Activation of Brain Acetylcholine Receptors by Neuromuscular Blocking Drugs

A Possible Mechanism of Neurotoxicity

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Background: Neuromuscular blocking drugs cause excitement and seizures when introduced into the central nervous system. We examined the possibility that these drugs produce paradoxical activation of acetylcholine or glutamate receptors, the chief types of brain receptors involved in excitatory neurotransmission.

Methods: Because activation of central glutamate or acetylcholine receptors causes calcium influx into postsynaptic neurons, we measured intracellular calcium concentration ([Ca\(^{2+}\)]) as an index of receptor activation. Changes in [Ca\(^{2+}\)] were compared in brain slices exposed to neuromuscular blocking drugs or acetylcholine and glutamate receptor agonists. [Ca\(^{2+}\)] was measured with the fluorescent dye fura-2.

Results: Pancuronium and vecuronium caused sustained increases in [Ca\(^{2+}\); in approximately the same potency ratio as for seizure activity in vivo (concentrations at which the increase in [Ca\(^{2+}\)] was 95% of maximal: 10 and 400 \(\mu\)M, respectively). Atracurium and laudanosine did not increase [Ca\(^{2+}\)] in cortical slices. Increases in [Ca\(^{2+}\)] caused by both pancuronium and vecuronium were prevented by the non-subtype-specific nicotinic acetylcholine receptor antagonist d-tubocurarine and were reduced 44–73% by atropine. Blockade of glutamate receptors or voltage-gated calcium or sodium channels had no effect on calcium influx.

Conclusions: The results suggest that the acute excitement and seizures caused by introduction of pancuronium and vecuronium into the central nervous system is due to accumulation of cytosolic calcium caused by sustained activation of acetylcholine receptor ion channels. (Key words: Brain; intracellular calcium concentration. Measurement techniques, fluorometry: fura-2. Neuromuscular relaxants. Neuromuscular blocking drugs: atracurium; pancuronium; vecuronium. Receptors: nicotinic acetylcholine.)

NONDEPOLARIZING neuromuscular blocking drugs, because they are highly ionized, normally do not cross the blood–brain barrier.1 d-Tubocurarine has been found in human cerebrospinal fluid after a single intravenous dose. Critically ill patients, however, can be susceptible to pathophysiologic disruptions of the blood–brain barrier, thereby allowing neuromuscular blocking agents into the central nervous system (CNS).1 Vecuronium and its 3-acetyl metabolite were found in the cerebrospinal fluid of two patients with sepsis who had undergone prolonged administration of vecuronium.2

Several studies suggest that neuromuscular blocking agents introduced into the CNS are pharmacologically active: autonomic dysfunction, weakness, prolonged neuromuscular blockade, neuronal death, and seizures all have been observed.3,6 In a recent study,7 vecuronium, pancuronium, and atracurium caused dose-dependent CNS excitement progressing to seizures in rats. These drugs also caused an increase in intracellular calcium concentrations ([Ca\(^{2+}\)]) in rat cerebrocortical brain slices.7 Because an increase in [Ca\(^{2+}\)] is involved in the pathophysiology of seizures, in relation to cellular depolarization, hyperexcitability, or neurotransmitter release or as a consequence of the seizure itself,8,9 it is possible that the increase in [Ca\(^{2+}\)] induced by these agents is the cause of the CNS excitement. There is also substantial evidence that increases in [Ca\(^{2+}\)], by excess activation of ion channels linked to excitatory neurotransmitter receptors (particularly glutamate receptors) for even brief periods leads to neuronal injury and death.9,11

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The possibility that neuromuscular blocking drugs cause an increase in $[\text{Ca}^{2+}]_i$ and seizures by activating ion channels associated with excitatory neurotransmitter receptors has not been investigated. Activation of either brain nicotinic acetylcholine receptors or glutamate receptors are known to cause seizures and are associated with an increase in $[\text{Ca}^{2+}]_i$. Of note, the ion channel of the nicotinic acetylcholine receptor is seven times as permeable to calcium as the neuromuscular junction acetylcholine receptor. Activation of the N-methyl-D-aspartate (NMDA) subtype of cortical glutamate receptor causes a large influx of calcium. Thus, increase in cytosolic calcium is a marker for activation of the chief classes of excitatory receptors in the CNS.

The goals of this study were (1) to compare the seizure induction potency of neuromuscular blocking drugs with ability to increase $[\text{Ca}^{2+}]_i$ in brain slices; (2) to test the hypothesis that the sustained increase of $[\text{Ca}^{2+}]_i$ in cerebrocortical slices induced by neuromuscular blocking drugs is caused by activation of ion channels associated with cortical acetylcholine receptors or glutamate receptors; and (3) to explore the possibility that anticonvulsant drugs prevent increase in $[\text{Ca}^{2+}]_i$ caused by the neuromuscular blockers.

**Materials and Methods**

Cortical brain slices were used to determine the relationship between concentration of neuromuscular relaxant and increase in $[\text{Ca}^{2+}]_i$, as well as to identify the causes of increased $[\text{Ca}^{2+}]_i$.

**Preparation of Cortical Brain Slices**

After institutional review board approval, Sprague-Dawley rats (20–30 days old) of either sex were anesthetized with 3% enflurane and oxygen. They were decapitated, and their brains were rapidly dissected and placed in 3–5°C artificial cerebrospinal fluid (Earle's balanced salt solution [Sigma, St. Louis, MO] saturated with 95% $\text{O}_2$, 5% $\text{CO}_2$, and 10 mM glucose, pH 7.35–7.45). Ionized calcium was 1.04–1.08 mM. Cortical brain slices, 350 µm thick, were then prepared with a vibrating tissue slicer.

**Measurements of Cytosolic Calcium in Cortical Slices**

The slices were prepared for measurements of $[\text{Ca}^{2+}]_i$ by incubation in artificial CSF containing 3 µM fura-2 acetoxyethyl ester (Molecular Probes, Eugene, OR). After at least 1 h, individual slices were rinsed in dye-free buffer, mounted on a mesh baffle, and placed in a standard fluorometer cuvette. The cuvette holder was thermostatted to 37°C throughout the experiment. Measurements of $[\text{Ca}^{2+}]_i$ were made with a Hitachi F-2000 fluorometer as described in detail by Bickler. Briefly, $[\text{Ca}^{2+}]_i$ was determined by the ratio of the intensity of 510-nm light emitted by cytosolic fura-2 during alternate stimulation of the slice with 340- and 380-nm light. Calibration was achieved by measuring the fluorescent signals from the fura-2 when $[\text{Ca}^{2+}]_i$ was manipulated to equilibrate with extracellular calcium (saturating the fura-2 with calcium) and at zero $[\text{Ca}^{2+}]_i$ in the presence of calcium ionophore A23187 (Sigma) or 0.01% Triton-X 100, calcium-free medium, and 1 mM EGTA (ethylene glycol-b-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid). Background fluorescence was subtracted by quenching fura-2 fluorescence with 5 mM MnCl$_2$.

**Experimental Procedures**

After a 5–10 min period to ensure baseline $[\text{Ca}^{2+}]_i$, stability, neuromuscular relaxants were introduced into the cuvette by injection. In studies involving pretreatment with antagonists such as d-tubocurarine or the anticonvulsant phentoin, these compounds were introduced 10 min before the neuromuscular relaxant being tested. In addition to neuromuscular relaxants and antagonists of acetylcholine receptors, we also examined agents that act as agonists at nicotinic acetylcholine receptors, such as nicotine and acetylcholine, to determine if the magnitude and time course of increase in $[\text{Ca}^{2+}]_i$ differed from that seen with the neuromuscular relaxants. Several glutamate receptor agonists (L-glutamate and NMDA) and the NMDA antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) were also studied to determine if glutamate receptor activation might be involved in neuromuscular relaxant–induced increase in $[\text{Ca}^{2+}]_i$. The convulsant pentylentetrazole, which inhibits γ-aminobutyric acid A receptors, was also examined. Calibration of $[\text{Ca}^{2+}]_i$ was done after completion of test drug exposure.

**Statistical Analysis**

Statistical analysis of the effect of the different drugs on change in $[\text{Ca}^{2+}]_i$ was evaluated by analysis of vari-
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Fig. 1. Concentration–response curves for increase in [Ca\(^{2+}\)]\(_i\) by pancuronium and vecuronium in cortical brain slices. Each data point represents the maximal change in [Ca\(^{2+}\)]\(_i\) in a single brain slice.

The concentration at which sustained increases in [Ca\(^{2+}\)]\(_i\) were observed was about 50 \(\mu M\) for pancuronium and 350 \(\mu M\) for vecuronium. Mean 95%-maximal increases in [Ca\(^{2+}\)]\(_i\) caused by pancuronium and vecuronium were 118 \(\pm\) 85.3 and 69 \(\pm\) 42 nm, respectively. The concentrations producing 95%-maximal increases were then used for subsequent studies with antagonists and anticonvulsants.

The source of increased [Ca\(^{2+}\)]\(_i\) caused by the neuromuscular blocking drugs was predominately influx from the extracellular medium. Calcium-free artificial cerebrospinal fluid reduced the increases in [Ca\(^{2+}\)]\(_i\), caused by 125 \(\mu M\) pancuronium and 400 \(\mu M\) vecuronium by 86 \(\pm\) 12% and 90 \(\pm\) 17% \(n = 4\) for both, respectively.

Changes in [Ca\(^{2+}\)]\(_i\), caused by different neuromuscular blocking drugs are summarized in table 1. Atracurium (50–1,200 \(\mu M\)) and laudanosine (50–1,400 \(\mu M\)) did not cause a change in [Ca\(^{2+}\)]\(_i\). For atracurium, this is 23 times the seizure threshold concentration estimated for rats \textit{in vivo}.\(^7\) Pentylentetrazol, a commonly used drug for inducing seizure activity that acts by binding at the \(\gamma\)-aminobutyric acid\(_a\) receptor, caused an increase in [Ca\(^{2+}\)]\(_i\) of approximately 100 nm.

Table 1. Change in Cytosolic Calcium Concentration Caused by Neuromuscular Blocking Drugs, Acetylcholine Receptor Agonists, Glutamate Receptor Agonists, and Convulsants in Cortical Brain Slices

<table>
<thead>
<tr>
<th>Agent</th>
<th>(\Delta [Ca^{2+}] (nm))</th>
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<tbody>
<tr>
<td>Neurumuscular blocking drugs</td>
<td></td>
</tr>
<tr>
<td>Pancuronium 113 (\mu M)</td>
<td>118 (\pm) 55 (7)*</td>
</tr>
<tr>
<td>Vecuronium 400 (\mu M)</td>
<td>69 (\pm) 42 (8)*</td>
</tr>
<tr>
<td>Atracurium (\leq 1,400 \mu M)</td>
<td>0 (\pm) 0 (23)</td>
</tr>
<tr>
<td>(d)-Tubocurarine 112 (\mu M)</td>
<td>0 (\pm) 0 (14)</td>
</tr>
<tr>
<td>Convulsants</td>
<td></td>
</tr>
<tr>
<td>Pentylentetrazol 100 (\mu M)</td>
<td>75 (\pm) 20 (3)*</td>
</tr>
<tr>
<td>Laudanosine (\leq 1,400 \mu M)</td>
<td>0 (\pm) 0 (10)</td>
</tr>
<tr>
<td>Cholinergic agonists</td>
<td></td>
</tr>
<tr>
<td>Acetylcholine 400 (\mu M)</td>
<td>100 (\pm) 25 (3)*</td>
</tr>
<tr>
<td>Nicotine 300 (\mu M)</td>
<td>125 (\pm) 35 (3)*</td>
</tr>
<tr>
<td>Glutamatergic agonists</td>
<td></td>
</tr>
<tr>
<td>Glutamate 500 (\mu M)</td>
<td>130 (\pm) 43 (10)*</td>
</tr>
<tr>
<td>(N)-methyl-o-aspartate 100 (\mu M)</td>
<td>280 (\pm) 100 (9)*</td>
</tr>
<tr>
<td>Kainate 100 (\mu M)</td>
<td>50 (\pm) 30 (5)*</td>
</tr>
</tbody>
</table>

Mean baseline [Ca\(^{2+}\)] prior to agonist addition was 135 \(\pm\) 20 nm (pooled data from 96 slices). Values in parentheses are number of slices studied.

* Change in calcium significantly different from zero (ANOVA, \(P < 0.05\)).
Effects of Glutamate and Acetylcholine Receptor Agonists

Agonists for brain acetylcholine and glutamate receptors both caused increases in \([Ca^{2+}]_i\). The glutamate receptor agonists glutamate (500 \(\mu M\)) and NMDA (100 \(\mu M\)) produced large increases in \([Ca^{2+}]_i\) in cortical brain slices, as did 100 \(\mu M\) kainic acid (table 1). An example of the increase in \([Ca^{2+}]_i\) caused by NMDA is shown in figure 2. Acetylcholine (300–500 \(\mu M\)) and nicotine (500 \(\mu M\)) also increased cytosolic calcium (fig. 1). After addition of neuromuscular blocking drugs, acetylcholine (500 \(\mu M\)) failed to elicit further increases in \([Ca^{2+}]_i\).

Effects of Glutamate Receptor Antagonists

MK-801 and 6-cyano-7-nitroquinoxaline-2,3-dione, potent NMDA and AMPA (\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid)/kainate antagonists, respectively, did not prevent increase in \([Ca^{2+}]_i\) caused by 100 \(\mu M\) pancuronium or 400 \(\mu M\) vecuronium. MK-801 (100 \(\mu M\)) blocked NMDA-induced increases in \([Ca^{2+}]_i\) (data not shown).

Effects of Nicotinic and Muscarinic Acetylcholine Receptor Antagonists

Pretreatment of the brain slices with 100 \(\mu M\) \(\alpha\)-bungarotoxin or 100 \(\mu M\) neuronal bungarotoxin, which block specific subtypes of neuronal nicotinic acetylcholine receptors, had no effect on the increase in \([Ca^{2+}]_i\) caused by vecuronium or pancuronium. However, pretreatment with \(\alpha\)-tubocurarine (112 \(\mu M\)), a non–subtype-specific neuronal nicotinic acetylcholine receptor antagonist, blocked the increase in \([Ca^{2+}]_i\) with 392 \(\mu M\) vecuronium (increase 0 \(\pm\) 15% of control) and reduced the increase in \([Ca^{2+}]_i\) caused by 125 \(\mu M\) pancuronium by 92% \((P = 0.0004)\) (examples in fig. 3). At a vecuronium concentration of 780 \(\mu M\), however, 112 \(\mu M\) \(\alpha\)-tubocurarine had no inhibitory effect (table 2). \(\alpha\)-Tubocurarine 112 \(\mu M\) alone had no effect on \([Ca^{2+}]_i\).

Pretreatment of slices with 100 \(\mu M\) atropine reduced the increase in \([Ca^{2+}]_i\) caused by pancuronium by 55% \((P < 0.05)\). However, atropine had no effect on vecuronium-induced \([Ca^{2+}]_i\) increase (table 2).

Effects of Voltage-gated Calcium and Sodium Channel Antagonists

To examine the possibility that increased \([Ca^{2+}]_i\) might be caused by activation of L-type voltage-gated calcium channels secondary to cell depolarization, we attempted to prevent calcium influx with nimodipine. However, 100–400 \(\mu M\) nimodipine had no effect on
the increase in $[\text{Ca}^{2+}]$ induced by vecuronium and pancuronium ($P > 0.05$ in both cases).

For similar reasons, we examined the possibility that activation of voltage-gated sodium channels might be involved in the sequence of events leading up to increases in cytosolic calcium. We found, however, that tetrodotoxin ($5$–$10 \mu\text{M}$), a potent sodium channel blocker, did not prevent pancuronium or vecuronium from increasing cytosolic calcium.

**Effects of Phenytoin**

Pretreatment with the anti-convulsant phenytoin $30 \mu\text{g/ml (119} \mu\text{M})$ reduced the increase in $[\text{Ca}^{2+}]$, caused by vecuronium by $86\%$ ($P < 0.05$), and that caused by pancuronium by $80\%$ ($P < 0.03$), (table 2).

**Discussion**

The results suggest that pancuronium and vecuronium cause sustained increases in cytosolic calcium by initiating prolonged activation of nicotinic acetylcholine receptors. These drugs increase cytosolic calcium in brain slices with roughly the same relative potency (calcium increase threshold concentration for vecuronium eight times higher than that for pancuronium) as for induction of seizures in vitro (pancuronium 12 times as potent as vecuronium). These data are therefore consistent with the hypothesis that neuromuscular blocking drugs elicit seizures by causing uncontrolled increases in cytosolic calcium. Clearly, further work is needed before a link between seizures and changes in intracellular calcium caused by these drugs can be firmly established.

**Interaction of Brain Nicotinic Acetylcholine Receptors and Neuromuscular Relaxants**

We propose that neuromuscular blocking drugs may activate, rather than inhibit, particular subtypes of nic-

Table 2. Antagonism of Muscle Relaxant-Induced Elevations in Cytosolic Calcium ($\Delta[\text{Ca}^{2+}]$) by $d$-Tubocurarine and Phenyltoin: Cortical Brain Slices Exposed to $125 \mu\text{M}$ Pancuronium or $400$ or $785 \mu\text{M}$ Vecuronium (controls) and in Slices Pretreated for 10 Minutes with Either $112 \mu\text{M} d$-Tubocurarine, $125 \mu\text{M}$ Phenyltoin, or $164 \mu\text{M}$ Atropine before Pancuronium or Vecuronium

<table>
<thead>
<tr>
<th>Agent</th>
<th>$\Delta[\text{Ca}^{2+}]$</th>
<th>% Reduction from Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancuronium 125 $\mu\text{M}$ (control)</td>
<td>128 ± 49 (12)</td>
<td>93*</td>
</tr>
<tr>
<td>Pancuronium + $d$-tubocurarine</td>
<td>9 ± 13 (12)</td>
<td>93*</td>
</tr>
<tr>
<td>Pancuronium + atropine</td>
<td>58 ± 14 (10)</td>
<td>55*</td>
</tr>
<tr>
<td>Pancuronium + phenytoin</td>
<td>25 ± 20 (7)</td>
<td>80*</td>
</tr>
<tr>
<td>Vecuronium 390 $\mu\text{M}$ (control)</td>
<td>55 ± 25 (9)</td>
<td>100*</td>
</tr>
<tr>
<td>Vecuronium + $d$-tubocurarine</td>
<td>0 ± 15 (4)</td>
<td>100*</td>
</tr>
<tr>
<td>Vecuronium 785 $\mu\text{M}$ (control)</td>
<td>119 ± 39 (6)</td>
<td>7*</td>
</tr>
<tr>
<td>Vecuronium + $d$-tubocurarine</td>
<td>111 ± 77 (3)</td>
<td>7*</td>
</tr>
<tr>
<td>Vecuronium + atropine</td>
<td>74 ± 36 (6)</td>
<td>38*</td>
</tr>
<tr>
<td>Vecuronium + phenytoin</td>
<td>9 ± 9 (5)</td>
<td>92*</td>
</tr>
</tbody>
</table>

Values are mean ± SD with number of slices in parentheses.

* Significantly different from control (ANOVA, $P < 0.05$).
otinic acetylcholine receptors found in the CNS. This paradoxical effect may be the result of the substantial differences in subunit composition and pharmacology between central and neuromuscular nictinic acetylcholine receptors. One important difference is that the predominant brain subtype of nictinic acetylcholine receptors, that composed of α4 and β2 subunits, is seven times as permeable to calcium as the neuromuscular junction receptor.\(^\text{17}\) Whether neuromuscular blocking drugs increase calcium concentration directly by activating this receptor, or by other mechanisms, will require further study. The selectivity of neuromuscular blocking drugs for particular classes of neuronal nictinic acetylcholine receptors is shown by the failure of neuronal and α-bungarotoxin to prevent pancuronium and vecuronium from activating subtypes of acetylcholine receptors responsible for calcium influx. This is not surprising: nictinic acetylcholine receptors composed of α4 and β2 subunits are not inhibited by neuronal or α-bungarotoxin.\(^\text{20}\) Our results show that neuromuscular blocking drugs also interact with brain muscarinic acetylcholine receptors. For example, pancuronium appears to activate brain muscarinic receptors because atropine inhibits increase in \([\text{Ca}^{2+}]\).

Neuromuscular relaxants may act both as agonists and antagonists at brain acetylcholine receptors. For example, at low concentrations, d-tubocurarine antagonizes the activation of nictinic acetylcholine receptors by pancuronium and vecuronium. At higher concentrations, d-tubocurarine induces seizures when injected into the inferior colliculus in rats.\(^\text{12}\) It is possible that a given concentration of neuromuscular relaxant could act simultaneously as an agonist and antagonist at different subtypes of acetylcholine receptors, or in different brain regions expressing different subsets of receptors. It would be of interest to know if the concentration of d-tubocurarine that prevents calcium influx in brain slices also prevents the excitement and seizures caused by vecuronium or pancuronium when injected into the cerebral ventricles of intact rats.

The results suggest, but do not prove, that the action of pancuronium and vecuronium is on postsynaptic acetylcholine receptors. It is clear that acetylcholine receptors are present on both pre and postsynaptic membranes as well as on nonneuronal cells within the CNS.\(^\text{17}\)

Role of Calcium in the Pathogenesis of Seizures

Our results are consistent with the well established role of calcium in the pathophysiology of seizures.\(^\text{8,9}\)

It is unclear whether \([\text{Ca}^{2+}]\) initiates uncontrolled excitatory neurotransmission or is the consequence of excessive activation of postsynaptic excitatory receptors, or both. In either case, increases in cytosolic calcium probably occur via ion channels linked to receptors for excitatory neurotransmitters. It is not surprising that the cholinergic system modulates seizure activity, because its major role in the CNS is to modulate the excitability of neurons.\(^\text{21}\) Calcium signaling accompanying this activity might, under the right circumstances, contribute to excessive stimulation and ultimately to seizures.\(^\text{15}\)

The glutamatergic system might reasonably be thought to be involved in seizures caused by large increases in cytosolic calcium, because the NMDA subtype of glutamate receptors is highly permeable to calcium. Activation of these receptors may be important in certain types of seizures.\(^\text{14,15}\) Several anticonvulsants (e.g., carbamazepine) have been documented to inhibit the activity of the NMDA receptor.\(^\text{22}\) Glutamate receptors do not appear to be involved in the genesis of calcium fluxes observed with pancuronium or vecuronium because glutamate receptor antagonists do not inhibit calcium influx.

Effects of the Anticonvulsant Phenytoin on Calcium Uptake

The effect of phenytoin on calcium influx during exposure to pancuronium or vecuronium may be related to its suppression of neuronal excitability via a direct effect on voltage-gated sodium channels.\(^\text{23,24}\) Activation of voltage gated sodium channels might contribute indirectly to calcium influx in postsynaptic neurons via stimulation of release of excitatory neurotransmitters, or via activation of voltage-dependent calcium channels during sodium channel-mediated cell depolarization. However, because nimodipine (L-type voltage-gated calcium channel antagonist) did not influence calcium influx, it appears more likely that phenytoin’s prevention of calcium influx was due to suppression of excitability. Because tetrodotoxin (a potent and specific voltage-gated sodium channel blocker) had no effect on calcium influx caused by the neuromuscular relaxants, it is unlikely that phenytoin’s reduction in calcium influx is solely mediated by voltage-gated sodium channels.

Mechanisms of Seizures Not Related to Calcium

Clearly, not all neuromuscular relaxants having seizure activity \textit{in vivo} increase \([\text{Ca}^{2+}]\) in cortical slices
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from 20–30-day-old rats. Atracurium is more potent than either pancuronium or vecuronium in seizure induction when injected into the cerebral ventricles, yet it did not increase [Ca^{2+}]_i at concentrations approximately five times that required to induce seizures in vivo. In older rats, atracurium was found to increase [Ca^{2+}]_i, but we do not have an explanation for this maturational difference. Laudanosine, a metabolite of atracurium related to seizures in dogs, also failed to increase [Ca^{2+}]_i in brain slices. In the case of laudanosine, it is possible that seizures are confined to subcortical structures, or involve mechanisms distinct from those involving intracellular calcium.

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References