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Emergence of a spermine-sensitive, non-inactivating conductance in mature hippocampal CA1 pyramidal neurons upon reduction of extracellular Ca^{2+} : dependence on intracellular Mg^{2+} and ATP.

Christos Chinopoulos^{1,2}, John A Connor¹, and C William Shuttleworth¹

¹ Department of Neurosciences, University of New Mexico, Albuquerque, NM, USA

² Semmelweis University, Department of Medical Biochemistry, Neurochemical Group, Hungarian Academy of Sciences, Szentagothai Knowledge Center, Budapest, Hungary

Abstract

Large and protracted elevations of intracellular $[\text{Ca}^{2+}]$ and $[\text{Na}^{+}]$ play a crucial role in neuronal injury in ischemic conditions. In addition to excessive glutamate receptor activation, other ion channels may contribute to disruption of intracellular ionic homeostasis. During episodes of ischemia, extracellular $[\text{Ca}^{2+}]$ falls significantly. Here we report the emergence of an inward current in hippocampal CA1 pyramidal neurons in acute brain slices from adult mice upon reduction/removal of $[\text{Ca}^{2+}]_e$. The magnitude of the current was 100–300 pA at -65 mV holding potential, depending on intracellular constituents. The current was accompanied by intense neuronal discharge, observed in both whole-cell and cell-attached patch configurations. Sustained currents and increased neuronal firing rates were both reversed by restoration of physiological levels of $[\text{Ca}^{2+}]_e$, or by application of spermine (1 mM). The amplitudes of the sustained currents were strongly reduced by raising intracellular $[\text{Mg}^{2+}]$, but not by extracellular $[\text{Mg}^{2+}]$ increases. Elevated intracellular ATP also reduced the current. This conductance is similar in several respects to the “calcium-sensing, non-selective cation current” (csNSC), previously described in cultured mouse hippocampal neurons of embryonic origin. The dependence on intracellular $[\text{ATP}]$ and $[\text{Mg}^{2+}]$ shown here, suggests a possible role for this current in disruption of ionic homeostasis during metabolic stress that accompanies excessive neuronal stimulation.

Keywords

transient receptor potential; store-operated Ca^{2+} entry; spreading depression; stroke; TRPM7; Ca^{2+} paradox

An excessive increase in intracellular Ca^{2+} concentration has been repeatedly implicated as a key determinant of neuronal cell death pathways (Arundine and Tymianski 2003). Sustained cytosolic Ca^{2+} increases can be triggered by a variety of insults, and often involve persistent activation of the NMDA subtype of glutamate receptors. This leads to loading of mitochondria

Corresponding author: Christos Chinopoulos, MD, PhD, Semmelweis University, Department of Medical Biochemistry, Puskin street 9, Budapest, 1088, Hungary. Tel: +361 26 62 755 ext 4107; Fax: +361 26 70 031; email: cch@puskin.sote.hu.

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with large amounts of Ca^{2+} compromising oxidative phosphorylation, ultimately resulting in intracellular ATP depletion (Greene and Greenamyre 1996; Nicholls and Budd 2000).

Interstitial $[\text{Ca}^{2+}]_e$ levels drop during neurotransmission at central excitatory synapses, as a consequence of influx into both pre- and post-synaptic neurons (Nicholson *et al.* 1978). This is exacerbated in pathological states involving excessive glutamate receptor stimulation, such as during ischemia or traumatic brain injury, in which extracellular Ca^{2+} concentration can be reduced to approximately 0.1 mM (Harris *et al.* 1981; Silver and Erecinska 1990; Nilsson *et al.* 1996). In ischemia, $[\text{Ca}^{2+}]_e$ reductions produced by neuronal Ca^{2+} influx may also be compounded by the accumulation of extracellular lactate, which is an effective chelator of divalent cations (Martell and Smith 1977; Immke and McCleskey 2001).

Fluctuations in $[\text{Ca}^{2+}]_e$ are sensed by a range of plasma membrane Ca^{2+} -sensing receptors and ion channels (Hofer 2005). Furthermore, the voltage dependence of many types of monovalent ion channels is shifted in the absence of divalent cations. For example, the voltage-dependence of a number of voltage-gated Ca^{2+} and Na^+ channels may be shifted to more negative potentials by lowering $[\text{Ca}^{2+}]_e$, as a consequence of reduced shielding of negatively charged groups located at the membrane surface (Armstrong 1999; Armstrong and Cota 1999; Frankenhaeuser and Hodgkin 1957; Fukushima and Hagiwara 1985). Chloride channels are also sensitive to alterations in $[\text{Cl}^-]_e$ (Liu and Stimers 1998). In extreme cases, selectivity of voltage-dependent channels can also be lost when $[\text{Ca}^{2+}]_e$ is experimentally reduced to ultra-low ($<1 \mu\text{M}$) concentrations (Xiong and MacDonald 1999) and lead to significant additional cation influx.

Recently, a number of non-selective currents triggered by reductions in $[\text{Ca}^{2+}]_e$ have been described (Formenti *et al.* 2001; Udem *et al.* 2003; Xiong *et al.* 1997; Smith *et al.* 2004; Mubagwa *et al.* 1997; Smith *et al.* 2004). These channels can produce significant inward current and their lack of inactivation may lead to Na^+ overloading following extracellular Ca^{2+} decreases. While the channel identities carrying these currents are at present unknown, conductances attributed to several members of the “transient receptor potential” (TRP) family, such as TRPC1-3-6-7, TRPM7 and TRPV5-6 are known to be enhanced by $[\text{Ca}^{2+}]_e$ reductions (Lintschinger *et al.* 2000; Shi *et al.* 2004; Aarts *et al.* 2003; Owsianik *et al.* 2005). Relevant to this, it has been shown that TRPM7 channels underlie currents measured in cultured neurons that contribute to cell death in an *in vitro* model of ischemic neuronal damage (Aarts *et al.* 2003).

Since previous work in cultured hippocampal neurons demonstrated a non-selective cation current activated by $[\text{Ca}^{2+}]_e$ decreases (Xiong *et al.*, 1997), the present study was undertaken to determine whether *in situ* mature neurons express an analogous current since it is widely recognized that there can be significant differences in channel expression and functioning between cell culture models and mature neurons. We show that the CA1 pyramidal neurons in acute hippocampal slices from adult mice exhibit a significant inward current upon reduction of $[\text{Ca}^{2+}]_e$. Furthermore, this current is regulated by intracellular $[\text{Mg}^{2+}]$ and [ATP], making it a potential contributor to increased Na^+ influx during neuronal metabolic compromise.

EXPERIMENTAL PROCEDURES

Slice preparation

Male mice (FVB/N) were obtained from Harlan Laboratories (Indianapolis, IN) at 4–6 weeks of age and were housed in standard conditions (12 hr light/dark cycle) for at least 2 weeks before death. The currents triggered by extracellular $\text{Ca}^{2+}/\text{Mg}^{2+}$ removal (see Results) were not as readily observed in slices prepared from younger animals (<6 weeks), and therefore the study was restricted to the 6–8 week age group. All animal procedures were carried out in accordance with the National Institutes of Health and the University of New Mexico, Animal

Care and Use Committee Guidelines. Mice were anesthetized with a mixture of ketamine and xylazine (85 and 15 mg/ml, respectively; 0.2 ml, subcutaneously) and subsequently decapitated. The use of ketamine during brain slice preparation improves the viability and maintains *in situ* morphology of CA1 neurons (Feig and Lipton 1990; Aitken *et al.* 1995; Lipton *et al.* 1995), and is washed from slices before recording. Brains were removed and placed in ice-cold cutting solution (see below for composition), equilibrated with 95% O₂/5% CO₂. Coronal sections (350 µm) were cut with a Vibratome (Technical Products International, St. Louis, MO), and slices were transferred into artificial cerebrospinal fluid (ACSF, see below for composition), equilibrated with 95% O₂/5% CO₂ and kept at 34°C. Cutting and recording solutions were both 300–310 mOsm. After being warmed to 34°C for 1 hr, ACSF was changed again, and the slices were maintained at room temperature until used for recording. Individual slices were transferred to the recording chamber and perfused with warmed (32±0.5 °C), oxygenated ACSF at 2 ml/min.

Electrophysiological recordings

Somatic whole-cell and cell-attached recordings were made from visualized CA1 pyramidal neurons, using a Multiclamp 700A amplifier (Axon Instruments, Union city, CA). Signals were digitized at 10–25 kHz (Digidata 1322A), and acquired using Clampex version 9 software (Axon Instruments). Patch pipettes intended for whole-cell recordings had resistances of 2–4 MΩ, and 2–2.5 MΩ for cell-attached recordings. Seal resistances were typically 2–7 GΩ before whole-cell access. Immediately after obtaining whole-cell access, cells were discarded if the access resistance was more than 20 MΩ, or leak currents exceeded ±100 pA at –65 mV holding potential. All cells were dialyzed for 3–4 min before initiation of experimental manipulations. If the access resistance varied more than 20% from the beginning to the end of the experiment, the recordings were excluded from analyses. For cell-attached recordings, pipettes were filled with ACSF (including 2 mM CaCl₂ and 1 mM MgSO₄) and the pipette potential was held at 0 mV. “Leak” currents were not subtracted for any of the recordings.

Materials

All chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO). Slice cutting solution comprised (in mM): 220 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 6 MgSO₄, 26 NaHCO₃, 0.2 CaCl₂, 10 glucose, and 0.43 ketamine and was equilibrated with 95% O₂/5% CO₂. The use of ketamine is based on observations described in (Aitken *et al.* 1995), and ketamine is thoroughly washed from slices (>2 hours, multiple solution exchanges) before beginning experiments. Artificial cerebrospinal fluid (ACSF) comprised (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 2 CaCl₂, and 10 glucose was equilibrated with 95% O₂/5% CO₂. Cutting and recording solutions were both 300–310 mOsm. Nominally Ca²⁺-free solutions were prepared by omission of CaCl₂ (no added chelators) and the free extracellular Ca²⁺ concentration of nominally-Ca²⁺-free ACSF was estimated at 3.8 µM, using Fura 6F ratio fluorescence measurements, and assuming a K_d value of 2.47 µM (Chinopoulos *et al.* 2003). In some experiments [Ca²⁺]_e was buffered to very low concentrations (~1 nM) by the omission of extracellular CaCl₂ and the inclusion of 0.5 mM EGTA. Table 1 lists the composition of intracellular recording solutions. In experiments to examine the effects of intracellular [Mg²⁺] and [ATP], estimated free [Mg²⁺] was calculated by using Winmaxc software (Bers *et al.*, 1994). The ATP source contained less than 0.005% Mg²⁺ as a contaminant (Sigma, A7699, disodium salt), which was calculated to contribute less than 0.2 µM Mg²⁺ to pipette solutions when used at the highest concentration tested (4 mM).

Statistics

Data are presented as mean $\pm$ SEM; significant differences between group data were evaluated by using Mann-Whitney rank sum test, with $p < 0.05$ considered significant, unless otherwise indicated. For results showing representative recordings, each experiment was recorded from 4 to 17 neurons. Meta-analysis of electrophysiological recordings was performed using Clampfit software version 9.2 (Axon Instruments) and Mini Analysis version 5 (Synaptosoft Inc, Decatur, GA).

RESULTS

Non-inactivating inward current and neuronal discharge produced by reduction of extracellular divalent cation concentration

Figure 1A shows an example of the effects of reducing extracellular divalent cation concentration on currents measured from a CA1 pyramidal neuron. The neuron soma was voltage clamped at -65 mV, and the superfusion buffer was switched from normal ACSF (2 mM Ca^{2+} , 1 mM Mg^{2+}) to a modified ACSF containing no added Ca^{2+} or Mg^{2+} (nominally $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free). A K^{+} -based intracellular solution was used (termed “Int 2”). Approximately 50 sec after onset of exposure, a slowly-developing inward current was observed. This sustained current was also associated with intense neuronal discharge, as evidenced by superimposed large transient inward currents, (see also figure 8). Both the sustained inward current and neuronal discharge were abolished by restoration of normal extracellular $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentrations. Similar sustained currents were produced in 10 out of 12 cells examined under these conditions, and the mean peak amplitude of responders was -145 ± 18 pA. In the remaining two neurons, no inward current was observed throughout at least 6 min of divalent cation removal. In responding neurons, the delay before demonstrable onset of the inward current was variable (range: 50 – 250 sec). The time lag is likely due to the relatively slow exchange of solutions in the slice superfusion system, and also the range of depths from which CA1 neurons were recorded (<60 μm from the top surface of the slice).

A sustained inward current and neuronal discharge were also observed when K^{+} channels were blocked by substitution of intracellular K^{+} with Cs^{+} and TEA (solution “Int 6”). An example is shown in Figure 1B, and similar responses were observed in 7/9 neurons tested (12 ± 3 Hz firing rate). K^{+} channel block also increased the duration of current spikes (grey bars Figure 1B, inset), that followed extracellular divalent removal (compare with Figure 1A inset). This increased duration implies that a portion of the transient current was generated intrinsically in unclamped regions of the recorded neuron, and was not entirely due to presynaptic input.

Although $[\text{Ca}^{2+}]_e$ reduction triggered sustained inward currents in a high proportion of neurons (see above), a concomitant increase in current spike rate was not observed in every case. Some neurons (12 out of a total of 109 cells patched in the whole-cell mode under all intra- and extracellular conditions described throughout this study) showed no increase in spike rate, while 7 neurons showed an increase in rate of small excitatory currents (Figure 1C, inset) without the appearance of the large, spike-like events.

As a control for dialysis effects in the whole cell mode, figure 2 shows the effects of extracellular Ca^{2+} and Mg^{2+} reduction on recordings made in the cell-attached recording mode. While small sustained inward currents are not observable in the cell-attached mode, increases in the rate of large transient events (current spikes) should be detectable. As before, neurons were bathed in normal ACSF and then exposed to $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free solution. In most cases ($\sim 75\%$), tonic firing was observed following $\text{Ca}^{2+}/\text{Mg}^{2+}$ reduction (28 ± 1 Hz). Figure 2A shows an example where a progressive increase in tonic discharge rate was followed by a progressive decrease in amplitude of the membrane current observed through the window of membrane

capacitance; the decrease representing either a decrease of the spike upstroke slope, or increasing distance of spike generation from the soma. In a minority of cases (~15%) a bursting firing pattern was observed (figures 2C&D) following $\text{Ca}^{2+}/\text{Mg}^{2+}$ reduction, and the number of firing per cluster progressively increased over time in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free media. Similar responses (both tonic and burst-like) were recorded in the cell-attached mode following removal of extracellular Ca^{2+} only (not shown). In the remaining population of patched neurons, no spike activity was observed.

The non-inactivating current cannot be accounted for by seal deterioration upon reduction of extracellular divalent cations

Formenti and De Simoni (Formenti and De 2000) previously demonstrated that reduction of extracellular Ca^{2+} leads to a decrease in seal resistance and therefore “currents” may appear due to leak conductance from the pipette to the bath solution. The extent of this decrease depends on the value of the initial resistance. To address this possibility, and to quantify how much “leak-seal” current potentially contaminates our recordings, we examined the effects of extracellular divalent cation removal on seal resistance, when recording in the cell-attached mode (figure 3). Seal resistances were calculated from current-voltage (I–V) curves ($r^2 > 0.95$ in all cases). When the initial seal resistance was 1.96 ± 0.11 GOhm, ($n=5$, initial pipette resistance ~ 2 MOhm), removal of extracellular Ca^{2+} and Mg^{2+} produced a small decrease in seal resistance (to 1.72 ± 0.25 GOhm, $p=0.41$, not statistically significant, Student t-test, $p=0.411$, figure 3A). This translates to a difference in conductance from 510 to 581 pS. Assuming a resting membrane potential of -65 mV, and that the reversal potential of the “current” appearing due to deterioration of seal resistance is 0 mV, we estimate that the “leak-seal” conductance is $(581 \text{ pS} - 510 \text{ pS}) \cdot (-65 \text{ mV} - 0 \text{ mV}) = 4.6 \text{ pA}$. This is too small to alter the values obtained from whole-cell recordings, upon removal of extracellular Ca^{2+} , which is on the order of hundreds of pA (see figure 7). Note also that the extrapolated value for the holding current is approximately -35 pA at -65 mV, when the seal is ~ 2 GOhm, a value which is close to the recorded one in control ACSF (see figure 7). However, as pointed out previously (Chu *et al.* 2003), it can be argued that during the formation of the whole-cell patch configuration, the seal resistance is reduced due to a physical disruption of the patch membrane, rendering it more sensitive to the changes in $[\text{Ca}^{2+}]_e$. We tested this by forming seals with an initial resistance of ~ 500 MOhm. Removal of extracellular $\text{Ca}^{2+}/\text{Mg}^{2+}$ then led to a larger, and statistically significant decrease in seal resistance from 0.569 ± 16 to 0.411 ± 17 MOhm (Figure 3B, $n=5$, Student t-test, $p < 0.001$), as calculated from fitting a line on the slope of the I–V curve ($r^2 = 0.96$, both fittings). This translates to a difference in conductance from 1.76 to 2.43 nS. Applying the same calculations as above, the expected increase in “current” due to diminished seal formation, is ~ 44 pA, much larger than when the seal resistance was ~ 2 GOhm, and closer to the values obtained during our whole-cell recordings, comparing the holding current in the presence and absence of extracellular divalent cations. However, the values obtained from our whole-cell recordings are still significantly larger than 44 pA (figure 7). Moreover, in our hands immediately after whole-cell access, the holding current at -65 mV is significantly lower than 44 pA, therefore the extrapolated value of the seal resistance should be well above 500 MOhm. Hence, it seems reasonable to conclude that our measured conductances upon removal of extracellular divalent cations are due to biological phenomena, though most conservatively overestimated by ~ 40 – 50 pA.

Reduction of extracellular Ca^{2+} alone produces a sustained inward current and neuronal firing

Figure 4 shows the effects of removal of $[\text{Ca}^{2+}]_e$ alone, while maintaining $[\text{Mg}^{2+}]_e$ constant at 1 mM. This had an effect similar to removal of both Ca^{2+} and Mg^{2+} (compare with figures 1&2); current spikes have been thresholded out (by removing data points that overshoot below -0.35 nA) for visual clarity of the sustained current. This was reversed by re-introduction of

2 mM Ca^{2+} to the perfusate. Removal of Ca^{2+}_e accompanied by equimolar replacement of extracellular Mg^{2+} , in order to maintain a constant concentration of extracellular divalent cations and minimize possible changes in charge screening didn't affect the sustained inward current (data not shown).

Figure 5A compares the mean inward currents obtained by reduction of extracellular Ca^{2+} from 2 mM to either nominally Ca^{2+} -free or to 100 μM Ca^{2+} , in whole-cell recordings. In cell-attached recordings, reduction of $[\text{Ca}^{2+}]_e$ to 100 μM caused a trivial decrease in seal resistance, that would account to no more than 1–2 pA increase in “leak-seal” currents (not shown) and produced a demonstrable net inward current (white bar), though significantly less than that produced by reduction to nominally Ca^{2+} -free solution (~ 3.8 μM , grey bar). An example of the effects of reduction to 100 μM extracellular Ca^{2+} is shown in Figure 5B (representative of 5 out of 5 neurons tested). The neuron was voltage clamped at -65 mV in the whole cell configuration and superfusion with 100 μM $[\text{Ca}^{2+}]_e$ produced a small but discernible inward current and neuronal discharge. Reduction of $[\text{Ca}^{2+}]_e$ to very low concentrations (~ 1 nM) by addition of 0.5 mM EGTA produced much larger currents (~ -700 pA, not shown). Although we recorded the greatest responses in the presence of 1 nM $[\text{Ca}^{2+}]_e$, we chose to characterize further the sustained inward current by reducing extracellular Ca^{2+} to 3.8 μM . This is because many types of channels are influenced by reduction of $[\text{Ca}^{2+}]_e$ to nanomolar levels, reviewed by (Xiong and MacDonald 1999). Working with ACSF in which $[\text{Ca}^{2+}]_e$ would be adjusted to 0.1 mM is potentially more relevant to *in vivo* conditions, however the currents that it produces are much smaller, making it more very difficult to evaluate the significance of changes due to intracellular Mg^{2+} and ATP manipulation described below.

Characterization of the non-inactivating inward current, as a function of intracellular Mg^{2+} and ATP

In Figure 6, representative experiments are shown in which somata were voltage-clamped in the whole cell configuration (Cs^+ being the major intracellular cation in all cases), and voltage ramps (-40 to $+40$ mV) were applied starting from a holding potential of 0 mV (5–10 cells per condition, see figure 6 inset for voltage ramp waveform). This protocol avoids initiation of neuronal discharge, as Na^+ channels remain inactivated by holding at 0 mV, and by not exceeding -40 mV during application of the waveform. All panels show currents recorded in 2 mM extracellular Ca^{2+} (thin black curves), and currents following exposure to nominally Ca^{2+} -free solution (grey curves). $[\text{Mg}^{2+}]_e$ was held constant at 1 mM, but intracellular Mg^{2+} was progressively increased in separate sets of recordings in different neurons. When Mg^{2+} was omitted from the patch pipette (Panel A), removal of extracellular Ca^{2+} produced a robust current activation. This current was still present at very low Mg^{2+}_i levels in the patch pipette (calculated 0.08 mM free Mg^{2+}_i , Panel B), but was absent at 0.9 mM Mg^{2+}_i (Panel C). In addition, the current observed in low free Mg^{2+}_i was prevented if the intracellular solution contained 4 mM ATP (Panel D). The sustained current appearing upon removal of extracellular Ca^{2+} was reversed following reintroduction of Ca^{2+} to the bathing medium (panel A, dotted curve, and figure 7). The emergence of this current (when $[\text{Mg}^{2+}]_i$ is 0) shifted the reversal potential of the overall conductances of the cell by approximately 28 mV towards positive with Cs^+ as the major intracellular cation, and by approximately 31 mV when K^+ was the major cationic charge carrier (not shown). Subtraction of the I–V curve performed in the absence of extracellular $[\text{Ca}^{2+}]_e$ from that performed in the presence of $[\text{Ca}^{2+}]_e$, resulted in an I–V curve that reversed around -5 mV (panel A, thick black curve “d”). In conditions in which the conductance is not activated by removing $[\text{Ca}^{2+}]_e$ (i.e. in the presence of 0.9 mM $[\text{Mg}^{2+}]_i$) the I–V curves in the presence or absence of $[\text{Ca}^{2+}]_e$ are virtually identical. Therefore, the subtraction I–V curve appears to be due to the emergence of a conductance induced by removal of extracellular Ca^{2+} , rather than modification of existing voltage-dependent currents by cation removal. Figure 7 shows mean values of peak inward currents recorded from a population of

neurons. When Mg^{2+} was absent or very low (0.08 mM) in intracellular solutions, significant increases in peak current were observed with removal of Ca^{2+} alone, or removal of both Ca^{2+} and Mg^{2+} . This was the case in experiments where K^+ was the charge carrier (Figure 7A), and also when Cs^{2+} was the charge carrier (Figure 7B), and in all cases the effects of extracellular cation removal was reversible (hatched bars). With a higher intracellular Mg^{2+} concentration (0.9 mM), there was no significant increase in peak inward current following extracellular cation removal, compared with control values (black bars). No significant increase in peak inward current was observed following cation removal if intracellular ATP levels were elevated to 2.2 mM while maintaining low intracellular Mg^{2+} (0.07 mM).

Effect of extracellularly applied spermine

Currents measured in other type of neurons upon removal of extracellular Ca^{2+} were sensitive to inhibition by a high concentration of extracellular spermine (Formenti *et al.* 2001; Xiong *et al.* 1997). Figure 7 (C&D) shows the effects of extracellular spermine on currents produced by superfusion with nominally Ca^{2+} -free solution. The fact that substitution of $[K^+]_i$ with Cs^{2+} and TEA does not affect the appearance of the inward current supports the hypothesis that the conductance is non-selective for K^+/Cs^+ . In the experiments where spermine was applied, K^+ -based intracellular solution (Int 2) was used. As shown, 0.1 mM spermine had no significant effect on the sustained current ($n=4$), but 1 mM spermine virtually abolished the current ($n=4$, current spikes have been thresholded out for visual clarity).

Characterization and origin of the current spikes accompanying the sustained inward current

To investigate the basis of current spikes observed during Ca^{2+} removal, we examined spikes in current clamp recordings. As shown in figure 8A (left panel), reducing extracellular Ca^{2+} resulted in high frequency action potential firing and a significant depolarizing shift in the interspike voltage (fig. 8A, right panel, action potentials have been removed by threshold-exclusion of data more positive than 0 mV, for visual clarity). The decrease in the interspike voltage was inversely related to the $[Mg^{2+}]_i$. With 0.2 mM $[Mg^{2+}]_i$ in the recording pipette, membrane potential decreased to -57 ± 2 mV, and with 0 $[Mg^{2+}]_i$, it diminished further to -50 ± 3 mV ($n=5$, both conditions).

To test whether the currents measured under soma voltage clamp conditions were of an appropriate magnitude to produce such firing and depolarization, we applied a range of depolarizing currents to neurons bathed in normal ACSF. The range included the values determined for the population of neurons tested under soma voltage clamp (see Fig 7A). The firing rate/interspike depolarization relationships shown in Fig. 8B (left panel) were obtained by manually increasing the injected current from 0 to 150 pA (~ 20 pA increment steps/5 sec) and determining the parameters within a sampling interval of 1 sec. For comparison, the firing rate and depolarization profile of this same neuron during low- $[Ca^{2+}]_e$ exposure are given in Fig. 8B (right panel), and is representative of 5 independent experiments. The order of the protocol applied (injection of current versus superfusion with Ca^{2+} -free ACSF) did not influence the outcome. The upward slope of firing rate reflects the development of inward current under low Ca^{2+} exposure mimicked by gradual increase of applied current. The falloff in firing rate at high stimulus current is likely due to Na^+ conductance inactivation, as the interspike potential is quite depolarized (fig. 8B left panel, measured V_m marked above the plot). This is not the cause in the low Ca^{2+} condition judging from the interspike potential. Removal of extracellular Ca^{2+} caused a greater increase in firing frequency (25 ± 1 Hz, $n=5$), as compared to the frequency of current spikes appearing by injection of current through the pipette (21 ± 1 Hz, $n=5$, $p=0.009$ statistically significant). This difference could be due to additional synaptic inputs to the patched neurons from projecting neurons in the slice that would be excited by the nominally Ca^{2+} -free ACSF but not by injection of current into the postsynaptic neuron. Also the magnitude of the nominally Ca^{2+} -free induced current is

measured at a voltage-clamped soma. If the channels carrying the current are distributed over the neuron, this will be only a fraction of the total cellular current acting to initiate action potentials.

Inclusion of high [ATP] in the recording pipette abolished the sustained current observed in nominally Ca^{2+} -free solution (figure 8C, current spikes have been thresholded out for visual clarity). Firing frequency in the presence of ATP in the pipette upon superfusion with nominally Ca^{2+} -free ACSF was 26 ± 1 Hz ($n=4$, not significantly different compared to the absence of ATP in the pipette). This reflects the possibility that other sources of excitability participate in the high frequency firing. This hypothesis is also supported by the finding that the frequency of the spikes is diminished by blocking glutamatergic transmission with $50 \mu\text{M}$ CNQX + $100 \mu\text{M}$ APV in some, but not all cases (data not shown).

DISCUSSION

The results of this study demonstrate that extracellular Ca^{2+} reductions produce a sustained inward current in hippocampal CA1 pyramidal neurons in brain slices from adult mice, associated with an increased frequency in action potential generation. This current appears similar to Ca^{2+} -sensitive non-selective currents reported in cultured neuronal preparations or freshly isolated neurons (Formenti and De 2000; Udem *et al.* 2003; Xiong *et al.* 1997). A novel finding of the present study is the demonstration of a strong negative dependence on intracellular Mg^{2+} and ATP concentrations not examined in other preparations, suggesting that increasing metabolic stress could be an important trigger (or at least play a permissive role) for this influx pathway in mature neurons.

The onset rate and amplitude of sustained inward currents in the present study were significantly lower than those measured in cultured hippocampal neurons of embryonic origin (Xiong *et al.* 1997). On several occasions we observed that prolonged bathing of the slice to nominally Ca^{2+} -free solution (with or without extracellular Mg^{2+}) led to large and precipitous decreases in the measured resistance with a zero reversal potential of the associated currents. Reintroduction of extracellular Ca^{2+} abolished this phenomenon in about half of the cases. We have conservatively attributed such large responses to loss of seal formation, however, we do not exclude the possibility that initially, activation of major currents led to the large decrease in membrane resistance that itself quickly resulted in a loss of seal. As described in Results, changes in seal quality were considered, and do not appear to explain the currents observed with nominally Ca^{2+} -free solutions.

A small current activation was observed with Ca^{2+} reductions to 0.1 mM but for most characterization of the current described here, studies were performed with nominally Ca^{2+} -free solutions (containing $\sim 3.8 \mu\text{M}$ Ca^{2+}) that provided larger amplitude currents (Figure 5). If the current described here contributes to neuronal demise in injuries such as stroke, it is possible that adjuvant circumstances (e.g. an oxidative stress) create conditions by which channel activation could be significantly increased during the more modest Ca^{2+} reductions (see below).

Dependence on intracellular Mg^{2+} and ATP

The observation that currents were much larger without ATP in the recording pipette raises the possibility that metabolic state is a regulator of this inward current. Thus, this conduit may be a critical determinant in ischemic or other conditions involving excitotoxicity, in which intracellular ATP levels are significantly decreased (Greene and Greenamyre 1996; Nicholls and Budd 2000; Nicotera and Lipton 1999). The mechanism(s) underlying ATP effects here are currently unknown. A caveat here is that ATP is a weak chelator of Mg^{2+} , and therefore the equilibrium of the free Mg^{2+} ion to the Mg-ATP complex may be such that there is

considerably more free Mg^{2+} in the dialysed cytosol (Kozak and Cahalan 2003). Alternatively, the configuration of the Mg-ATP complex may be such that, the Mg^{2+} moiety is still able to act on its target.

Current spikes and action potentials

Intense neuronal firing upon reduction of extracellular Ca^{2+} has been previously observed in rat hippocampal slices (Konnerth *et al.* 1986). Robust increases in firing were observed in the present study even in the somata voltage-clamp mode, implying origin in distal axon and/or dendrites that may include synaptic responses from other neurons in the slice. Synaptic origin of some of the firing is suggested by the observation that increasing the extracellular Mg^{2+} to 3 mM upon removal of extracellular Ca^{2+} , substantially diminished spiking, while the sustained current was not affected. However, there appear to be additional mechanisms contributing to this intense neuronal discharge, since blocking excitatory neurotransmission with CNQX +APV did not always abolish the current spikes. The reason for this variance is beyond the scope of the present study and was not further investigated. However it was previously reported that NMDA receptors mediate epileptiform activity induced by the absence of extracellular Mg^{2+} in some conditions but not others, depending on the individual morphology of the dendritic projections (Thomson and West 1986). In addition, it is not obvious if the projecting non-dialysed neurons meet the intracellular requirements (reduction of intracellular Mg^{2+} and ATP) during reduction of extracellular Ca^{2+} , in order to exhibit the sustained current – or conditions might be different in nerve terminals – i.e. there may not be the same Mg^{2+} /ATP dependence there. For a comprehensive discussion on the relation of extracellular Ca^{2+} to seizure-like phenomena in slice, the reader is referred to (Heinemann *et al.* 1990).

Relationship to known currents and channels

At this juncture, it is not known how the other Ca^{2+} sensing mechanisms that are found in the hippocampus, such as voltage-dependent Ca^{2+} channels, mGluRs or hemi-gap channels (Hofer 2005) may contribute to the observations recorded here. One interesting possibility is that the sustained conductance that we record on the pyramidal neurons is due to one or more members of the transient receptor potential (TRP) family (Clapham *et al.* 2001). TRP channels are widely expressed in mammalian tissues, especially in neurons of the central nervous system including the hippocampus (Moran *et al.* 2004). Not infrequently, it has been observed that several TRP channels conduct better in the absence of divalent cations. Among them, TRPM7 deserves particular attention as a candidate for the conductance that we present here, due to 1) the strong negative feedback it receives by intracellular Mg^{2+} (irrespective of whether it is weakly chelated by ATP (Nadler *et al.* 2001;Kozak and Cahalan 2003), 2) its activation upon removal of external divalent ions (Runnels *et al.* 2001;Nadler *et al.* 2001), and 3) its sensitivity to inhibition by spermine (Kozak *et al.* 2002;Kerschbaum *et al.* 2003). However, in our case, 100 μ M spermine failed to inhibit the sustained current upon removal of extracellular Ca^{2+} , a concentration that should fully eradicate the TRPM7-mediated current (Kerschbaum *et al.* 2003), plus the current was insensitive to inhibition even by high extracellular $[Mg^{2+}]$. 1 mM spermine completely abolished the low- $[Ca^{2+}]_e$ -induced sustained current in adult pyramidal neurons in the slice preparation, consistent with a previous observation in cultured hippocampal neurons (Xiong *et al.* 1997). Nonetheless, possible candidates may also include many voltage-gated channels (see Introduction) as well as ligand gated ion channels, since low, but not zero $[Ca^{2+}]_e$ is known to elicit additional transmitter release, via modification of surface potential and subsequent Ca^{2+} influx (Piccolino and Pignatelli 1996). I_{crae} -conducting channels can also be regulated by extracellular divalent ions (Hoth and Penner 1993).

Consequences of current activation

$[Na^+]_i$ increases due to non-inactivating current may be appreciable. Relevant to this, an increase of greater than 10 mM $[Na^+]_i$ may up-regulate NMDA receptor activity (Yu and Salter 1998), and it was recently shown that extracellular Ca^{2+} depletion leads to $[Na^+]_i$ elevation, that in turn increases NMDA channel activity (Xin *et al.* 2005). Large $[Ca^{2+}]_i$ increases are known to be triggered by reintroduction of physiological Ca^{2+} concentrations to the extracellular milieu (reperfusion) after cardiac muscle tissue has experienced a prior $[Ca^{2+}]_e$ -free challenge, or at least a severe reduction in Ca^{2+}_e concentration (ischemia), (Zimmerman and Hulsman 1966) termed the “ Ca^{2+} Paradox”. We have recently reported that cultured cortical neurons exhibit paradoxical Ca^{2+} entry (Chinopoulos *et al.* 2004) and it is possible that this entry pathway may be a significant contributor to excitotoxic neuronal death. Evidence for the existence of the Ca^{2+} Paradox in brain has been summarized previously (Young 1986), and it was proposed recently that the phenomenon may be substantiated by one or more members of the TRP family of channels (Chinopoulos and Adam-Vizi 2006; MacDonald *et al.* 2006). Activation of NMDAR/non-NMDARs during hypoxia is associated with a severe Ca^{2+} influx, imposing downstream injurious effects on cellular functions. Notably though, the conductance mechanism presented here is not $[Ca^{2+}]_i$ activated, as there was 10 mM EGTA (free) at all times in the pipette solution available to chelate any incoming Ca^{2+} , verified by Fura 2 imaging (not shown). Overall, we demonstrate the presence of a sustained inward conductance in adult hippocampal CA1 pyramidal neurons upon reduction of $[Ca^{2+}]_e$, provided that intracellular Mg^{2+} /ATP concentrations are reduced. This could be an important causative mechanism in neuronal pathology encompassing diminished metabolic capacity in the presence of reduced levels of extracellular calcium, conditions that are both met in various disease states.

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Abbreviations

APV
2-Amino-5-phosphonopentanoic acid

csNSC
calcium-sensing non-selective channel

CNQX

6-Cyano-7-nitroquinoxaline-2,3-dione

mGluR

metabotropic glutamate receptor

NMDAR

NMDA receptor

TEA

tetraethylammonium chloride

TRP

Transient Receptor Potential

Figure 1. Removal of extracellular divalent cations produces a non-inactivating inward current accompanied by neuronal firing

A: Whole cell recording, with K^+ -containing intracellular solution, in normal ACSF (2mM Ca^{2+} , 1mM Mg^{2+}). After onset of a sustained inward current triggered by $\text{Ca}^{2+}/\text{Mg}^{2+}$ removal (indicated by black bar), data acquisition was interrupted (at grey dotted bar) for application of voltage ramp protocols (see figure 6). Following resumption of continuous data acquisition, the current and spiking were terminated by restoration of Ca^{2+} and Mg^{2+} . Dashed line indicates zero current in this and all figures. **B:** Conditions of this experiment are identical to those in **A**, except that the intracellular solution contained Cs^+ as the major cation plus 2 mM TEA ("Int 6"), in lieu of K^+ . A similar inward current and spiking activity was recorded. **C:** The conditions

of this experiment are identical as for “**B**”; however, no neuronal firing is observed in this neuron. The inset is an expanded region of the area indicated by the black box, to show small excitatory currents generated by $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free solution in this neuron.

Figure 2. Increased activity produced by removal of divalent cations, recorded in cell-attached mode

A: Recording of a CA1 pyramidal neuron perfused in the absence of extracellular divalent cations, as indicated by the top black bar. **B:** Expanded regions corresponding to those depicted in the recording shown in A, showing a tonic firing pattern (seen in ~75 % of neurons tested). **C&D:** Identical experimental conditions as in A, but in this example, a burst firing pattern was seen following removal of extracellular divalent cations (seen in ~15% of neurons tested).

Figure 3. Effects of reduction of extracellular divalent cations on seal resistance

A: Current-voltage relationships from cell-attached recordings in the presence (black circles) or absence (grey triangles) of extracellular divalent cations (mean $\pm$ SEM of 5 cells each). Seal resistance (R_s) was adjusted by mouth suction to $\sim 2 \text{ GOhm}$ and divalent cations removal had a minor effect on seal resistance. **B:** Recordings performed as for **A**, except that R_s was adjusted by mouth suction to $\sim 0.5 \text{ GOhm}$, and resulted in significantly greater decreases in seal resistance following divalent cations removal.

Figure 4. Inward currents produced by removal of Ca^{2+} alone

Whole cell recording (holding potential $= -65 \text{ mV}$), showing inward current produced by Ca^{2+} removal, retaining extracellular Mg^{2+} constant at 1 mM. Current spikes are truncated.

Figure 5. Effects of moderate decreases in extracellular Ca^{2+}

A: Mean differences of peak minus the base leak amplitudes of sustained inward currents, generated by nominally Ca^{2+} -free solution (grey bar) or $0.1 \text{ mM } \text{Ca}^{2+}_e$ (white bar). $[\text{Mg}^{2+}]_e$ was constant at 1 mM . **B:** Representative recording of a neuron perfused with ACSF containing $100 \mu\text{M } [\text{Ca}^{2+}]_e$. The intracellular solution contains K^+ as the major cation (Int “2”). Neuronal discharge also accompanies the sustained inward current.

Figure 6. Inhibition of currents evoked by Ca^{2+} removal by intracellular $[Mg^{2+}]$ and [ATP]
 Currents were evoked by voltage ramps (-40 to $+40$ mV, see inset for waveform) from a holding potential of 0 mV. The intracellular solution contained Cs^{2+} as the major cation. Thin black curves indicate superfusion with normal ACSF, and grey curves indicate superfusion in nominally Ca^{2+} -free ACSF (1 mM Mg^{2+}). Panels A–C show the effects of increasing Mg^{2+}_i concentrations. D shows the effects of addition of ATP to the intracellular solution. The calculated free $[Mg^{2+}]_i$ and [ATP] are shown for each panel (calculated with Winmaxc, see Methods). The dotted curve on panel “A” indicates reintroduction of $[Ca^{2+}]_e$ to the perfusate (curve “c”). The thick black curve on panel A is the result of subtracting curve “b” from curve “a”.

Figure 7. Estimates of currents induced by removal of extracellular Ca^{2+} at -65 mV holding potential in whole cell recordings, and effect of spermine

A: The intracellular solution contains K^+ as the major cation (Int “1–4”). **B:** The intracellular solution contains Cs^+ as the major cation (Int “5–8”). Hatched bars signify reintroduction of Ca^{2+} (and Mg^{2+} if it was removed previously) to the perfusate. *, significant, ($p < 0.05$), Mann-Whitney rank sum test. Each bar represents the mean value of 5–9 cells. Non-responding cells were not included in the calculations of the mean currents. Free $[Mg^{2+}]_i$ and [ATP] concentration are shown on the corresponding group of bars. The total $[Mg^{2+}]_i$ and [ATP] are identical to that shown for figure 6. The currents measured with 0.08 mM free $[Mg^{2+}]_i$ is statistically significantly higher than in the presence of 0.07 mM free $[Mg^{2+}]_i$ plus 2.2 mM

ATP, irrespective if the major intracellular cation is K^+ or Cs^+ ($p < 0.005$). **C**: Whole cell recordings of neurons perfused with either nominally Ca^{2+} -free ACSF (grey bar) as compared to the presence of 0.1 mM spermine in the perfusate (grey-white hatched bar), or 1 mM spermine (white-black hatched bar). The intracellular solution for these experiments is "Int 6". In panel **D**, a representative recording of a voltage clamped neuron in the whole cell mode is shown, where 1 mM spermine ameliorates against the $-low-[Ca^{2+}]_e$ -induced current; current spikes are truncated.

Figure 8. Characterization of neuronal firing accompanying the sustained inward current

A: Left graph: Current clamp recording of a patched neuron in the whole cell mode, during superfusion with nominally $[\text{Ca}^{2+}]_e$ -free ACSF; right graph: Interspike voltage (mV) of the recording depicted on the left graph. **B: Left graph:** Quantification of current spike frequency as a function of interspike Vm, for the same neuron as shown in **A**. **B, Right graph:** Quantification of current spike frequency over gradual injection of current on the patched neuron through the pipette. Top x-axis depicts the measured Vm during injection of current. **C:** Voltage clamp recording of a patched neuron in the whole cell mode, during superfusion with nominally $[\text{Ca}^{2+}]_e$ -free ACSF, but in the presence of intracellular Mg^{2+} and ATP ("Int 4"). Current spikes are truncated.

Table 1

Intracellular solutions used for electrophysiological recordings.

	CaCl ₂	MgCl ₂	NaCl	KCl	Glucose	EGTA	K gluconate	CsCl	Cs MES	ATP DiNa ⁺
Int 1 [*]	1	-	15	40	10	11	70	-	-	-
Int 2	1	0.2	15	40	10	11	70	-	-	-
Int 3	1	2	15	40	10	11	70	-	-	-
Int 4	1	2	7	40	10	11	70	-	-	4
Int 5	1	-	15	-	10	11	-	40	70	-
Int 6	1	0.2	15	-	10	11	-	40	70	-
Int 7	1	2	15	-	10	11	-	40	70	-
Int 8	1	2	7	-	10	11	-	40	70	4

^{*}Int: Intracellular solution. All values are given in mM. pH was adjusted to 7.25 with KOH or CsOH (with buffer HEPES, 10 mM), depending whether the major cation was vK⁺ or Cs⁺, respectively. Intracellular solutions containing CsCl and Cs-MES were also supplemented with 2 mM TEA chloride. Cs-MES: Cs⁺ methanesulfonate. The free [Ca²⁺]_i was estimated to be 10–30 nM, using the Winmaxc software (Bers *et al.* 1994) for all solutions.