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Regulation of Calcium Channels in Brain: Implications for the Clinical Neurosciences

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Calcium is a major second messenger in neurons and modulates many neuronal functions, including protein phosphorylation, phospholipid metabolism, cytoskeletal activity, and neurotransmitter release. These important events, which regulate neuronal activity, are directly dependent on the influx of extracellular calcium through voltage-sensitive calcium channels (VSCCs) in the neuronal membrane. Modulation of VSCC function represents an important strategy for regulating neuronal excitability. Although substantial evidence supports the ability of dihydropyridines to block VSCCs and contractility in cardiovascular tissue, their ability to block the majority of neuronal VSCCs remains controversial. Benzodiazepines, and other anticonvulsants, block depolarization-dependent ^{45}Ca uptake through VSCCs in brain synaptosome preparations. In addition, benzodiazepines reduce voltage-gated calcium conductance as determined by voltage clamp studies of identified invertebrate neurons. Inhibition of VSCC activity may be an important mechanism by which these compounds produce their anticonvulsant and sedative effects. Intrasomal injection of calcium-calmodulin-dependent protein kinase modulates calcium conductance in invertebrate neurons, suggesting that protein phosphorylation may be an endogenous regulatory mechanism of VSCC activity. Developing novel pharmacological approaches to regulating VSCCs and understanding the endogenous regulatory mechanisms may lead to new therapeutic approaches to the treatment of neurological diseases.

Calcium is an important mediator of molecular events in neurons [1]. An increase in cytoplasmic Ca^{2+} in response to depolarization has been shown to modulate numerous neuronal functions, including synaptic morphological changes, stimulation of protein phosphorylation, and neurotransmitter release [2]. These events are directly dependent on the entry of extracellular Ca^{2+} ions into the presynaptic nerve terminal through specific voltage-sensitive Ca^{2+} channels (VSCC) [3]. This central role of Ca^{2+} channel function in neuronal activity, and in stimulus-secretion coupling phenomena, has led to extensive investigation of Ca^{2+} channel function in a wide variety of tissues [1,4]. These studies have led to the understanding that VSCCs are a heterogenous population of membrane channels with different pharmacological and physiological properties. In spite of extensive biochemical and electrophysiological characterization of Ca^{2+} channels in brain, however, the molecular nature and pharmacological properties of neuronal Ca^{2+} channels remain largely unclear.

VOLTAGE-SENSITIVE CALCIUM CHANNELS

The properties of Ca^{2+} channels in some non-neuronal tissues, particularly in the heart and vascular smooth muscle, have been extensively studied [5]. Ca^{2+} regulates muscle function and contractility in both smooth and cardiac muscle. As in brain, some

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Ca^{2+} channels in these tissues are voltage-sensitive, although Ca^{2+} influx can also be modulated by neurotransmitters, hormones, and various drugs through receptor-operated channels [6]. Both of these types of Ca^{2+} channels are blocked by polyvalent cations such as Mn^{2+}, Co^{2+}, and La^{3+}. Several distinct classes of organic Ca^{2+} channel blockers have been identified in cardiovascular systems, including verapamil, diltiazem, and the dihydropyridines, nitrendipine, nisoldipine, and nimodipine. The ability of the dihydropyridines to act as VSCC antagonists has led to their clinical use in the treatment of cardiac arrhythmias, hypertension, and other cardiovascular disorders. Furthermore, these drugs have been used as specific pharmacological probes for the molecular study of Ca^{2+} channels in heart and smooth muscle preparations. High-affinity [^{3}H]nitrendipine binding sites, which have been identified and characterized in ileal smooth muscle, have binding properties that correlate well with nitrendipine inhibition of Ca^{2+} flux [7]. Although some variations between preparations have been observed, the pharmacological correlation between nitrendipine binding and nitrendipine inhibition of Ca^{2+} uptake suggests that [^{3}H]nitrendipine binding sites may represent VSCCs in cardiovascular and smooth muscle tissue.

In brain and other neuronal tissues, voltage-sensitive Ca^{2+} channels are also blocked by polyvalent cations such as Mn^{2+}, Co^{2+}, and La^{3+}. Because of the success of verapamil and the dihydropyridines as Ca^{2+} channel probes in cardiac and smooth muscle, these compounds have been employed to study Ca^{2+} channels in brain. In brain preparations, specific high-affinity [^{3}H]nitrendipine binding sites have been observed which have pharmacological properties almost identical to those found in the heart [8]; however, the effectiveness of these drugs as Ca^{2+} channel blockers in brain seems to be substantially different. Organic Ca^{2+} antagonists are relatively ineffective blockers of voltage-sensitive Ca^{2+} uptake in synaptosomes [9] at concentrations where binding to [^{3}H]nitrendipine receptors is maximal. Although some studies have appeared which show dihydropyridine inhibition of Ca^{2+} uptake in cultured neuronal cell lines, nitrendipine does not block neurotransmitter release in these cells or neurons. Recent studies have shown that fast-phase Ca^{2+} uptake (<1 second) in synaptosomes is inhibited by nitrendipine in Na^+-free media [10], but its effect was incomplete and may not be associated with inhibition of neurotransmitter release. These observations suggest that the functional characteristics of Ca^{2+} channels in neuronal tissue are extremely complex and that multiple types of Ca^{2+} channels, which have different pharmacological properties, may exist. Future studies of VSCCs in neuronal tissue will center on electrophysiological characterization where subtle functional differences can be detected.

Although the molecular properties of Ca^{2+} channels in brain are controversial, the central role of VSCCs in brain function remains unchallenged. Pharmacological manipulation of VSCC activity potentially provides an important mechanism for modulating neuronal function, as has been successfully achieved with Ca^{2+} channel modulators in cardiovascular systems. This review will examine the evidence that certain anticonvulsant compounds, including the benzodiazepines, diphenylhydantoin and carbamazepine, modulate Ca^{2+} channel function in brain, and that this action may represent an important mechanism by which these compounds produce their anticonvulsant and sedative effects.

ANTICONVULSANT INHIBITION OF SYNAPTOSOMAL CALCIUM UPTAKE

Anticonvulsants are a complex class of neuroactive compounds which have numerous effects on cells, including alteration of growth properties, energy metabolism,

enzyme activity, and neurotransmitter secretion. Since anticonvulsants produce multiple clinical effects as well, it is important to examine the effects of these compounds on the biochemical level to identify their precise mechanisms of action. Benzodiazepines (BZs) have been shown to bind to high-affinity BZ receptors in brain [11]. These central BZ receptors exist as a complex with GABA receptors and chloride ionophores such that, on BZ binding to its receptor, a hyperpolarizing increase in chloride flux occurs. The pharmacological characteristics of BZ binding to the high-affinity central BZ receptor correlate well with BZ anxiolytic effects and BZ inhibition of pentylenetetrazol-induced seizure activity, suggesting that these effects are mediated through BZ binding at this receptor site. However, other therapeutic effects of BZs do not correlate with binding to this site, such as BZ inhibition of maximal electric shock-induced seizures, suggesting that BZs have other physiological effects. We have investigated the possibility that the neuronal stabilizing properties of BZs and other anticonvulsants are produced by modulation of Ca^{2+} uptake parameters in neurons.

Several lines of evidence suggest that anticonvulsants modulate Ca^{2+}-dependent mechanisms in neurons and led to investigation of the role of anticonvulsants in altering VSCC function. Several types of anticonvulsant compounds, including the benzodiazepines, diphenylhydantoin and carbamazepine, have been shown to block Ca^{2+} uptake in nerve terminal preparations [12,13,14]. In addition, these compounds inhibit Ca^{2+}-dependent neurotransmitter release in synaptosomes [2], indicating that they regulate functionally significant Ca^{2+} influx. Since this action may represent a physiologically relevant anticonvulsant or sedative mechanism, we have examined more precisely the ability of the benzodiazepines to act as Ca^{2+} channel blockers in brain.

To investigate the effects of BZs on synaptosomal Ca^{2+} uptake, ^{45}Ca flux was monitored under both depolarized and non-depolarized conditions [14]. Under these experimental conditions, Ca^{2+} flux consists of both Ca^{2+} uptake into the synaptosomal cytoplasm as well as Ca^{2+} efflux. BZs, in micromolar concentrations (50–200 μM), block the ^{45}Ca uptake component of Ca^{2+} flux and show no effect on Ca^{2+} efflux under these conditions (Fig. 1). BZs inhibit the rapid phase of depolarization-sensitive ^{45}Ca uptake (<5 seconds) and have no significant effect on control (non-depolarized) synaptosomes. Synaptosomal depolarization can be induced either by the presence of high K^+ levels (30–70 mM) or veratridine (50 μM), and both result in Ca^{2+} accumulation which is BZ-sensitive. Under these depolarizing conditions, synaptosomal Ca^{2+} uptake proceeds down its concentration gradient through voltage-sensitive Ca^{2+} channels and Na^+ channels. The Na^+ channel blocker, tetrodotoxin (TTX), effectively blocks veratridine-induced ^{45}Ca uptake, but TTX does not inhibit high K^+-induced ^{45}Ca uptake, nor does it affect BZ inhibition of high K^+-induced ^{45}Ca uptake. Thus, BZ-sensitive ^{45}Ca uptake in depolarized synaptosomes is not mediated by TTX-sensitive Na^+ channels and is probably associated with voltage-sensitive Ca^{2+} channels. In support of this conclusion, we observed that BZ-sensitive ^{45}Ca uptake is also sensitive to the Ca^{2+} channel blockers Mn^{2+} and Co^{2+}. In summary, the biochemical evidence suggests that the nature of BZ inhibition of Ca^{2+} accumulation in synaptosomes represents blockage of Ca^{2+} uptake induced by depolarization and mediated by voltage-sensitive Ca^{2+} channels. Thus, BZs act like Ca^{2+} channel blockers in synaptosomes.

BENZODIAZEPINE INHIBITION OF CALCIUM CONDUCTANCE IN LEECH NEURONS

Since numerous factors can influence ^{45}Ca uptake in synaptosomal fractions, we

initiated a series of electrophysiological investigations to complement the biochemical studies using synaptosomes [15]. These investigations were designed to test the hypothesis that benzodiazepines block voltage-gated Ca^{2+} channels in intact invertebrate neurons. We employed established techniques in identified cells selected for their well-studied Ca^{2+} currents. Our initial efforts in this direction involved the use of intracellular recording techniques in identified nociceptive neurons (N cells) of the leech *Macrobdella decora.*

To determine the electrophysiological effects of BZs on Ca^{2+} conductance, we studied the actions of these compounds on the long-lasting regenerative Ca^{2+} potential revealed by blocking outward K^+ currents in identified N cells [15]. These neurons possess Mn^{2+}- and Co^{2+}-sensitive divalent cation potentials which have the same properties as Ca^{2+} channels described in other neuronal preparations from vertebrate and invertebrate phyla. We observed that, in μM concentrations, BZs reversibly inhibit voltage-dependent Ca^{2+} conductance in a dose-dependent manner, indicating that BZs act as Ca^{2+} channel blockers (Fig. 2). Like Mn^{2+}, BZs inhibit the maximum rate of depolarization (V_{max}) and duration of the Ca^{2+} potentials. This inhibitory effect was observed at BZ concentrations that do not significantly affect the resting membrane potential or V_{max} of the Na^+-dependent action potential. These findings suggest that BZs are not acting non-specifically or like local anesthetics, but may selectively affect the Ca^{2+} channel in these neurons of the leech. The apparent K_i for BZ inhibition of the N cell Ca^{2+} conductance correlated well with the IC_{50} values for BZ inhibition of synaptosomal ^{45}Ca uptake. These findings are consistent with the hypothesis that BZs regulate voltage-sensitive Ca^{2+} channels and that this effect may play a role in anticonvulsant regulation of neuronal excitability.

BENZODIAZEPINES REDUCE VOLTAGE-GATED CALCIUM CONDUCTANCE IN *HERMISSENDA* NEURONS

The data from the electrophysiological studies in the leech do not preclude the possibility that BZs may have effects on other ion conductances. It is important to investigate the effects of BZs on other model systems using voltage clamp technology to determine more accurately their role as neuroactive agents. Thus, we have examined the effects of BZs on Ca^{2+} conductance under voltage clamp conditions in identified neurons from the sea snail *Hermissenda crassicornis.*

We used conventional two-electrode voltage clamp techniques to examine the effects of BZs on a Ca^{2+} conductance (I_{Ca}) in identified *Hermissenda* neurons [16]. I_{Ca} was measured in a giant pedal neuron (LP1) bathed in 0 Na^+ artificial seawater containing 100 mM tetraethylammonium (TEA) and 3 mM 4-aminopyridine (4-AP) to eliminate Na^+ and K^+ conductances. Ba^{2+} replaced Ca^{2+} as the current carrier to maximize current carried and eliminate Ca^{2+}-activated outward K^+ conductance (I_C). Previous studies have demonstrated that current carried under these conditions is sensitive to other ionic blockers of neuronal voltage-gated Ca^{2+} channels, including Mn^{2+} and Co^{2+}. We observed that micromolar concentrations of bath-applied diazepam or medazepam substantially reduced I_{Ca} in a dose-dependent manner. Inhibition of I_{Ca} was reversible (1–2 minutes). Vehicle (ethanol, 0.5 percent) alone had no effect. Similar results were obtained in other identified cells in the pedal ganglion and in photoreceptor cells of the *Hermissenda* eye. These data suggest that BZs reduce the inward Ca^{2+} current in *Hermissenda* neurons. This result is consistent with the biochemical and electrophysiological data previously obtained, indicating that BZs

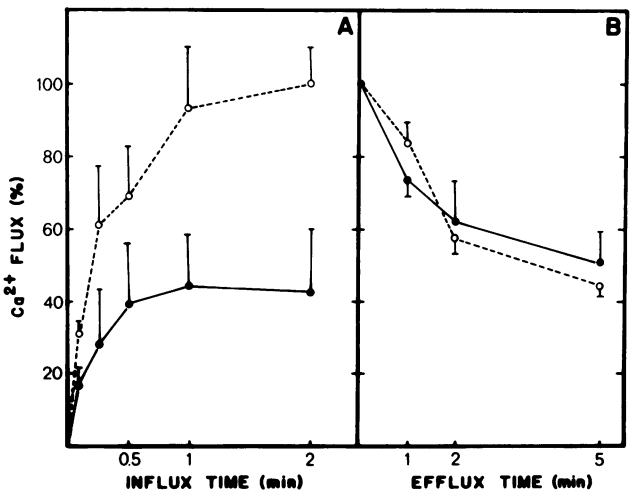

FIG. 1. Effects of diazepam (DZ) on synaptosomal ^{45}Ca uptake (**A**) and efflux (**B**). **A.** Data show ^{45}Ca uptake in depolarizing (70 mM K^+) conditions in the presence (*solid circles*) and absence (*open circles*) of 150 μM DZ. DZ inhibits Ca^{2+} uptake at all time points studied. **B.** The effect of DZ on ^{45}Ca efflux from preloaded synaptosomes was determiend in the presence (*solid circles*) and absence (*open circles*) of 150 μM DZ. No effect of DZ on Ca^{2+} efflux is evident. All data are expressed as percentages of maximal ^{45}Ca (n = 8; error bars, mean ± SEM). Taken from [14].

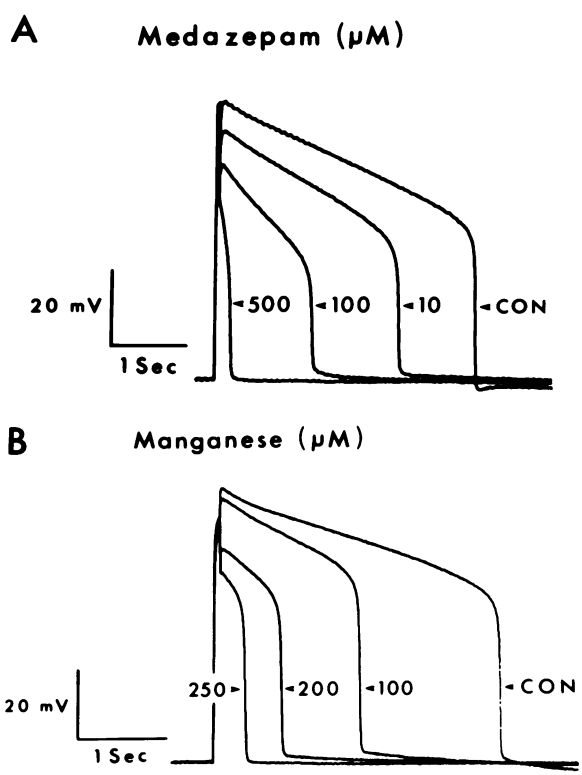

FIG. 2. Effects of medazepam (MDZ) and Mn^{2+} on Sr^{2+} potentials in lateral N cells of the leech. Dose-dependent inhibition of the divalent cation conductance by MDZ (**A**) and Mn^{2+} (**B**) was obtained in Na^+-free/TEA/Ringers solution. A similar pattern of Ca^{2+} channel inhibition was observed. Taken from [15].

function as neuronal Ca^{2+} channel blockers. Thus, based on ^{45}Ca uptake studies in synaptosomes, intracellular recording of Ca^{2+} potentials in the leech, and voltage clamp recording of Ca^{2+} conductance in *Hermissenda,* BZs inhibit neuronal VSCC. Future studies will examine the role of other putative Ca^{2+} channel blockers in these model systems, as well as BZ effects on other ionic currents.

EFFECTS OF CaM KINASE II ON CALCIUM CONDUCTANCES IN *HERMISSENDA* NEURONS

Although substantial evidence suggests that neuronal Ca^{2+} channels are sensitive to BZs, the mechanism by which these compounds exert their inhibitory effect is unknown. The BZs may act directly on the channel itself or might affect VSCC via a known regulator of channel function. Recent studies have examined putative mechanisms that might be involved in the *in vivo* regulation of VSCC function. It has been well-described that cellular second messengers, such as cAMP and Ca^{2+}, and neurotransmitters and hormones acting through these messengers, can modulate ion channel function [6]. Initial efforts to identify the endogenous mechanisms that regulate ion channels investigated the role of cAMP in regulating conductances in heart cells and demonstrated that cAMP-dependent protein kinase may mediate the effects of cAMP on these conductances [4]. This work established the concept that the activity of specific ion channels in the membrane can be modulated by intracellular second messengers activating protein phosphorylation pathways.

Since Ca^{2+} plays a major role in regulating excitability in the nervous system, subsequent studies have investigated the ability of Ca^{2+}-regulated protein kinases, and particularly calcium-calmodulin-stimulated protein kinase (CaM kinase II), to modulate ion channel activity [17]. The recent purification and characterization of CaM kinase II makes it possible to determine directly the effects of purified kinase on identified Ca^{2+} conductances in intact neurons. The potential role of CaM kinase II in regulating Ca^{2+} conductances is particularly interesting in light of the fact that BZs inhibit CaM kinase II activity [18,19] and may thus be a mechanism for BZ inhibition of Ca^{2+} channel function.

Results from our laboratory, in collaboration with Dr. Daniel Alkon, have shown that injection of highly purified CaM kinase II reduces Ca^{2+} current in *Hermissenda* photoreceptor cells [17]. In these studies, CaM kinase II is injected intracellularly into *Hermissenda* neurons, and its effect on ion conductances is documented electrophysiologically using voltage clamp techniques. I_{Ca} was observed with 3 mM 4-AP, 100 mM TEA, and Ba^{2+} substituting for Ca^{2+} as the current carrier, as performed previously. A single injection of CaM kinase II was followed by a significant reduction of I_{Ca}. This reduction was enhanced by the presence of increased intracellular Ca^{2+} levels, presumably due to increased kinase activity under those conditions. Similar results were not obtained when heat-inactivated enzyme was injected. These effects of CaM kinase II appear to be an amplification of a regulatory mechanism already existing within the *Hermissenda* photoreceptor cell. These observations are consistent with the hypothesis that CaM kinase II may modulate VSCC activity in neurons. A hypothetical model might suggest that neuronal firing leads to increased intracellular Ca^{2+} levels and formation of the activated Ca^{2+}-calmodulin complex. This process activates CaM kinase II which can then phosphorylate either the VSCC itself or a regulator of VSCC activity to reduce channel activity. Although the effects of CaM kinase II were

not exclusive to I_{Ca} (two outward K^+ currents were also affected), this may represent an important mechanism by which neurons regulate excitability.

SUMMARY

The variation in potency of dihydropyridines and other Ca^{2+} channel blockers between neuronal and non-neuronal tissues suggests that VSCCs are a heterogenous population of membrane channels with different pharmacological properties. Whether these differences reflect diversity of the molecular composition of VSCCs, a complex set of conformational states, or alterations in the regulation of VSCC activity remain to be determined. Benzodiazepines and other anticonvulsants are effective inhibitors of some types of Ca^{2+} channel activity as determined in biochemical and electrophysiological models. This action may represent the biochemical mechanism by which these compounds elicit their sedative and anticonvulsant properties. In addition, protein phosphorylation regulates certain types of Ca^{2+} channels as demonstrated by CaM kinase II modulation of Ca^{2+} conductance in invertebrate neurons. Understanding the molecular mechanisms mediating Ca^{2+} flux and how it is regulated endogenously and by neuroactive compounds is an important goal of neuroscience and may provide new therapeutic interventions for neurological diseases.

REFERENCES

1. Rubin RP: The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol Rev 22:389–428, 1972
2. DeLorenzo RJ: Calmodulin in neurotransmitter release and synaptic function. Fed Proc 41:2265–2272, 1982
3. Katz B, Miledi R: Further study of the role of calcium in synaptic transmission. J Physiol 207:789–801, 1970
4. Tsien RW: Calcium channels in excitable cell membranes. Ann Rev Physiol 45:341–358, 1983
5. Fleckenstein A: Specific pharmacology of calcium in myocardium, cardiac pacemakers and vascular smooth muscle. Ann Rev Pharmacol 17:149–166, 1977
6. Reuter H: Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 301:569–574, 1983
7. Bolger GT, Gengo P, Klockowski R, et al: Characterization of binding of the calcium channel antagonist, [^{3}H]nitrendipine, to guinea pig ileal smooth muscle. J Pharmacol Exp Therap 225:291–299, 1983
8. Gould RJ, Murphy KMM, Snyder SH: [^{3}H]Nitrendipine-labeled calcium channels discriminate inorganic calcium agonists and antagonists. Proc Natl Acad Sci USA 79:3656–3660, 1982
9. Nachshen DA, Blaustein MP: Effects of some organic "calcium antagonists" on calcium influx in presynaptic nerve terminals. Molec Pharmacol 16:579–586, 1979
10. Turner TJ, Goldin SM: Calcium channels in rat brain synaptosomes: Identification and pharmacological characterization: High affinity blockade by organic calcium channel blockers. J Neurosci 5:841–849, 1985
11. Braestrup C, Squires RF: Pharmacological characterization of benzodiazepine receptors. Eur J Pharmacol 48:263–270, 1978
12. Leslie SW, Friedman MB, Coleman RR: Effects of chlordiazepoxide on depolarization-induced calcium influx into synaptosomes. Biochem Pharmacol 29:2439–2443, 1980
13. Ferrendelli JA, Daniels-McQueen S: Comparative actions of phenytoin and other anticonvulsants on potassium- and veratridine-stimulated calcium uptake in synaptosomes. J Pharmacol Exp Therap 220:29–34, 1982
14. Taft WC, DeLorenzo RJ: Micromolar benzodiazepine receptors regulate voltage-sensitive calcium channels in nerve terminal preparations. Proc Natl Acad Sci USA 81:3118–3122, 1984
15. Johansen J, Taft WC, Yang J, et al: Inhibition of calcium conductance in identified leech neurons by benzodiazepines. Proc Natl Acad Sci USA 82:3935–3939, 1985

16. Taft WC, Forman RR, Alkon DL, et al: Benzodiazepines reduce voltage-gated calcium conductance in Hermissenda neurons. Trans Amer Soc Neurochem 17:250, 1986
17. Sakakibara M, Alkon DL, DeLorenzo RJ, et al: Modulation of calcium-mediated inactivation of ionic currents by calcium/calmodulin-dependent protein kinase II. Biophys J 50:319–328, 1986
18. Taft WC, Goldenring JR, Buckholz TM, et al: Benzodiazepine inhibition of purified CaM kinase II. Pharmacologist 27:185, 1985
19. Taft WC, Goldenring JR, DeLorenzo RJ: Molecular mechanisms of neuronal excitability: Possible involvement of CaM kinase II in seizure activity. In Molecular Mechanisms of Neuronal Responsiveness. In press