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Emerging Physiological Significance of R-Type Calcium Currents

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EMERGING PHYSIOLOGICAL SIGNIFICANCE OF R-TYPE CALCIUM CURRENTS

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Introduction

R-type channels are voltage dependent calcium channel subtypes. To date, the only variant of the R-type calcium channel that has been isolated and cloned is the $Ca_V2.3$ variant, encoded by the gene Cchra1 (Yamazaki et al., 1998). The channel was named "R-type" because it was initially characterized by its resistance to nifedipine (a blocker of L-type calcium channels), Ω-conotoxin GVIA (an N-type calcium channel blocker) and Ω -Aga IVA (a blocker of P/Q-type calcium channels) (Hille 2001). The $Ca_V2.3$ calcium channel is selectively inhibited by the peptide SNX-482, which was isolated from venom of the African tarantula *Hysterocrates gigas* (Newcomb et al., 1998). R-type calcium currents are typically characterized by high voltage activation and rapid kinetics of deactivation (Foehring et al., 2004). Recent studies have begun to show the channel's physiological significance. Several pathophysiological states and diseases have been linked to abnormal R-type calcium currents. Recently $Ca_V2.3$ has been identified as a regulator of physiological changes following subarachnoid hemorrhage (SAV) (Ishiguro et al., 2005), blood insulin regulation (Jing et al., 2005), juvenile myoclonic epilepsy (Suzuki et al. 2004), and regulation of synaptic transmission (Cohen et al., 2004). Mechanisms of the channel's regulation, reasons for its association with pathophysiological states, and a pharmacological inhibitor that is effective at therapeutic doses are still being investigated. This thesis will review many of the recent findings about the physiological effects of R-type calcium channels, with special emphasis given to the possible relationship between these channels and epilepsy. It will then present original research regarding the effects of the novel anti-epileptic drug topiramate on R-type calcium channels.

Review of Recent Findings

Role of $Ca_V2.3$ in cerebral aneurysm and subarachnoid hemorrhage

Survival rates following subarachnoid hemorrhage (SAH) and cerebral aneurysms hover around 50%. This is partially due to constriction of the cerebral arteries following SAH. The constricted arteries carry insufficient blood to adequately feed the brain. Ishiguro et al. (2005) suggested that the arterial constriction is due to an increased expression of R-type calcium channels in the surface membrane of vascular smooth muscle cells following the hemorrhaging. L-type channels are thought to be the most common type of calcium channels in arterial myocytes and the dominant contributor to the inward Ca^{2+} current that regulates vascular tone. However, within five days following SAH, a new current is introduced. It was hypothesized that this was due to the increased expression of the $Ca_V2.3$ channel because the new current was blocked by SNX-482. The novel calcium channel was isolated and mRNA encoding the pore forming subunit was analyzed by RT-PCR. As hypothesized, the mRNA sequence matched the known sequence for the $Ca_V2.3$ channel. Furthermore, SNX-482 was found to dilate the arteries that had previously been constricted following SAH. This observation suggests that it is the $Ca_V2.3$ channel which directly causes vasoconstriction of the cerebral arteries. It is thought that following SAH, currently unknown mechanisms stimulate the expression of $Ca_V2.3$ channels in the damaged blood vessels.

Role of $Ca_V2.3$ in glucose regulation: insulin and glucagon secretion

Pancreatic beta cells secrete insulin, which regulates blood glucose levels. Insulin is typically released in two phases: the first phase has a rapid onset and is transient; the

second phase has a slower onset and is prolonged. Type II diabetes is characterized by a single rapid phase insulin release, in which the longer second phase is absent. Insulin exocytosis is stimulated by the opening of voltage-gated calcium channels, increasing the intracellular Ca^{2+} concentration. It is believed that L-type calcium channels (specifically $Ca_V1.3$) regulate the first phase of insulin release. However, knockout mice lacking $Ca_V2.3$ show symptoms of type II diabetes (Matsuda et al., 2001). Jing et al. (2005) noted a 21% decrease in insulin exocytosis from beta cells of the $Ca_V2.3$ knockout mice compared with control mice. To assess the role of $Ca_V2.3$ in dynamic insulin release, they performed *in situ* pancreatic perforations. There was little difference between the knockout mice and the control mice during the initial phase of insulin release. However, following the initial insulin peak, insulin exocytosis dropped in the knockout mice but not in the control mice. This led the team to conclude that $Ca_V2.3$ is a key regulator in second phase insulin release.

Pereverzev et al. (2005) showed that $Ca_V2.3$ also has minor involvement in glucagon secretion. Glucagon release was slightly impaired in $Ca_V2.3$ -/- knockout mice compared to wild-type mice. Their experiments showed a greater inhibition of glucagon release *in vitro* than *in vivo*. They hypothesize that Ca^{2+} influx through $Ca_V2.3$ stimulates glucagon release.

The effects of μ-opioid receptors on $Ca_V2.3$

Morphine is suspected to produce analgesic effects by inhibiting voltage-gated calcium channels of primary afferent neurons, and thus reducing nociceptive neurotransmitter release. Yokoyama et al. (2004) suggested that Cav2.3 is immune to morphine effects, and that an increase of Cav2.3 channels is directly linked to morphine tolerance. Results of their experiments showed that Cav2.3 -/- mice have greater resistance to developing morphine tolerance, as shown by response to painful stimuli during extended morphine treatment. They suggested that morphine tolerance might be linked to an increase in $Ca_V2.3$ channels following exposure. Furthermore, they found that blocking the $Ca_V2.3$ channel alone could prevent morphine tolerance. They hypothesized that $Ca_V2.3$ interacts either directly or indirectly with DAG, a receptor that is thought to be the one of the primary targets of morphine. Studies showing this relationship have not been completed. The ability to inhibit morphine tolerance would reduce the amount of morphine required for pain treatment and allow for extended morphine treatments (Yokoyama et al., 2004). In a separate study, the same group also found $Ca_V2.3$ -/- mice to have reduced sensitivity to anesthetics such as halothane (Takei et al., 2003).

Ortiz-Miranda et al. (2005) showed that a μ-opioid receptor agonist called DAMGO (D-ALA2, MePhe4, Glyol(5)enkephalin) inhibits oxytocin release from neurohypophysial cells. It had previously been established that $Ca_V2.3$ regulates oxytocin secretion, and they hypothesized that DAMGO preferentially blocks the $Ca_V2.3$ channel. Experiments in which cells were treated with both SNX- 482 and DAMGO showed that the two agents were not additive, suggesting that both compete for the same target, presumably the

$Ca_V2.3$ channel. After isolating R-type calcium currents, they were able to show that DAMGO selectively inhibited the current. They believe that the blocking of $Ca_V2.3$ channels by μ-opioid receptors may be a general mechanism of opiates (Ortiz-Miranda et al., 2005).

The potential role of $Ca_V2.3$ in epilepsy

$Ca_V2.3$ has been recently implicated in epilepsy. Suzuki et al. (2004) recently identified the EFHC1 gene. This gene is thought to encode protein containing an E-F hand motif. EFHC1 appears to be very closely linked to a $Ca_V2.3$ channel. When the EFHC1 protein was precipitated with an antibody, the $Ca_V2.3$ channel precipitated with it. Mutations in the EFHC1 gene were found in several different cases of juvenile myoclonic epilepsy in unrelated families. Apoptosis increased when cells that naturally express $Ca_V2.3$ were transfected with EFHC1. Conversely, cell survival rates increased when cells expressing both EFHC1 and $Ca_V2.3$ were administered the R-type blocker SNX-482. No difference in apoptosis rate was observed when the cells were treated with agents that block $Ca_V2.1$ and $Ca_V2.2$. The team hypothesized that EFHC1 increases Ca^{2+} flow through $Ca_V2.3$ channels and stimulates apoptosis in developing nervous systems. Neurons with a mutant form of EFHC1 fail to stimulate sufficient Ca^{2+} influx through $Ca_V2.3$; this prevents normal apoptosis, leading to excess neurons. Excess neurons are thought to be unstable and fire spontaneously, possibly leading to grand mal episodes. However, the mechanism

by which EFHC1 causes epilepsy is not fully understood. Its association with epilepsy could be the result of downstream targets other than $Ca_V2.3$. Interestingly, epilepsy has not been observed in $Ca_V2.3$ -/- mice. The team hypothesized that this could be due to insufficient observation, or species-independent responses.

Kuzmiski et al. (2005) suggested another possible relationship between $Ca_V2.3$ and epilepsy. They observed that the anti-epileptic drug topiramate depressed plateau potentials in hippocampal CA1 pyramidal neurons by an unknown mechanism. Plateau potentials are thought to contribute to epileptic activity in the hippocampus. After treatment with a series of channel blocking agents, including Ω-conotoxin MVIIC, Ω-conotoxin GVIA, and Ω-agatoxin, they attributed the depression to a topiramate-induced reduction of blocker-resistant calcium currents. To follow up this experiment, they transfected $Ca_V2.3$ into tsA-201 Human Embryonic Kidney Cells, a cell line that normally does not express high voltage-activated calcium channels. In this cell line, they once again showed that topiramate blocked the calcium current.

They continued to hypothesize that flow through R-type calcium channels accounts for about 80% of the current required to achieve the plateau potential. They attributed the remaining 20% to low-voltage activated T-type calcium channels, which are also resistant to traditional calcium channel blockers, and to other unknown mechanisms. It is suggested that the development of agents that block $Ca_V2.3$ currents at therapeutic concentrations may be used as a way of treating epilepsy (Kuzmiski et al., 2005).

Original Research to Investigate the Effects of Topiramate on $Ca_V2.3$

Introduction

Topiramate is a novel antiepileptic drug that has also been found effective against migraines, bulimia, bipolar disorder, and post-traumatic stress disorder (Russo et al., 2004). It has been shown to have an antiepileptic effect in 40-50% of people who take it. Those benefited by the drug have a 50 % reduction in partial seizures and an 89-100% reduction in tonic-clonic seizures. Side effects include cognitive difficulties, ataxia, dizziness, headaches, fatigue, gastrointestinal upset, and renal calculi. Topiramate is known to block sodium channels and inhibit γ-aminobutyric acid receptors, induce cytochrome P450, and attenuate kainate-induced responses. However, the exact mechanisms of topiramate remain unknown (Dichter et al., 1996). Thapliyal et al. (unpublished results) showed that topiramate leads to a 15% reduction of $Ca_V2.3$ Ca^{2+} current in HEK 293 cells. Fraser and MacVicar (1996) suggested that the muscarinic agonist carbachol induces epilepsy-like plateau potentials in hippocampal neurons. Interestingly Kuzmiski et al. (2005) showed that these plateau potentials were partially caused by $Ca_V2.3$ and were blocked by topiramate. In Melliti et al., (2000) it was shown that carbachol can lead to stimulation of $Ca_V2.3$. Therefore, we sought to investigate whether topiramate blocks Ca^{2+} current through $Ca_V2.3$ by preventing the muscarinic-induced stimulation of $Ca_V2.3$.

Methods

Cell Culture

Human Embryonic Kidney 293 (HEK293) cells were obtained from American Type Culture Collection. They were cultured in 90% Dulbecco Modified Eagle Medium 11995 (Gibco) with 10% Fetal Bovine Serum (Hyclone Labs, Logan, Utah) and 50 μg/ml gentamicin (Gibco). Cultures were incubated at 37° Celsius in a water-saturated atmosphere containing 5% CO_2. The cells were passaged weekly by being trypsinized for 30 seconds and replated on to culture dishes.

Transfection

Cells were transfected using $CaPO_4$ precipitation between 12 and 24 hours after plating with plasmids encoding $Ca_V2.3$ (GenBank accession number X67856), $\alpha_2\delta$ (M86621), $ß_3$ (X64300). Cells were also transfected with plasmids encoding enhanced green fluorescence protein (EGFP). In addition, the cells were transfected with either the acetylcholine M1 muscarinic receptor (M1R) or Neurokinin 1 receptor (NK1R) (NM015727).

Electrophysiological recordings

Large-bore patch pipettes were pulled from 100 μl borosilicate glass micropipettes (VWR). The pipette resistance was between 1 and 4 MΩ. Pipettes were filled with a solution containing 155 mM CsCl, 10 mM Cs_2EGTA, 4mM MgATP, 0.32 mM LiGTP, and 10 mM HEPES brought to a pH of 7.4 using CsOH. Aliquots of the pipette solution were stored at -80° Celsius.

The bath solution contained 145 mM NaCl, 10 mM $CaCl_2$, 2 mM KCl, and 10 mM HEPES. The pH of the bath solution was brought to 7.4 with 1 N NaOH.

Transfected cells were identified by the presence of green fluorescence when stimulated by 488 nm wavelength light and by the presence of voltage-dependent calcium current. Data were only recorded from cells meeting both of these criteria. Patch recordings were generated using the protocol described in Melliti et al. (2000). Data were collected using the whole-cell ruptured-patch technique with seal of greater than one GΩ. The steady holding potential was - 90 mV. Depolarizations to +30 mV were delivered every 1-10 seconds. Data were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and pCLAMP 7.0 software (Axon Instruments)

Results

Recordings from cells transfected with $Ca_V2.3$, $\alpha_2\delta$, $ß_3$, and NK1R were taken in the whole-cell ruptured-patch configuration. Cells were held at -90 mV and stimulated every 1-10 seconds. The results of this experiment can be seen in Figure 1.

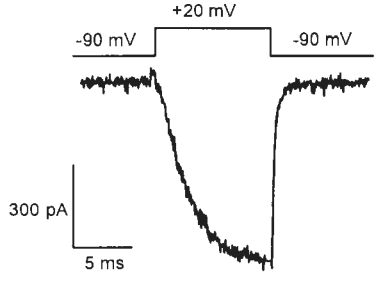
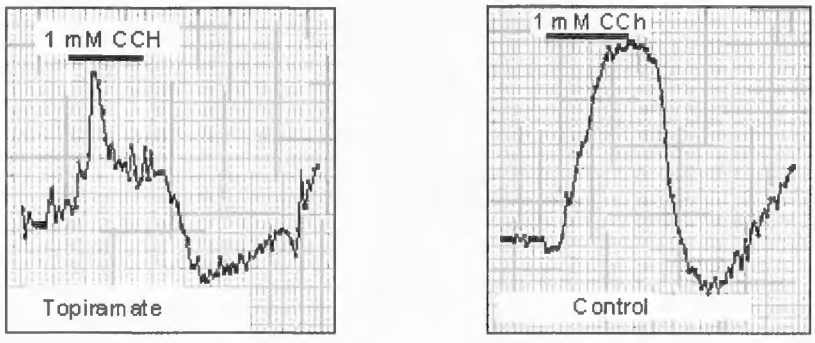


Figure 1: A representative Ca^{2+} current from a HEK293 cell expressing $Ca_V2.3$. Tail current has been removed for presentation.

The effect of the drug topiramate was determined by using HEK cells transfected with $Ca_V2.3$, $\alpha_2\delta$, $ß_3$, M1, and EGFP. Cells were incubated with 50 μM topiramate (in media containing 0.1% DMSO) for 1-3 hours before patching. The bath solutions also contained 50 μM topiramate and 0.1% DMSO. Cells were stimulated with 1 mM carbachol for 1 minute before being washed out. Control cells were exposed to the same concentration of



A **B**

Figure 2: Peak $Ca_V2.3$ currents of cells treated with topiramate and then exposed to CCh for ~2 minutes. Both the topiramate-treated cell (A) and the control cell (B, not treated with topiramate) show both inhibition and stimulation of $Ca_V2.3$ current when subjected to CCH.

DMSO without topiramate. These data are preliminary; not enough data have been collected to make statistically valid statements about the overall effect of topiramate on $Ca_V2.3$ via M1R. However, topiramate failed to consistently block carbachol-induced stimulation of $Ca_V2.3$ current. It is possible that topiramate reduces channel activation, without blocking it directly. The results of these experiments can be seen in Figure 2.

Discussion

Recent studies have shown that R-type calcium currents play key roles in several neurological disorders, including epilepsy. Kuzmiski et al., (2005) showed that $Ca_V2.3$ is involved in the generation of hippocampal plateau potentials, and that $Ca_V2.3$ is one of the targets of topiramate. Although our data fail to show that topiramate consistently

block stimulation of $Ca_V2.3$ through M1R, topiramate might reduce stimulation. However, topiramate might also reduce calcium current and block plateau potentials through another mechanism; possibly topiramate simply blocks the pore-forming subunit of $Ca_V2.3$. Metz et al. (2005) showed that R-type calcium currents responsible for epilepsy-like after-depolarizations are resistant to SNX-482, suggesting that additional R-type calcium channel isoforms might be involved. At this point, the data are preliminary, and it is difficult to make sound conclusions. Future experiments will seek to further establish the relationship between topiramate and $Ca_V2.3$. A possible experiment could involve transfection with a single plasmid encoding $Ca_V2.3$, $\alpha_2\delta$, $ß_3$, and M1. This might enable more control over the relative amount of each subunit expressed in the cell. Also, lower concentrations of carbachol could be used to stimulate the channel without inhibiting it, thus simplifying the results and removing compounding factors.

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