltage clamp mode. Data were collected using a Dagan 3900A patch clamp amplifier (Dagan Corporation, Minneapolis, MN) linked to a computer with a Digidata 1322 data acquisition system and pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). All electrophysiological experiments were performed at room temperature 20–24 hours following dissociation, which allowed the cells time to recover from enzymatic dissociation and adhere to the bottom of the dish (Mynlieff, 1997). Recording electrodes (3–7 MΩ) were made from borosilicate glass capillaries on a Flaming/Brown Micropipette Puller model P-87 (Sutter Instrument Co., Novato, CA). The internal solution used to fill the patch electrodes contained 140 mM Cs-aspartate, 5 mM $MgCl_2$, 10 mM Cs_2EGTA, 10 mM HEPES, 2 mM ATP-Na_2, and 0.1 mM GTP (pH of 7.4 with CsOH and osmolarity between 310–320 mOsm). The external solution used for calcium current measurements contained 10 mM $CaCl_2$, 145 mM TEACl, 10 mM HEPES, and 1 μM tetrodotoxin (Sigma-Aldrich, St. Louis, MO) to block Na^+ currents (pH of 7.4 with CsOH and osmolarity between 300–310 mOsm). The cells were held at −80 mV and depolarized with a 300 ms pulse to +10 mV. Whole-cell currents were electronically filtered at 1 kHz and digitized at 2 kHz. Linear components of leak current were subtracted post-hoc by the passive resistance protocol in pClamp 9.0.

(RS)-baclofen (Tocris, Ellisville, MO) and 8-Bromoadenosine 3′, 5′-cyclic monophosphate (8-Br-cAMP; Sigma-Aldrich, St. Louis, MO) were directly dissolved in the external calcium solution. GF-109203X (AG Scientific, INC., San Diego, CA) and phorbol-12-myristate-13-acetate (PMA, Calbiochem, La Jolla, CA) were dissolved in dimethyl sulfoxide (DMSO) at 1000 – 5000 fold the final concentration. Rp-cAMPS triethylammonium salt hydrate, H-89 dihydrochloride hydrate, protein kinase A inhibitor fragment 6–22 amide, and chelerythrine chloride (Sigma-Aldrich, St. Louis, MO) were dissolved in distilled water at 333 – 10,000 fold the final concentration. All concentrated stock solutions of the drugs were stored at −20°C until use for up to two months. Most of these compounds were diluted to their final concentration in the external calcium solution on the day of use. Kinase inhibitors dissolved in the external solution were bath applied for approximately 5–10 minutes prior to activation of $GABA_B$ receptors

by baclofen as well as being included in the baclofen solution itself. Baclofen and kinase activators dissolved in the external calcium solution were applied to cells using a U-tube delivery system, constructed with PE-10 polyethylene tubing housed in a piece of glass tubing, which allowed for quick application and washout of compounds that were gravity fed onto the cell and removed by vacuum suction. A blue dye, Fast Green FCF (Sigma-Aldrich, St. Louis, MO), was added to the drug solution to visualize application of the drug onto the cell and to ensure complete washout of the drug off the cell. Protein Kinase A fragment 6–22 was included in the internal solution. Protein kinase C fragment 19–36 (Sigma-Aldrich, St. Louis, MO) was dissolved in 1% acetic acid and included in the internal solution producing minimal change in the pH of the internal solution (<0.01). PTX (Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water at 500 fold the final concentration with bovine serum albumin (4 mg/ml) added for stabilization. Cultures were exposed to 200 ng/ml PTX for a minimum of 16 hours starting two hours after dissociation.

2.3 Data Analysis and Statistics

For analysis, the magnitudes of the currents in response to a 10 mV depolarizing pulse were plotted as a scatter graph vs. time. The linear regression and 95% confidence intervals were determined for the control current data points and were used to determine the effect of application of 10 μM baclofen on calcium current amplitude as described in Carter and Mynlieff (2004; see also Fig. 2). The percent change with baclofen application was determined by comparison to the regression line. The cells were grouped according to whether baclofen application caused a deviation in the magnitude of the calcium current when compared to the linear regression line determined by the control data. The change in current magnitude was considered significant if it fell outside the 95% confidence interval. If the current with baclofen application fell above the 95% confidence interval the cells were grouped as demonstrating an increase in current and if it fell below the 95% confidence interval the cells were grouped as demonstrating a decrease in current. In the cells where the current with baclofen application fell within the 95% confidence interval, the cells were grouped together as demonstrating no change.

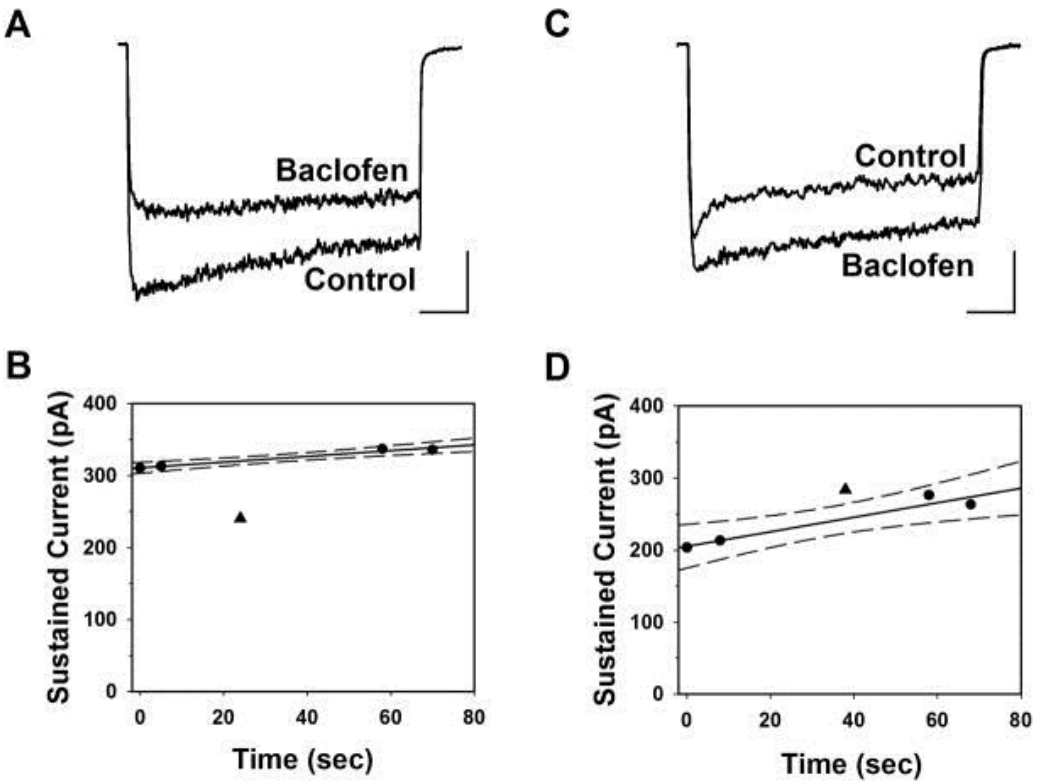

Figure 2 Illustration of baclofen effects on whole-cell calcium currents in hippocampal neurons. Calcium currents were elicited by a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV. The currents in A and B demonstrate an attenuation with 10 μM baclofen application. The currents in C and D demonstrate an enhancement of current with 10 μM baclofen application. For the graphs in B and D, the amplitude of the sustained current at the end of the 300 ms pulse were measured and plotted over time. The control currents (circles) were fit with a linear regression and a 95% confidence interval. Currents in the presence of 10 μM baclofen (triangles) were compared to the linear regression line. Scale bars in A and C are 100 pA and 50 ms.

Data were organized in contingency tables and a chi-square test was used to determine whether the distribution of the responses to baclofen (increase, decrease, and no change) varied in the absence or presence of various kinase inhibitors or activators. If the chi-square test demonstrated significance, pairwise comparisons were made using a Fisher's exact test.

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Section 3 - RESULTS

3.1 The enhancement of calcium current by $GABA_B$ receptors is still observed in the presence of PTX

Since one of the most common mechanisms of channel modulation by $GABA_B$ receptor activation occurs through direct modulation by a $G_{i/o}$ protein, the first experiment performed was overnight treatment of hippocampal cultures obtained from 7 day old rat pups with PTX to inactivate $G_{i/o}$ proteins by ADP-ribosylation. PTX (200 ng/ml) was added to the cultures two hours after dissociation. The effect of 10 μM baclofen on peak calcium current, activated by a depolarization to +10 mV, was measured the following day in cells treated with PTX and compared to cells without PTX treatment. The distribution of the responses (percent cells demonstrating a decrease, increase, and no change in peak current) differed in cultures that were treated with PTX in comparison to non-treated cultures ($P = 0.009$ using a Chi-square). Previous pharmacological experiments in our laboratory clearly demonstrated that the attenuation of current was due to attenuation of N-type current and the enhancement of current was due to enhancement of L-type current (Carter and Mynlieff, 2004). In cultures that were not treated with PTX, 33.3% of cells demonstrated a decrease, 38.5% of cells demonstrated no change, and 28.2% of cells demonstrated an increase in peak calcium current in response to 10 μM baclofen (N=39). In cultures that were treated with PTX, no cells demonstrated a decrease, 73.7% of cells demonstrated no change, and 26.3% of cells demonstrated an increase in peak calcium current in response to 10 μM baclofen (N=19). Enhancement of calcium current in response to 10 μM baclofen was still seen in cells treated with PTX, which suggests that enhancement of L-type calcium current is not mediated by a PTX sensitive G-protein (Fig. 1). However, it was demonstrated that the inhibition of N-type calcium current by $GABA_B$ receptors in the neonatal rat hippocampus is mediated through a $G_{i/o}$ protein, because following PTX treatment no cells demonstrated inhibition of their current ($P = 0.005$ using a Fisher's Exact test for pairwise comparisons). The average enhancement of the peak current in response to baclofen was very similar with (22.9 ± 4.0, N=5) or without (18.6 ± 3.1, N=11) PTX pretreatment.

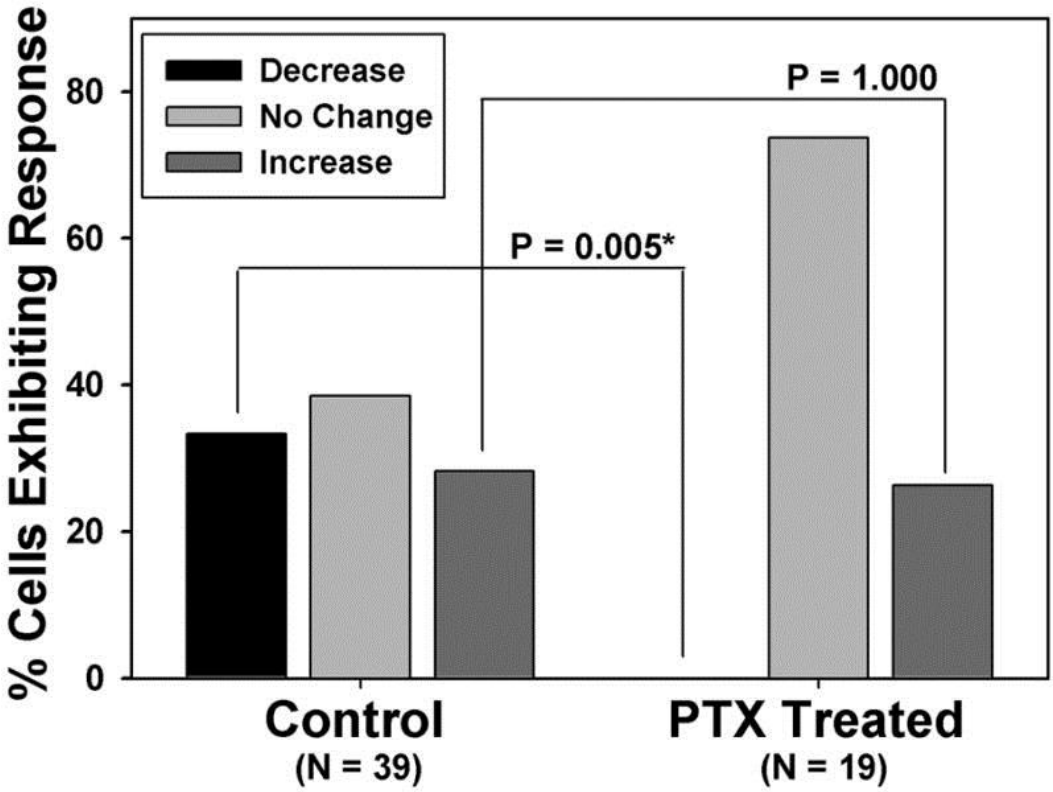

Figure 1 Effect of PTX on the baclofen responses in hippocampal neurons. The peak component of calcium current was measured at the beginning of a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen. Data were collected from 39 control cells and 19 cells in the presence of PTX (200 ng/ml, P = 0.009 using a Chi-square). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

3.2 Hippocampal neurons in culture demonstrate a variety of responses to $GABA_B$ receptor activation

For all PKC and PKA antagonists and agonists, the sustained component of the current was analyzed to maximize the contribution of L-type calcium current. The peak current is a combination of several different types of calcium current, which can hamper the interpretation of these data. Since L-type calcium channels exhibit slow inactivation kinetics, the sustained current component at the end of the 300 ms depolarization pulse to +10 mV was measured to minimize the contribution of T-type, N-type, P/Q-type, and R-type calcium current

while maximizing the contribution of L-type calcium current to the total measurement. Previous studies in which nimodipine, an L-type calcium channel antagonist, blocked the ability of baclofen to facilitate sustained current confirmed that the facilitatory effect of $GABA_B$ receptor activation on calcium currents was entirely mediated through L-type channels (Carter and Mynlieff, 2004). Control experiments were performed on cultures obtained from 6–8 day old rat pups for comparison to currents recorded in the presence of various antagonists or agonists. Fig. 2 illustrates how the currents were analyzed for classification of the responses. The amplitude of the sustained current in the presence of baclofen was compared to a linear regression line determined by data points collected in the absence of baclofen as described in the methods. The cell in fig. 2A and 2B demonstrated a decrease in voltage-dependent current when baclofen (10 μM) was applied while the cell in fig. 2C and 2D demonstrated an increase in voltage-dependent calcium current when baclofen was applied. The distribution of cells demonstrating a decrease, an increase, and no change in the sustained component of high voltage-activated calcium current in response to 10 μM baclofen was very similar to the responses we previously measured in cultures obtained from day 7 rat pups for the PTX studies. 34.1% of the cells demonstrated a decrease, 36.4% demonstrated an increase, and 29.5% demonstrated no change in the sustained component in response to baclofen (N=44). The average magnitudes of the changes in current are given in Table 1.

Classification	% Change	N
Decrease	15.7 ± 2.6	15
Increase	19.0 ± 2.9	16
No Change	0.9 ± 0.7	13

Table 1 Percent change in sustained current magnitude with $GABA_B$ receptor activation.

The sustained component of calcium current was measured at the end of a 300 ms depolarization to +10 mV from a holding potential of −80 mV in 44 cells. Control currents were measured both before and after baclofen application and a linear regression was fit to the data to account for run-up or run-down of the currents with a

95% confidence interval (as shown in fig. 2B and 2C). A change in current magnitude was considered significant if it fell outside the 95% confidence interval. To calculate the percent change, the actual magnitude of current in the presence of baclofen (10 μM) was compared to the value of the linear regression at that time point. Data are expressed as mean ± sem.

3.3 PKC inhibitors block the ability of baclofen to enhance sustained current

It is well documented that voltage-dependent calcium currents run-down or run-up in recordings made from cultured cells, which makes the ability to hold a cell through numerous drug applications and washes limited. Therefore, all experiments with kinase inhibitors and activators were carried out as population studies, comparing the distribution of responses in the presence and absence of each compound tested. In addition to the comparison of the percent of cells demonstrating each response to baclofen application, the average percent change in the magnitude of sustained current was also compared back to the average percent change seen in hippocampal cultures isolated from 6–8 day old rats that were treated with baclofen in the absence of any kinase inhibitors or activators.

Although over 12 different isoforms of PKC have been classified, broad-spectrum PKC inhibitors were used as a first step in determining the involvement of PKC in the signal transduction pathway of the enhancement of calcium current by $GABA_B$ receptor activation (for review see Tanaka and Nishizuka, 1994; Nishizuka, 1995; Webb et al., 2000; Zarate and Manji, 2009). Several PKC inhibitors with different chemical structures were used for verification of the results. Each of the inhibitors used has been shown to be a potent and selective inhibitor of PKC. Since the same population of control cells was used for comparison with cells treated with various PKC and PKA inhibitors/activators, the initial statistical analysis was performed on a contingency table including the distribution of responses in the control population as well as the distribution of responses in the presence of each inhibitor or activator ($P < 0.001$, Chi-square test). The first PKC inhibitor tested was the PKC fragment 19–36, which corresponds to a conserved region of the regulatory domain of PKC. Since the PKC fragment 19–36 is not membrane permeable, it was included in the internal pipette solution. The distribution of the responses differed in

the presence of the PKC fragment 19–36 in comparison to the control cells (Fig. 3; P = 0.003 using a Chi-square). In control cultures 36.4% of the cells demonstrated an increase in sustained calcium current in response to 10 μM baclofen (N=44). When the PKC fragment 19–36 (2 μM) was included in the recording pipette, there was a significant decrease in the percent of cells demonstrating an increase in sustained calcium current in response to 10 μM baclofen with only 7.4% of cells demonstrating an increase (N=27, P = 0.010 using a Fisher's exact test for pairwise comparisons). The mean enhancement of the sustained current was only 6.7% in 2 out of 27 cells treated with the PKC fragment 19–36 and baclofen in comparison to the average enhancement of 19.0 ± 2.9% seen in 16 out of 44 cells treated with baclofen alone (Table 2).

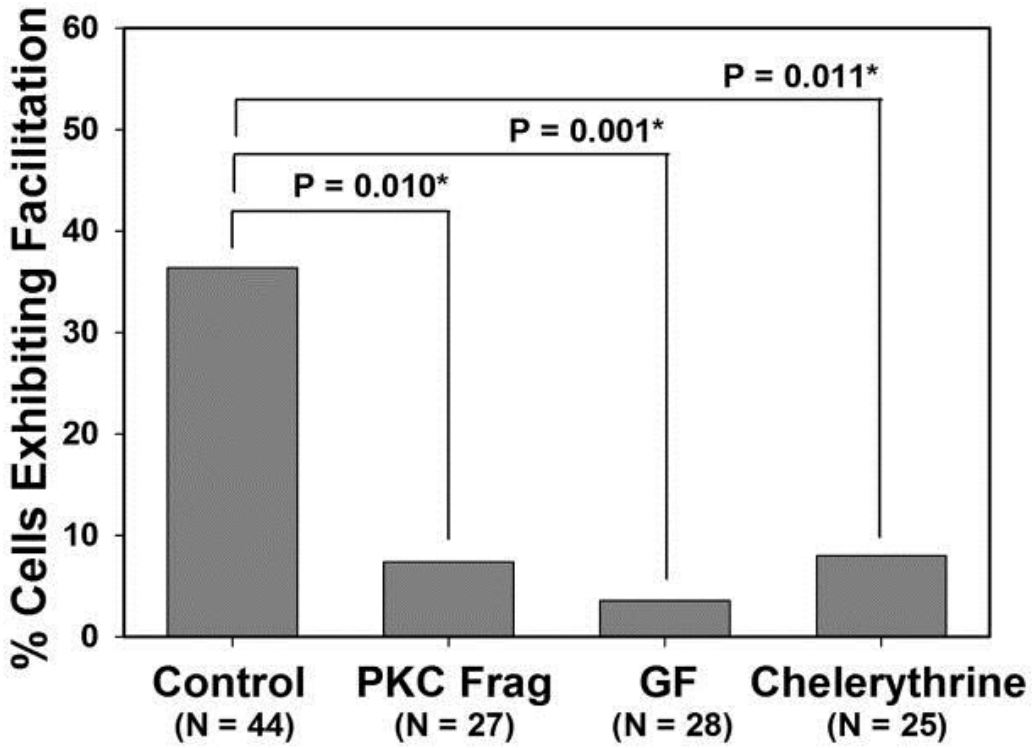

Figure 3 Effect of the PKC inhibitors, PKC fragment 19–36, GF-109203X, and chelerythrine chloride on the baclofen responses in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV. The bars represent the percent of cells demonstrating an increase in sustained calcium current in response to 10 μM baclofen. Data were collected from 44 control cells, 28 cells in the presence of the PKC fragment 19–36 (2 μM), 28 cells in the presence of GF-109203X

(500 nM), and 25 cells in the presence of chelerythrine chloride (5 μM). P = 0.003 using a Chi-square when comparing all of the groups together. Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

Activator	Inhibitor	%Enhancement	N Responding	N Tested
Baclofen	--	19.0 ± 2.9	16	44
PMA	--	21.7 ± 5.2	8	26
8-Br-cAMP	--	15.6	1	24
PKC Inhibitors				
Baclofen	PKC Fragment	6.7	2	27
Baclofen	GF-109203X	7.5	1	28
Baclofen	Chelerythrine Cl	11.2	2	25
PKA Inhibitors				
Baclofen	RP-cAMPS	11.2 ± 0.6	4	25
Baclofen	H-89	42.2	1	28
Baclofen	PKA Fragment	22.9	2	33

Table 2 Percent enhancement of sustained current magnitude with various kinase activators and inhibitors.

The sustained component of calcium current was measured at the end of a 300 ms depolarization to +10 mV from a holding potential of −80 mV. Cells were treated with 10 μM baclofen alone or in combination with various kinase inhibitors: PKC fragment 19–36 (2 μM), GF-109203X (500 nM), chelerythrine chloride (5 μM), H-89 (1 μM), Rp-cAMPS (30 μM), and PKA fragment 6–22 (500 nM). PKC and PKA were activated in the absence of baclofen by PMA (1 μM) and 8-Br-cAMP (500 μM), respectively. Data are given as mean ± sem when N>2.

The PKC inhibitor, GF-109203X (also known as bisindolylmaleimide I and Gö 6850) was also used to determine the involvement of PKC in the signal transduction pathway of $GABA_B$ receptor enhancement of L-type calcium current. GF-109203X is a competitive inhibitor for ATP binding and is highly selective for PKC (Toullec et al., 1991; Martiny-Baron et al., 1993). Since GF-109203X is membrane permeable, the compound was included in both the

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external bath calcium solution and in the baclofen solution. The distribution of the responses differed in the presence of GF-109203X (500 nM) in comparison to the control cells (P = 0.003 using a Chi-square). Focusing on the enhancement of sustained current, 36.4% of the control cells demonstrated enhancement of sustained calcium current when treated with 10 μM baclofen, while only 3.6% of cells demonstrated enhancement of sustained calcium current treated with 10 μM baclofen when GF-109203X was included in the external recording solution (Fig. 3; P = 0.001 using a Fisher's exact test for pairwise comparison). Only a single cell out of the 28 cells recorded from demonstrated a 7.51% increase in sustained calcium current with baclofen application when GF-109203X (500 nM) was included in the recording solutions, which is lower than the average percent increase for the 16 control cells demonstrating enhancement (19.0 ± 2.9%; Table 2).

The last PKC inhibitor used to investigate the involvement of PKC in the signal transduction pathway of $GABA_B$ receptor enhancement of L-type calcium current was chelerythrine chloride. Chelerythrine chloride is membrane permeable and therefore was included in both the external calcium solution and in the baclofen solution. The distribution of the responses differed in the presence of chelerythrine chloride (5 μM) in comparison to the control cells (P = 0.003 using a Chi-square). Only 8.0% of cells demonstrated an increase in sustained calcium current in response to 10 μM baclofen in the presence of chelerythrine chloride, which was significantly lower than the percent of cells demonstrating an increase in sustained calcium current in response to 10 μM baclofen without kinase inhibitors in the recording solutions (N=25; Fig. 3; P = 0.011 using a Fisher's exact test for pairwise comparisons). The 2 cells that demonstrated enhancement of sustained calcium current when treated with 10 μM baclofen in the presence of chelerythrine chloride exhibited an average percent increase of 11.2%, which is lower than the average percent increase of the 16 control cells that demonstrated enhancement in response to 10 μM baclofen (19.0 ± 2.9%; Table 2).

In the presence of all three of the PKC inhibitors, PKC fragment 19–36, GF-109203X, and chelerythrine chloride, baclofen caused a similar attenuation of current in a subset of cells as seen with baclofen alone, indicating that PKC was not involved in the attenuation of

current by baclofen (average attenuation ranged from 13.6% to 22.0% in the presence of the inhibitors). All three PKC inhibitors blocked the ability of baclofen to enhance sustained calcium current in response to baclofen application. These data all support the hypothesis that PKC is involved in the pathway of L-type calcium current enhancement by $GABA_B$ receptor activation.

3.4 Activation of PKC by PMA mimics the effect of baclofen to enhance sustained current

If a novel or conventional PKC isoform is involved in the signal transduction pathway of L-type calcium current enhancement by $GABA_B$ receptors, activation of PKC with a phorbol ester should mimic the enhancement of sustained calcium current seen with baclofen application. The phorbol ester PMA is a non-selective, general PKC activator and has been shown to activate both the conventional and novel PKC isoforms, but not the atypical PKC isoforms (Riedel et al., 1993a, 1993b; Goode et al., 1994; Shieh et al., 1995, 1996). The distribution of the responses differed when PMA (1 μM) was applied to cells in comparison to control cells where 10 μM baclofen was applied ($P = 0.003$ using a Chi-square). The distribution of responses differs between baclofen and PMA because PMA does not mimic the attenuation of calcium current seen with baclofen. There was only a single cell demonstrating a small decrease (8.74%) in response to PMA application, which suggests that the decrease of calcium current in response to $GABA_B$ receptor activation is not through a PKC mediated pathway ($P = 0.003$ using a Fisher's exact test for pairwise comparisons). However, as seen with baclofen application, a third of the cells (30.8%) demonstrated enhancement of sustained calcium current when 1 μM PMA was applied to hippocampal cultures isolated from rat pups that were 6–8 days old (N=26; Fig. 4). The average percent increase of the 8 cells that demonstrated enhancement of sustained calcium current in response to PMA application was 21.7 ± 5.2%, which is consistent with the 16 control cells that demonstrated a 19.0 ± 2.9 % increase with 10 μM baclofen application (Table 2). Thus, the enhancement of sustained current is very similar whether the currents were measured in the presence of baclofen or PMA. The distribution of responses with PMA also appears very similar to that of baclofen in PTX treated cells (Fig. 1). PTX eliminated the effect of

baclofen on N-type calcium current allowing only the L-type calcium current response to remain intact. The response to PMA should be similar if activation of PKC only affects the L-type calcium channels and not N-type calcium channels. This experiment using the PKC activator PMA supports the hypothesis that PKC is involved in the signal transduction mechanism of L-type calcium current enhancement by $GABA_B$ receptors.

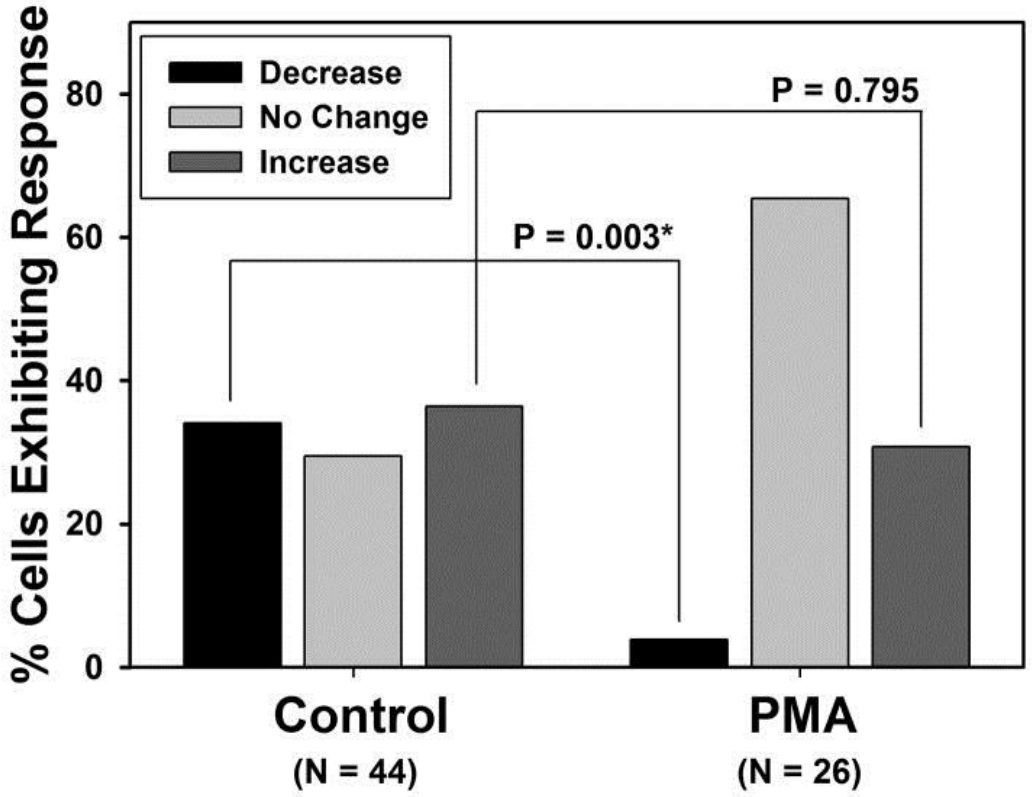

Figure 4 Effect of the PKC activator PMA in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen or 1 μM PMA. Data were collected from 44 cells with baclofen and 26 cells with PMA application (P = 0.003 using a Chi-square). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

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3.5 PKA inhibitors had varying effects on the enhancement of L-type current by $GABA_B$ receptors

In addition to PKC, Shen and Slaughter (1999) showed that the PKA inhibitor, Rp-cAMP (membrane impermeable, included in the recording pipette; 50 μM), was able to suppress L-type calcium current enhancement by $GABA_B$ receptors in the salamander retina. Therefore, three different PKA inhibitors, Rp-cAMPS (membrane permeable form), H-89, and PKA fragment 6–22, as well as a PKA activator, 8-Br-cAMP, were used to address the involvement of PKA in the signal transduction pathway of L-type calcium current enhancement by $GABA_B$ receptor activation in rat hippocampus.

In analyzing all three of the PKA inhibitors together and comparing the distribution with the control cells, there is a significant difference in the distribution of the responses to baclofen in the absence and presence of PKA inhibitors (Fig. 5; P = 0.002 using a Chi-square). However, there were no significant differences in the percentage of cells demonstrating enhancement of sustained calcium current with 10 μM baclofen application when Rp-cAMPS (30 μM, N=25) was present (control = 36.4% and Rp-cAMPS = 16.0%; Fig. 5). The average enhancement of sustained current in the presence of Rp-cAMPS (11.2 ± 0.65, N=4) was slightly lower than in control cells (Table 2). In contrast to Rp-cAMPS, the percent of cells demonstrating enhancement of sustained calcium current in response to application of baclofen differed significantly in the presence of H-89 (1 μM, N=28) in comparison to the control cells (3.6%; Fig. 5; P = 0.001 using a Fisher's exact test for pairwise comparisons). This was similar to the results obtained with the PKA fragment 6–22, where only 6.1% of the cells demonstrated an enhancement of the sustained component of current when 500 nM PKA fragment 6–22 was included in the recording pipette (N=34). Thus, both H-89 and PKA fragment 6–22 blocked the ability of baclofen to enhance L-type calcium current, but baclofen was still able to enhance sustained current in the presence of Rp-cAMPS. It is important to note that cells demonstrating an increase in sustained current in the presence of both H-89 and PKA fragment 6–22 had increases of comparable magnitude to cells without any PKA inhibitors (H-89, 42.4%; PKA fragment 6–22, 22.9%; Table 2)). Thus, the data obtained with PKA inhibitors is inconclusive as to the role PKA

plays in sustained calcium current by the $GABA_B$ agonist baclofen. The attenuation of current by baclofen was not blocked since there was no significant difference in the magnitude of the attenuation in the subset of cells demonstrating attenuation when the PKA inhibitors were present in comparison to cells treated with baclofen alone (average attenuation ranged from 13.8% to 21.8% in the presence of the inhibitors).

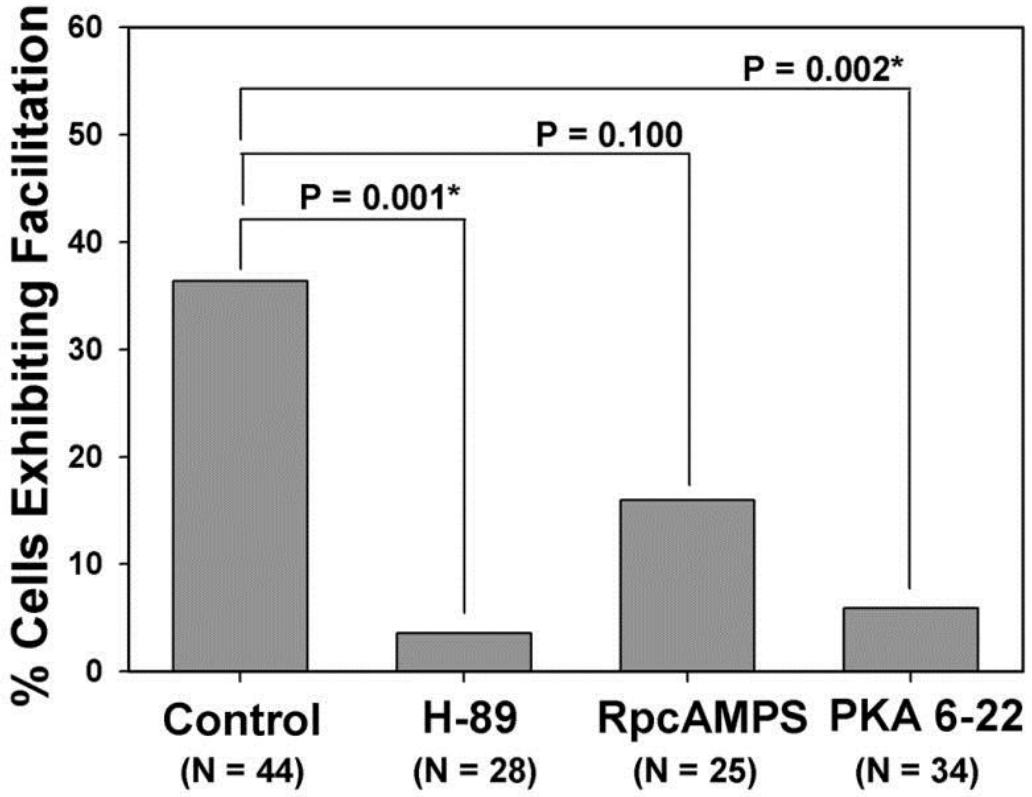

Figure 5 Effect of the PKA inhibitors, H-89, Rp-cAMPS, and PKA fragment 6–22 on the baclofen responses in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV. The bars represent the percent of cells demonstrating an increase in sustained calcium current in response to 10 μM baclofen. Data were collected from 44 control cells, 28 cells in the presence of H-89 (1 μM, P = 0.001), 25 cells in the presence of Rp-cAMPS (30 μM, P = 0.100), and 34 with PKA fragment 6–22 (500 nM, P=0.002). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

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3.5 8-Br-cAMP does not modulate calcium currents in the neonatal rat hippocampus

To determine whether PKA activation mimics the modulatory effects of baclofen, the cell-permeable cAMP analogue 8-Br-cAMP was used. Initial experiments were performed with 1 mM (EC_{50} = 166μM; Ogreid et al., 1989). 8-Br-cAMP was applied to the cells the same way baclofen was applied. Out of 10 cells, 4 cells demonstrated a decrease in sustained current whereas 6 cells demonstrated no change in sustained current amplitude. No enhancement of sustained current was seen with this concentration. However, the cells did not tolerate this concentration well so that it was difficult to hold the cells through multiple applications and washes. Therefore subsequent experiments were performed with 500 μM 8-Br-cAMP. The distribution of the responses differed when 500 μM 8-Br-cAMP was applied to the cells in comparison to the control cells in which 10 μM baclofen was applied (Fig. 6; $P < 0.001$ using a Chi-square). Most of the cells (91.7%) demonstrated no effect in response to 500 μM 8-Br-cAMP application, suggesting that PKA is not involved in the signal transduction mechanism of either the enhancement of L-type calcium current or attenuation of N-type calcium current by $GABA_B$ receptor activation.

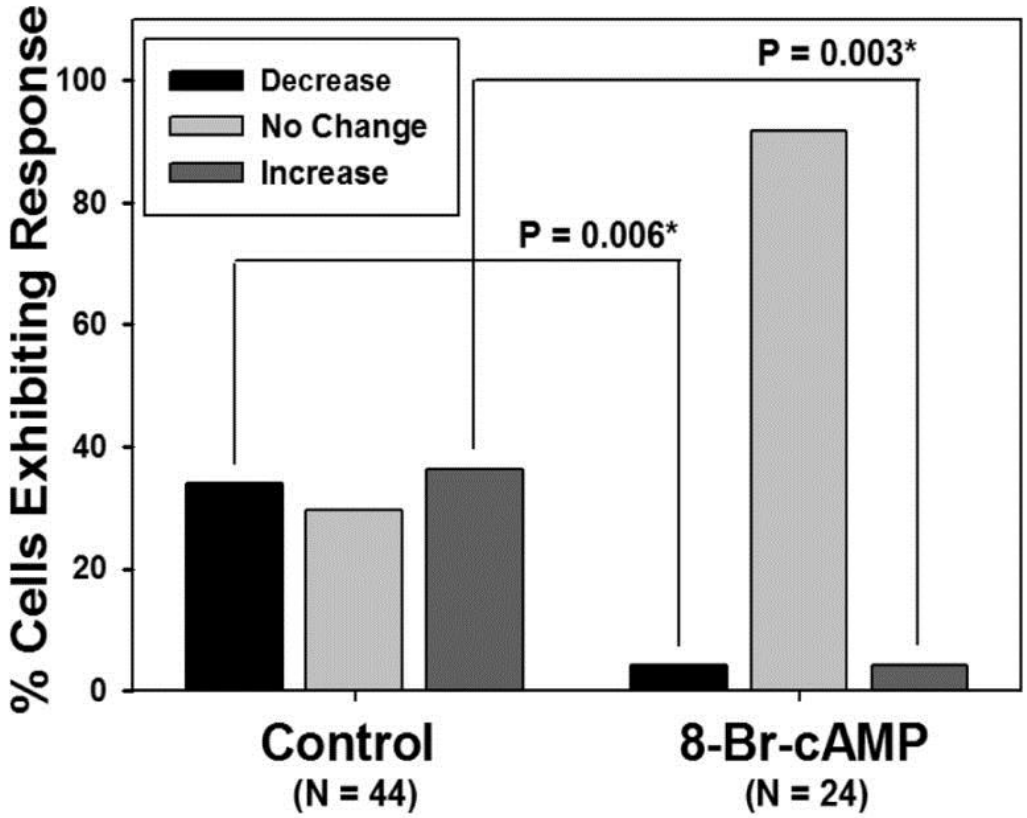

Figure 6 Effect of the PKA activator 8-Br-cAMP in hippocampal neurons. The sustained component of calcium current was measured at the end of a 300 ms depolarizing pulse to +10 mV from a holding potential of −80 mV. Cells were classified as demonstrating a decrease (black bar), no change (light gray bar), or an increase (dark gray bar) in response to 10 μM baclofen or 500 μM 8-Br-cAMP. Data were collected from 44 cells with baclofen and 24 cells with 8-Br-cAMP application ($P < 0.001$ using a Chi-square). Pairwise comparisons were done with a Fisher's Exact Test. * indicates a statistically significant difference.

Section 4 - DISCUSSION

The importance of calcium influx in neuronal function makes it a prime site for modulation by neurotransmitters and second messengers. Many inhibitory interneuron subtypes within the hippocampus utilize GABA to regulate the overall excitability of the hippocampus. Our laboratory has demonstrated that activation of $GABA_B$ receptors can enhance calcium influx through L-type calcium channels and attenuate calcium influx through N-type channels during development of the rat hippocampus (Carter and Mynlieff, 2004; Bray

and Mynlieff, 2009). Using calcium imaging, a similar enhancement of calcium current by $GABA_B$ receptor activation was recently reported in hippocampal neurons isolated from embryonic tissue (Park et al., 2010). $GABA_B$ receptors are metabotropic and thus regulate various channels through activation of G-proteins. There are several mechanisms of calcium channel modulation through direct and indirect signaling pathways. One of the most common signaling pathways associated with $GABA_B$ receptor activation is through $G_{i/o}$ proteins (Morishita et al., 1990; Campbell et al., 1993; Menon-Johansson et al., 1993; Greif et al., 2000; Mannoury La Cour et al., 2008). Both N-type and P/Q-type calcium current inhibition generally occurs through direct modulation by Gβγ subunits that are sensitive to PTX suggesting the involvement of $G_{i/o}$ proteins (Kleuss et al., 1991; Herlitze et al., 1996; Ikeda et al., 1996; Furukawa et al., 1998; Zamponi and Snutch, 1998; Kajikawa et al., 2001; Mirshahi et al., 2002). In the current study, treatment with PTX in hippocampal cultures isolated from 7 day old rats blocked the ability of baclofen to decrease calcium current, which has been shown to be through N-type calcium channels (Carter and Mynlieff, 2004). This is consistent with other studies that have demonstrated the involvement of $G_{i/o}$ proteins in the attenuation of N-type calcium current by $GABA_B$ receptor activation in other brain regions (Amico et al., 1995; Santos et al., 1995; Harayama et al., 1998; Bertrand et al., 2003). The signal transduction mechanism of L-type calcium current enhancement by $GABA_B$ receptors in the rat hippocampus is not known. Enhancement of L-type calcium current by $GABA_B$ receptors was still seen in the presence of PTX, indicating that $G_{i/o}$ proteins are not involved in the signaling pathway of L-type current enhancement by $GABA_B$ receptors.

L-type calcium channels have several consensus sites for phosphorylation by protein kinases, particularly PKC and PKA (Obenauer et al., 2003). Therefore, the involvement of both PKC and PKA in the $GABA_B$ mediated enhancement of L-type calcium current was investigated using inhibitors and activators for each kinase. Several broad-spectrum PKC inhibitors were used to verify the involvement of PKC. All three PKC inhibitors used, PKC fragment 19–36, GF-109203X, and chelerythrine chloride blocked the ability of baclofen to enhance L-type current in hippocampal cultures isolated from neonatal rats. These PKC inhibitors were chosen based on their specificity to several if not all of the PKC isoforms, their cell

permeability, and their varying chemical structures. To confirm the involvement of PKC, a phorbol ester, PMA, was used to activate PKC and the effects on sustained calcium current were measured. Enhancement was observed with PMA application in approximately the same percentage of cells as with baclofen application. All of these data suggest that PKC is involved in the signal transduction pathway of L-type calcium current enhancement by $GABA_B$ receptors in neonatal rat hippocampus.

Involvement of PKC in the signaling pathway implies that activation of $GABA_B$ receptors is coupled to G-proteins known to activate phospholipase C such as the α subunit of those in the G_q family or the βγ subunit of G_i proteins (Camps et al., 1992; Clapham and Neer, 1997; Hansen et al., 2003; Murthy et al., 2004). The lack of effect of PTX treatment on L-type calcium current enhancement rules out the possibility that G_i is mediating the PKC effect. Mannoury La Cour et al. (2008) used an antibody-capture/scintillation proximity assay to investigate coupling between $G_{q/11}$ and $GABA_B$ receptors in the hippocampus. These data appear to be in contradiction with the activation of PKC by $GABA_B$ receptors. However, the coupling with $G_{q/11}$ was the only G-protein in the G_q family tested in this study. There are three other distinct G-proteins, G_{14}, G_{15}, and G_{16} that may mediate this response and were not tested by Mannoury La Cour et al. (2008). In addition, the coupling of $GABA_B$ to $G_{i/o}$ had an EC_{50} on the order of 300–500 μM for GABA and ~50 μM for baclofen, which are significantly higher than values reported for $GABA_B$ receptor responses mediated by $G_{i/o}$ (Dolphin and Scott, 1987; Harrison, 1990; Sodickson and Bean, 1996). Shen and Slaughter (1999) reported that the PKC mediated enhancement of L-type current occurred with nanomolar concentrations of baclofen in the salamander retina. Experiments using calcium imaging to measure increases in intracellular calcium due to influx across voltage-dependent channels suggest that baclofen enhances L-type calcium current in the nanomolar range in our hippocampal cells, whereas attenuation of N-type calcium current requires higher concentrations (unbpublished). It is possible that the antibody-capture/scintillation proximity assay is not sensitive enough to demonstrate coupling at low concentrations of agonist.

The data obtained with the PKA inhibitors and activator are more difficult to interpret. Two of the PKA inhibitors, H-89 and PKA

fragment 6–22, blocked the baclofen mediated enhancement of L-type current whereas a third type of inhibitor, Rp-cAMPS did not. There are a number of potential explanations for the variety of responses seen. Rp-cAMPS and H-89 inhibit PKA through two very different mechanisms. Rp-cAMPS prevents dissociation of the catalytic subunit from the regulatory subunit by competitively binding to the cAMP sites on the regulatory subunit. In comparison, H-89 associates with the ATP binding site on the catalytic subunit and blocks the phosphorylation processes of PKA. Although H-89 is a potent inhibitor for PKA (Chijiwa et al., 1990), it has been shown to inhibit several other kinases including, PKG (IC_{50} = 340 nM), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII; IC_{50} = 11 μM), and PKC (IC_{50} = 14 μM; Davies et al., 2000; for review see Lochner and Moolman, 2006). Thus, it is possible that H-89 is inhibiting other kinases causing variation in the calcium current responses with baclofen application when compared to Rp-cAMPS. However, the PKA fragment 6–22 should be very specific to PKA with little effect on other kinases.

The variability of the PKA inhibitor data is not in contradiction with studies in salamander retina. Shen and Slaughter (1999) demonstrated that $GABA_B$ receptor activation can lead to the enhancement of L-type calcium current in a subset of salamander retinal neurons. Approximately 43% of the cells they tested showed an enhancement of current in response to application of 500 nM baclofen. In their study, the enhancement of L-type current was blocked in the presence of the PKC inhibitors GF-109203X and PKC fragment 19–36. In comparison to the PKC inhibitors, the PKA inhibitor Rp-cAMP only partially suppressed the enhancement of L-type current. Their study suggested that PKC is more important than PKA in the signaling pathway of $GABA_B$ receptor modulation of L-type current in the salamander retina (Shen and Slaughter, 1999). Our data with the PKC activator PMA, suggest that activation of PKC alone is sufficient to enhance L-type calcium current. Direct activation of PKA with 8-Br-cAMP did not result in the enhancement of L-type calcium current suggesting that activation of PKA alone is not sufficient to enhance L-type calcium current. It is possible that activation of $GABA_B$ receptors activates both G_q to turn on PKC and G_s to turn on PKA. Since all PKC inhibitors blocked the enhancement and the PKC activator mimicked the enhancement, it is likely that PKC activation is necessary to enhance L-type calcium current in neonatal hippocampal neurons. The

inhibition by two of the PKA inhibitors may reflect a supportive role of PKA in this process, since the PKA activator alone was not sufficient to produce the response. PKA and PKC would lead to phosphorylation on different residues of the calcium channel and thus, it is possible that phosphorylation at the PKA site makes phosphorylation at the PKC site more likely but in and of itself, is not capable of enhancing the current. For example, current through TRPV4 channels is enhanced by phosphorylation of different residues by PKC and PKA (Fan et al., 2009). Alternatively, there could be some interaction between the kinases prior to direct phosphorylation of the channel. Either kinase may not directly phosphorylate L-type calcium channels, but rather phosphorylate another intermediate effector protein or even each other. For example, in isolated rat ventricular myocytes activation of α_{1A} adrenoceptors leads to enhancement of L-type current. This enhancement of L-type calcium current is mediated through a PKC and CaMKII pathway (O-Uchi et al., 2008). Thus, it is possible that PKC or PKA may phosphorylate and activate CaMKII to enhance L-type current in the rat hippocampus. Both the regulatory subunit of PKA and CaMKII β-subunit exhibit consensus sequences for PKC mediated phosphorylation, whereas PKC α and β both exhibit consensus sequences for PKA mediated phosphorylation (Obenauer et al., 2003).

Although inhibitors of specific isoforms of PKC were not utilized in this study, the age of the pups and the specificity of PMA give some indication as to which isoforms may be involved. A study by Roisin and Barbin (1997), which explored the presence of several PKC isoforms during hippocampal development, suggests that PKCγ is likely not involved in the signaling pathway in this first postnatal week. PKCγ levels remain relatively low for the first postnatal week and only begin to rise following postnatal day 7. All of the other PKC isoforms tested, α, β, ε, and ζ were highly expressed by postnatal day 7. Therefore, each of these isoforms could be involved in the pathway of $GABA_B$ receptor mediated enhancement of L-type calcium current. Activation of PKC with PMA mimicked the enhancement seen with baclofen application, which suggests that either conventional or novel PKC isoforms are involved in the pathway rather than the atypical isoforms further reducing the potential PKC candidates to α, β, and ε.

In conclusion, the current study has demonstrated that attenuation of N-type calcium current by $GABA_B$ receptor activation is

mediated through a PTX sensitive G-protein pathway ($G_{i/o}$) and does not involve either PKC or PKA. Enhancement of L-type calcium current was still observed in the presence of PTX, which demonstrated that $G_{i/o}$ proteins are not involved in the pathway. Enhancement of L-type calcium current was not seen in the presence of PKC inhibitors and was observed with direct activation of PKC with a phorbol ester suggesting that PKC is involved in the pathway of L-type calcium current enhancement by $GABA_B$ receptor activation in the rat hippocampus. Enhancement was still observed in the presence of Rp-cAMPS, but not in the presence of two other PKA inhibitors, H-89 or PKA fragment 6–22, suggesting that PKA may be involved in the response. However, direct activation of PKA with a cAMP analogue, 8-Br-cAMP, did not result in the enhancement of L-type calcium current suggesting that activation of PKA alone is not sufficient to enhance L-type calcium current.

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Footnotes

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