Neuroprotective Effects of Increased Extracellular Ca\(^{2+}\) During Aglycemia in White Matter

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Brown, Angus M., and Bruce R. Ransom. Neuroprotective effects of increased extracellular Ca\(^{2+}\) during aglycemia in white matter. J Neurophysiol 88: 1302–1307, 2002; 10.1152/jn.00113.2002. We investigated the effects of extracellular [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_o\)) on aglycemia-induced dysfunction and injury in adult rat optic nerves. Compound action potentials (CAPs) from adult rat optic nerve were recorded in vitro, and the area under the CAP was used to monitor nerve function before and after 1 h periods of aglycemia. In control artificial cerebrospinal fluid (ACSF) containing 2 mM Ca\(^{2+}\), CAP function fell after 29.9 ± 1.5 (SE) min and recovered to 48.8 ± 3.9% following aglycemia. Reducing bath [Ca\(^{2+}\)] during aglycemia progressively improved recovery. For example, in Ca\(^{2+}\)-free ACSF, the CAP recovered to 99.1 ± 3.8%. Paradoxically, increasing bath [Ca\(^{2+}\)] also improved recovery from aglycemia. In 5 or 10 mM bath [Ca\(^{2+}\)], CAP recovered to 78.8 ± 9.2 or 91.6 ± 5.2%, respectively. The latency to CAP failure during aglycemia increased as a function of bath [Ca\(^{2+}\)] from 0 to 10 mM. Increasing bath [Mg\(^{2+}\)] from 2 to 5 or 10 mM, with bath [Ca\(^{2+}\)] held at 2 mM, increased latency to CAP failure with aglycemia and improved recovery from this insult. [Ca\(^{2+}\)]\(_o\), recorded with calcium-sensitive microelectrodes in control ACSF, dropped reversibly during aglycemia from 1.54 ± 0.03 to 0.45 ± 0.04 mM. In the presence of higher ambient levels of bath [Ca\(^{2+}\)] (i.e., 5 or 10 mM), the aglycemia-induced decrease in [Ca\(^{2+}\)]\(_o\) declined, indicating that less Ca\(^{2+}\) left the extracellular space to enter an intracellular compartment. These results indicate that the role of [Ca\(^{2+}\)], and divalent cations in general, during aglycemia is complex. While extracellular Ca\(^{2+}\) was required for irreversible aglycemic injury to occur, higher levels of [Ca\(^{2+}\)] or [Mg\(^{2+}\)] increased the latency to CAP failure and improved the extent of recovery, apparently by limiting Ca\(^{2+}\) influx. These effects are theorized to be mediated by divalent cation screening.

INTRODUCTION

Hypoglycemia is a common occurrence in diabetic patients and can damage both white matter and gray matter brain tissue (Auer 1986; Siesjo 1988), and in vivo evidence suggests that aglycemia can occur in the brain as a consequence of severe systemic hypoglycemia (Silver and Erecinska 1994). Prior studies from our laboratory have indicated that aglycemic injury to the adult rat optic nerve (RON), a representative central white matter tract, requires the presence of extracellular Ca\(^{2+}\) (Brown et al. 2001a). The degree of injury, as determined by the area under the evoked compound action potential (CAP), increased with bath [Ca\(^{2+}\)] (Brown et al. 2001a) as was the case for anoxia-mediated white matter injury (Brown et al. 2001b; Stys et al. 1990). Evidence indicates that the extracellular Ca\(^{2+}\) acts as a source for toxic Ca\(^{2+}\) influx into intracellular compartments mediated by reverse Na\(^{+}\)-Ca\(^{2+}\) exchange (Brown et al. 2001b; Stys et al. 1992) and voltage-gated Ca\(^{2+}\) channels (Brown et al. 2001b). The intracellular Ca\(^{2+}\) overload leads to axonal death and loss of nerve function.

In this report, we studied the effects of bath [Ca\(^{2+}\)] on aglycemic injury in the adult RON. Based on previous studies (Brown et al. 2001b; Stys et al. 1990), we expected that increasing [Ca\(^{2+}\)] during aglycemia would worsen subsequent recovery. Our results indicated, however, that increasing bath [Ca\(^{2+}\)] above 2 mM resulted in increased CAP recovery after aglycemia, the first recorded report of increased extracellular [Ca\(^{2+}\)] being neuroprotective. Analysis of this finding has led us to conclude that Ca\(^{2+}\) effects on white matter injury are complex and include both direct mediation of intracellular damage as well as biophysical effects on transmembrane voltage gradients affecting ion channel gating.

METHODS

Preparation

All experiments were carried out in accordance with the guidelines for Animal Care of the University of Washington. Long Evans rats aged 45+ days old were deeply anesthetized with CO\(_2\) then decapitated. The optic nerves (5–10 mm long) were cut at the optic chiasm and behind the orbit and placed in an interface perfusion chamber (Medical Systems, Greenvale, NY) (see also Stys et al. 1990). RONs were maintained at 37°C and perfused with artificial cerebrospinal fluid (ACSF) bubbled with 95% O\(_2\)–5% CO\(_2\), and in vivo evidence suggests that the extracellular components mediated by reverse Na\(^{+}\)-Ca\(^{2+}\) exchange (Brown et al. 2001b; Stys et al. 1992) and voltage-gated Ca\(^{2+}\) channels (Brown et al. 2001b). The intracellular Ca\(^{2+}\) overload leads to axonal death and loss of nerve function.

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no osmotic compensation was made but the change in osmolarity was only about 3%. Ca\(^{2+}\)-free ACSF was made by omitting CaCl\(_2\) and adding 5 mM ethylene glycol-bis(\(\beta\)-aminoethyl)-\(N,N',N'\)-tetraacetic acid (EGTA). Introduction of test ACSF containing test divalent cation solution occurred 20 min prior to the 1-h period of aglycemia to allow the extracellular space (ECS) and bath solution to equilibrate (Brown et al. 2001a,b).

**Electrodes**

Ion-sensitive microelectrodes were made with double-barreled piggyback glass (WPI, PB150F-6) according to the method of Borrelli et al. (1985) with slight modifications. Electrodes were pulled on an upright puller producing tips of about 1 \(\mu\)m in diameter (Narashige, East Meadow, NY: PP83). These were subsequently beveled to a tip diameter of 2–5 \(\mu\)m (Sutter, Novato, CA: BV-10). The tip of the ion-sensitive barrel was filled with hexamethyldisilazane (Fluka, Ronkonkoma, NY: 52619) and baked at 160°C for 1 h. The indifferent barrel was filled with 150 mM NaCl, and the ion-sensitive barrel was back-filled with (in mM) 120 NaCl, 3 KCl, 20 HEPES, and mM CaCl\(_2\) adjusted to pH 7.2 with 1 M HCl. The ion-sensitive barrel was filled at the tip by back injection with a short (100–400 \(\mu\)m) column of Ca\(^{2+}\)-sensitive liquid ion sensor (Fluka, Cocktail A 21098). Electrodes were calibrated in a solution containing 120 mM NaCl, 3 mM KCl, 20 mM HEPES, and Ca\(^{2+}\) concentrations of 20 \(\mu\)M, 200 \(\mu\)M, and 2 mM. All electrodes were individually calibrated and only those showing stable, near Nernstian responses (i.e., 25–30 mV) to decade changes in [Ca\(^{2+}\)] were used for experimental measurements. Electrodes were recalibrated after each experiment, and data from electrodes with greater than a 5 mV deviation in response to decade changes in [Ca\(^{2+}\)] were discarded. The average between the initial and final calibration responses was used to evaluate the experimental measurement. The latency to drop of [Ca\(^{2+}\)] was measured by defining baseline [Ca\(^{2+}\)], as the average value during the first 40 min of the experiment. Threshold for recording a [Ca\(^{2+}\)] “decline” was set at a drop of 0.05 mM [Ca\(^{2+}\)], below this baseline. The ion-sensitive barrel was connected to an Axoclamp 2A amplifier (Axion Instruments) via a high-impedance headstage (HS-2 \(\times \) 0.0001 M), and the indifferent signal was subtracted from the ion-sensitive signal using a differential amplifier (Stanford Research Systems, Sunnyvale, CA: SRS 560). The signal was amplified 100 times, filtered at 1 Hz, and acquired at 1 Hz. CAPs were recorded from a suction electrode connected to a separate Axoclamp 2A amplifier, and the signal was amplified 500 times (SRS 560), filtered at 30 kHz, and acquired at 20 kHz.

**Data analysis**

Data were acquired on-line (Axon Instruments, Digidata 1200A) using proprietary software (Axon Instruments, Axotape). CAP area was calculated using pClamp (Axon Instruments), and the ion-sensitive signal was converted to [Ca\(^{2+}\)], using a template created in Excel (Microsoft, Redmond, WA) based on the Nernst equation. Data are presented as means ± SE. Significance was determined by ANOVA using Tukey’s posttest.

**Results**

**Bath [Ca\(^{2+}\)] during aglycemia determines latency to onset of CAP failure and CAP recovery**

The effects of 60 min of aglycemia on CAP area in adult RONs are shown in Fig. 1. Latency to the onset of CAP failure was defined as the time from aglycemia onset to the point when CAP area dropped below 95% of its normalized baseline value (Wender et al. 2000). Latency to the start of CAP failure in aglycemia depended on bath [Ca\(^{2+}\)] (Fig. 1A). In control ACSF containing 2 mM Ca\(^{2+}\) and 2 mM Mg\(^{2+}\), the latency to CAP failure was 30.5 ± 1.3 min (n = 8). As the baseline [Ca\(^{2+}\)] in the ACSF was decreased to 1, 0.5, or 0 mM Ca\(^{2+}\) (Mg\(^{2+}\) was constant at 2 mM), the latency to CAP failure decreased to 20.0 ± 2.1 min (n = 6, P < 0.001 compared with 30.5 min), 10.3 ± 0.7 min (n = 6, P < 0.001 compared with 20.0 min), or 6.0 ± 1.7 min (n = 6, P < 0.05 compared with 10.3 min), respectively. Thus in these experiments, decreasing Ca\(^{2+}\) correspondingly reduces the total divalent ion concentration [compare with Fig. 3C in Brown et al. (2001a), where decreases in Ca\(^{2+}\) were compensated by equimolar substitution with Mg\(^{2+}\) to ensure the total divalent ion concentration stayed constant]. Although the latency to CAP failure changed with [Ca\(^{2+}\)], it did not appear that the rate of CAP decline varied, once initiated. CAP recovery after aglycemia was also dependent on bath [Ca\(^{2+}\)] (Fig. 1). In 2 mM Ca\(^{2+}\) CAP recovered to 48.8 ±
3.9% (n = 6). The degree of CAP recovery increased as bath Ca\textsuperscript{2+} was decreased. In 1, 0.5, or 0 mM Ca\textsuperscript{2+}, CAP recovery was 62.2 ± 6.0, 69.5 ± 9.7, or 99.1 ± 3.8%, respectively. Specimen records of CAPs before, during, and after 60 min of aglycemia at different test concentrations of Ca\textsuperscript{2+} are shown in Fig. 1B. Control CAPs in each condition exhibit the standard three peaks (Foster et al. 1982). These data indicated that aglycemia-mediated irreversible injury to the adult RON required extracellular Ca\textsuperscript{2+} and that the degree of injury was related to the bath [Ca\textsuperscript{2+}], strongly suggesting that during aglycemia Ca\textsuperscript{2+} moves from the ECS to intracellular compartments.

**Increased bath Ca\textsuperscript{2+} above 2 mM improves CAP recovery**

We explored the effects of bath [Ca\textsuperscript{2+}] above 2 mM during aglycemia on latency to CAP failure and CAP recovery (Fig. 2). Solutions containing the test concentration of Ca\textsuperscript{2+} were introduced 20 min prior to the 1-h period of aglycemia, after which nerves recovered for 1 h in control ACSF containing 2 mM Ca\textsuperscript{2+}. In these experiments, [Mg\textsuperscript{2+}] was held constant at 2 mM. Because aglycemia-induced white matter injury depends on Ca\textsuperscript{2+} influx, we expected greater injury with higher ambient [Ca\textsuperscript{2+}]. However, as the [Ca\textsuperscript{2+}] of the bath was increased from 2 to 5 or 10 mM, the degree of CAP recovery from aglycemia increased from 48.8 ± 3.9% (n = 6) to 74.3 ± 7.5% (n = 7) or 91.1 ± 5.2% (n = 6), respectively (Fig. 2A).

**Increasing bath Mg\textsuperscript{2+} increases latency to CAP failure during aglycemia**

The effect of [Ca\textsuperscript{2+}] on latency to onset of CAP failure during aglycemia was unexpected. We hypothesized that Ca\textsuperscript{2+} might be exerting this action due to “charge screening effects” (Hille 2001). As charge-screening effects are not solely exhibited by Ca\textsuperscript{2+} but are shared by all divalent cations (Hille 2001), we predicted that increasing the concentration of a divalent cation other than Ca\textsuperscript{2+} should also increase latency to CAP failure. We tested this idea by altering ACSF [Mg\textsuperscript{2+}]. Charge screening effects are well documented for Mg\textsuperscript{2+} (Hille et al. 1975), and Ca\textsuperscript{2+} channels are not permeable to Mg\textsuperscript{2+} nor are they blocked by this ion. In this series of experiments, bath Ca\textsuperscript{2+} was kept constant at 0.2 mM to minimize the contribution of Ca\textsuperscript{2+} to charge screening. As Mg\textsuperscript{2+} was increased from 1 to 2 or 5 mM the latency to CAP failure increased significantly from 1.3 ± 1.0 min (n = 2) to 12.9 ± 1.1 min (n = 4) or 25.7 ± 1.3 min (n = 6), respectively (Fig. 3). These results are consistent with a charge screening effect (see DISCUSSION).

**Effects of increasing bath Mg\textsuperscript{2+} on CAP recovery**

Increasing Mg\textsuperscript{2+} from 2 to 5 or 10 mM, keeping Ca\textsuperscript{2+} constant at 2 mM, resulted in increased latency to CAP failure and increased CAP recovery (Fig. 4). In 2 mM Mg\textsuperscript{2+} ACSF, the latency to CAP failure was 29.9 ± 1.5 min and CAP recovery was 48.8 ± 3.9% (n = 6) of control. Increasing Mg\textsuperscript{2+} to 5 or 10 mM increased latency to CAP failure to 43.8 ± 2.8 or 47.9 ± 7.8 min, respectively, and increased CAP recovery to 97.7 ± 7.1 or 102.0 ± 7.1%, respectively (Fig. 4A). Thus increasing [Mg\textsuperscript{2+}], like increasing [Ca\textsuperscript{2+}], enhances CAP recovery from 60 min of aglycemia. In fact, CAP recovery in both 5 and 10 mM Mg\textsuperscript{2+} was greater than in 5 mM Ca\textsuperscript{2+} ACSF (94.9 ± 8.4 vs. 78.9 ± 9.2%, P < 0.05) or 10 mM Ca\textsuperscript{2+} (102.0 ± 7.0 vs. 91.1 ± 5.2%, not significant; see Fig. 5B).

\[ [Ca^{2+}]_o \text{ changes during aglycemia in nerves bathed in various bath [Ca}^{2+}] \]

The pattern of changes in [Ca\textsuperscript{2+}]\textsubscript{o} before, during, and after a 1-h period of aglycemia was studied while varying bath [Ca\textsuperscript{2+}]. The level to which [Ca\textsuperscript{2+}]\textsubscript{o} fell during aglycemia was determined by bath [Ca\textsuperscript{2+}] (Fig. 6). In all these experiments, Mg\textsuperscript{2+} was constant at 2 mM. In 2 mM Ca\textsuperscript{2+}, the baseline [Ca\textsuperscript{2+}]\textsubscript{o} of 1.55 ± 0.02 mM fell to 0.45 ± 0.04 mM at the end of 1 h of aglycemia and recovered to 1.44 ± 0.05 mM (n = 6). In 5 or 10 mM Ca\textsuperscript{2+}, baseline [Ca\textsuperscript{2+}]\textsubscript{o} was 3.36 ± 0.15 mM (n = 4) or 7.90 ± 0.11 mM (n = 4), respectively, and fell to...
2.93 ± 0.08 mM or 7.74 ± 0.21 mM, respectively, during 1 h of aglycemia (Fig. 6A). The lower the bathing [Ca$^{2+}$], the shorter the latency to [Ca$^{2+}$]o decrease and the greater the degree of [Ca$^{2+}$]o decrease.

The latency to the onset of [Ca$^{2+}$]o decline following application of aglycemia progressively increased as bath [Ca$^{2+}$]o increased. In 2 mM Ca$^{2+}$, the latency to [Ca$^{2+}$]o decrease below baseline was 40.7 ± 1.5 min (n = 6), which rose to 44.0 ± 0.6 min (n = 4, not significantly different from 40.7 min) in 5 mM and 49.7 ± 2.2 min (n = 4, not significantly different from 44.0 min) in 10 mM [Ca$^{2+}$].

The relationship between the total amount of Ca$^{2+}$ entering the intracellular compartments and injury was examined. We

![Graph showing latency to CAP failure](44x561)

FIG. 3. Increasing [Mg$^{2+}$] from 1 to 2 or 5 mM progressively increased the latency to CAP failure. Bath [Ca$^{2+}$] was held constant at 0.2 mM. Data are averaged from 6 nerves in each condition.

![Graph showing effect of bath Mg$^{2+}$ on CAP responses to aglycemic injury](44x561)

FIG. 4. Effect of bath [Mg$^{2+}$] on CAP responses to aglycemic injury. A: the long bar indicates the period of superfusion with ACSF containing variable [Mg$^{2+}$]. The short bar indicates 1 h of aglycemia. Higher [Mg$^{2+}$] caused increased latency to CAP failure during aglycemia and improved CAP recovery. B: individual CAPs recorded at the times indicated in A as letters above traces. Scale bars are 1 mV and 1 ms for all traces.

![Graph showing effects of aglycemia on change in [Ca$^{2+}$]o as a function of baseline [Ca$^{2+}$]o in various bath [Ca$^{2+}$]. [Ca$^{2+}$]o was recorded with Ca$^{2+}$-sensitive microelectrodes in bath [Ca$^{2+}$] of 2, 5, or 10 mM. A: the integral of [Ca$^{2+}$]o decrease below baseline was used to estimate the total amount of Ca$^{2+}$ influx during aglycemia. B: plot of CAP recovery vs. integral of [Ca$^{2+}$]o decrease (2 mM, □; 5 mM, ○; or 10 mM, ◇). Aglycemia-induced injury increased in a linear fashion as a function of the integral of [Ca$^{2+}$]o decrease.

![Graph showing effects of aglycemia on change in [Ca$^{2+}$]o as a function of baseline [Ca$^{2+}$]o in various bath [Ca$^{2+}$]. [Ca$^{2+}$]o was recorded with Ca$^{2+}$-sensitive microelectrodes in bath [Ca$^{2+}$] of 2, 5, or 10 mM. A: the integral of [Ca$^{2+}$]o decrease below baseline was used to estimate the total amount of Ca$^{2+}$ influx during aglycemia. B: plot of CAP recovery vs. integral of [Ca$^{2+}$]o decrease (2 mM, □; 5 mM, ○; or 10 mM, ◇). Aglycemia-induced injury increased in a linear fashion as a function of the integral of [Ca$^{2+}$]o decrease.
have used the integral of [Ca$^{2+}$]$_b$ below baseline during aglycemia as a crude qualitative measure of net amount of Ca$^{2+}$ influx (Brown et al. 2001a). Fig. 6A illustrates (□) how the integral of [Ca$^{2+}$]$_b$ decrease during aglycemia was measured. Data shown are the averages of nerves exposed to aglycemia at 2 mM ($n = 6$), 5 mM ($n = 4$), or 10 mM ($n = 4$) bath [Ca$^{2+}$]. The relationship between integral of Ca$^{2+}$ decrease during aglycemia and CAP recovery is illustrated in Fig. 6B, demonstrating that there is a linear relationship between injury and the amount of Ca$^{2+}$ leaving the ECS, presumably entering intracellular compartments.

**DISCUSSION**

The brain requires both glucose as an energy source and oxygen to oxidize the glucose to maintain the transmembrane ion gradients on which excitability depends (Clarke and Sokoloff 1999). Only recently have the effects of energy deprivation on CNS axons [i.e., white matter (WM)], independent of the cell bodies been assessed. The importance of a continual supply of glucose and oxygen to WM has been demonstrated by the irreversible injury that occurs to the adult RON, when either glucose (Brown et al. 2001a) or oxygen (Brown et al. 2001b; Stys et al. 1990) is withdrawn.

Prior studies have indicated that central axonal injury from energy deprivation is dependent on the presence of extracellular Ca$^{2+}$ (Brown et al. 2001a). Our results show that the effects of [Ca$^{2+}$] on WM injury during aglycemia are complex. While lowering bath [Ca$^{2+}$] from 2 mM is progressively protective, so too is raising [Ca$^{2+}$] from 2 mM. These contrary findings are best explained by two interesting actions of extracellular Ca$^{2+}$. On the one hand, Ca$^{2+}$ acts as an intracellular agent of destruction, and in this context, lowering its concentration in the extracellular space is highly protective (Brown et al. 2001a). On the other hand, Ca$^{2+}$ and other divalent cations have powerful biophysical effects on membrane potential and ion channel gating (Hille 2001) such that higher extracellular concentrations have the net effect of decreasing excitability (Frankenhauser 1957) and blocking the toxic Ca$^{2+}$ influx previously mentioned. These antagonistic actions are strikingly diverse either side of 2 mM; the toxic role of this ion predominates from near 0 to 2 mM, while the protective role dominates the picture at [Ca$^{2+}$]$_b$’s above 2 mM. These data indicate the importance of maintaining constant extracellular divalent cation concentration to avoid such effects.

Thus if Ca$^{2+}$ enters intracellular compartments, its toxic actions result in cell death. However, the extracellular actions of Ca$^{2+}$ on ion channel gating render cells less excitable with increasing extracellular [Ca$^{2+}$]. In this way, extracellular Ca$^{2+}$ can be viewed as a regulator of voltage-gated Ca$^{2+}$ channel gating. In addition to the effects on Ca$^{2+}$ channels, extracellular Ca$^{2+}$ has two other effects relevant to this study. First, increasing Ca$^{2+}$ results in a depolarization of resting membrane potential. Indeed, pioneering studies on squid axons showed that decreasing Ca$^{2+}$ in the perfusate resulted in spontaneous firing of action potentials, whereas the squid axons perfused in Ca$^{2+}$ containing perfusate was quiescent (Frankenhauser 1957; Frankenhauser and Hodgkin 1957). Second, in a study examining the relationship between membrane potential and energy deprivation in adult RON, inhibition of glycolysis or oxidative phosphorylation led to delayed membrane depolarization. Omitting Ca$^{2+}$ from the perfusate resulted in both and accelerated membrane depolarization and an increased rate of depolarization increased (Leppanen and Stys 1997).

These effects of divalent cations have been incorporated into our model illustrated in Fig. 7. The resting membrane potential of the adult RON has been calculated at −80 mV (Stys et al. 1997) in ACSF containing 2 mM Ca$^{2+}$ (black trace), and under similar conditions the threshold for activation of Na$^{+}$ channels is −60 mV (Campbell and Hille 1976). On removal of glucose from the ACSF, the membrane potential is maintained at rest, probably due to the nerves’ ability to utilize energy stores (Wender et al. 2000). When these stores are depleted, the membrane potential depolarizes. Once the depolarization reaches threshold the Na$^{+}$ channels inactivate resulting in CAP failure (at the time indicated by the arrow). In our recording conditions and using the grease gap method (Leppanen and Stys 1997), this latency period was about 30 min. In 0 Ca$^{2+}$ ACSF (red trace) the membrane potential is depolarized slightly (Leppanen and Stys 1997), and threshold for Na$^{+}$ channel activation is hyperpolarized compared with 2 mM Ca$^{2+}$ (Campbell and Hille 1976). Removal of glucose results in accelerated depolarization (Leppanen and Stys 1997). This coupled to the relatively depolarized resting membrane potential and hyperpolarized threshold results in more rapid CAP failure. Conversely, in 5 mM Ca$^{2+}$ ACSF (blue trace), the resting membrane potential will be slightly hyperpolarized compared with control (Leppanen and Stys 1997), and threshold for Na$^{+}$ channel activation will be depolarized compared with 2 mM Ca$^{2+}$ (Campbell and Hille 1976). Thus CAP failure will be delayed compared with control conditions.

The consequences of these effects of divalent cations to injury are as follows. In Fig. 5, we showed that the drop in [Ca$^{2+}$]$_b$ is intimately related to CAP failure. Thus delaying the latency to CAP failure by increasing extracellular divalent cation concentration resulted in an increased latency after the onset of aglycemia before Ca$^{2+}$ entered intracellular compartments. We predict that the more Ca$^{2+}$ that leaves the ECS, the more injury should occur. This is seen if CAP recovery is compared between experiments where 5 mM Mg$^{2+}$:2 mM Ca$^{2+}$ ACSF or 5 mM Ca$^{2+}$:2 mM Mg$^{2+}$ were employed.

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**FIG. 7.** Schematic effect of bath [Ca$^{2+}$] on the electric field within the axon membrane during aglycemia. The effects of aglycemia on membrane potential in nerves bathed in [Ca$^{2+}$] of 0, 2, or 5 mM are shown. According to this model, higher [Ca$^{2+}$] neutralized the negative charge on fixed surfaces particles causing lower field strength within the membrane. Conversely low [Ca$^{2+}$] increased the field within the membrane. Transmembrane voltage would be the same in both conditions. Because channel activation depends on the field within the membrane, high [Ca$^{2+}$] "functionally" hyperpolarizes the membrane (---). At a given rate of depolarization, it takes longer to reach Na$^{+}$ channels threshold in high [Ca$^{2+}$] compared with low [Ca$^{2+}$].
There is an increased latency to CAP failure in both conditions that is not significantly different. However, the CAP recovery is significantly greater in 5 mM Ca$$^{2+}$$:2 mM Mg$$^{2+}$$. This is because there is a greater gradient for Ca$$^{2+}$$ influx than in 5 mM Mg$$^{2+}$$:2 mM Ca$$^{2+}$$ and hence more injury occurs. This difference is not as pronounced limits the duration of Ca$$^{2+}$$ influx.

Experiments investigating the effects of divalent cations on anoxic injury also support this theory. Increasing Mg$$^{2+}$$ in ACSF to 10 mM during a 60-min anoxic insult increased CAP recovery from 29.7 to 54.9% (Stys et al. 1990). Although the effect of Mg$$^{2+}$$ on latency to CAP failure was not described, we would expect that it would delay the failure of CAP and thus lead to a decreased amount of toxic Ca$$^{2+}$$ influx leading to improved CAP recovery. Additional supportive evidence from the author states CAP area falls to near zero within a minute of anoxia onset in 0 mM Ca$$^{2+}$$/5 mM EGTA versus 5–6 min in normal Ca$$^{2+}$$ (P. Stys, personal communication).

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