Ionic Mechanisms Underlying Burst Firing of Layer III Sensorimotor Cortical Neurons of the Cat: An In Vitro Slice Study

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Nishimura, Yoshihiro, Masaru Asahi, Koichi Saitoh, Hirofumi Kitagawa, Yuichi Kumazawa, Kunio Itoh, Min Lin, Takanobu Akamine, Hiroshi Shibuya, Toshihiro Asahara, and Tetsuro Yamamoto. Ionic mechanisms underlying burst firing of layer III sensorimotor cortical neurons of the cat: an in vitro slice study. J Neurophysiol 86: 771–781, 2001. We examined the ionic mechanisms underlying burst firing in layer III neurons from cat sensorimotor cortex by intracellular recording in a brain slice. Regular spiking was observed in 77.4% of 137 neurons in response to constant intracellular current pulses of 0.5- to 1-s duration. The rest of the neurons showed burst firing. An initial burst followed by regular-spike firing was seen in 71.0% of 31 bursting neurons. The rest of the bursting neurons (n = 9) exhibited repetitive bursting. In the bursting neurons, spikes comprising the burst were triggered from the afterdepolarization (ADP) of the first spike of the burst. We examined the ionic mechanisms underlying the ADP by applying channel-blocking agents. The ADP was enhanced (rather than blocked) by Ca\(^{2+}\) channel blockade. This enhancement of the ADP by Ca\(^{2+}\) channel blockade was apparent even after blockade of the afterhyperpolarization by apamin or intracellular Ca\(^{2+}\) chelation by EGTA. The firing rate of the regular-spiking cells was increased by apamin, intracellular EGTA or Ca\(^{2+}\) channel blockers. In 17.9% of the neurons examined (n = 56), these agents switched the regular-spiking pattern into a bursting one. Burst firing could not be changed to regular spiking by these agents. Four neurons that responded with a single initial burst in control solution responded with repetitive bursting after application of these agents. We conclude that the main function of Ca\(^{2+}\) influx in layer III neurons is to activate Ca\(^{2+}\)-dependent K\(^+\) conductance, which prevents or limits burst firing. At a time when spike amplitude was unchanged, the ADP was blocked and the burst firing changed to regular spiking by extracellularly applied tetrodotoxin (TTX) or intracellularly applied N-(2,6-dimethylphenylcarbamoylmethyl) triethyl ammonium bromide (QX314). We concluded that a TTX- and QX314-sensitive Na\(^+\) current underlies the ADP and therefore contributes to the burst firing of layer III neurons from the cat cortex.

INTRODUCTION

The major function of the motor cortex is an execution of skilled movements that is achieved through information processing in the columnar organization of the cortical neurons (Porter 1981). To understand this information processing, the integrative function of the major projecting pyramidal neurons should be considered. The integration of information in the pyramidal neurons is performed through the intrinsic mechanisms underlying the transformation of the graded synaptic inputs into the encoded action potentials, that is, the mechanisms controlling the input-output relation. Injected current pulses mimic the summed synaptic currents arriving at the soma. Therefore the input-output relation in the neuron may be understood by examining the firing properties induced by current injections.

The firing patterns of cortical pyramidal neurons in rodents were classified into regular spiking and intrinsic bursting (Connors et al. 1982). It was also reported that the bursting neurons were found in layers IV and V (Connors et al. 1982), and they play a specific role in information processing (Chagnac-Amitai and Connors 1989; Mason and Larkman 1990). Our previous study revealed the presence of bursting neurons in layer III pyramidal neurons of the cat sensorimotor cortex (Nishimura et al. 1996).

Kandel and Spencer (1961) hypothesized that burst firing results from afterdepolarization (ADP) “summation,” which maintains spike recruitment until the accumulated spike inactivation terminates the discharge. Furthermore, a number of other investigators have suggested that the voltage-gated inward currents, which are larger than the outward currents shortly after a spike, generating the ADP will produce burst firing (Azouz et al. 1996; Friedman and Gutnick 1987, 1989; Jensen et al. 1996; McCormick et al. 1985; Silva-Barrat et al. 1992; Wong and Prince 1978, 1981). We also found that the ADP following a spike plays an important role in generation of burst firing in cat layer III sensorimotor cortical neurons (Nishimura et al. 1996). Therefore it is important to study the mechanisms underlying the ADP to understand the mechanisms generating the burst-firing pattern of these neurons.

A body of evidence has implicated Ca\(^{2+}\) currents as playing a pivotal role in the generation of the ADP and/or burst firing (Friedman and Gutnick 1989; Jahnse and Llinas 1984; Kobayashi et al. 1997; McCormick and Pape 1990; Silva-Barrat et al. 1992; Wong and Prince 1978). In contrast, recent studies showed that the ADP and/or the burst firing were controlled mainly by activation of Na\(^+\) currents (Azouz et al. 1996; Deisz and Prince 1987; De Waele et al. 1993; Franceschetti et al. 1995; Guateto et al. 1996; Hoehn et al. 1993; Jensen et al. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
revealed the contribution of persistent Na+ currents (I\textsubscript{pap}) to the generation of ADP and burst firing in the ferret visual cortical neurons (Brumberg et al. 2000). To investigate the ionic basis of the ADP and the burst firing in layer III pyramidal neurons in this study, we examined the role of both Ca\textsuperscript{2+} and Na\textsuperscript{+} currents by observing the effect of specific channel-blocking agents on the ADP amplitude and on the evoked firing pattern.

**METHODS**

The procedures for making and maintaining slices and identifying the layer III pyramidal neurons were described in detail previously (Nishimura et al. 1996) and mentioned here briefly. A block of the brain including the precorpus cortex was removed after dissecting the skull in the cat, which was previously anesthetized with ketamine hydrochloride (20 mg/kg im). Parasagittal sections (400–500 µm) were made using a vibratome (DTK 1000, DOSAKA EM, Kyoto, Japan) and then stored in a chamber filled with artificial cerebrospinal fluid (ACSF, see following text) bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} continuously and maintained at 35°C. The slices were transported to the recording chamber in which the slice was maintained at the interface between ACSF and 95% O\textsubscript{2}-5% CO\textsubscript{2} saturated with water vapor. The composition of the ACSF was (in mM) 130 NaCl, 3 KCl, 2 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, and 10 dextrose. The ACSF was bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} to maintain pH at 7.4.

Intracellular recording and staining were performed using microelectrodes (1.5 mm OD) filled with 3 M-KCl (DC resistance of ~40 MΩ) or filled with 2% Biocytin (Sigma) dissolved in 0.5 M K-acetate (~150 MΩ). In some experiments, microelectrodes containing 2.7 M KCl plus 0.1 M ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA, Fluka Chemie AG, Switzerland) or containing 25 mM N-(2,6-dimethylphenylcarbamoylmethyl) triethyl ammonium bromide (QX314, Alamine Labs, Jerusalem, Israel) in 3 M KCl were used. Electrodes were inserted in the gray matter at ~700 µm below the pial surface, which is estimated to be layer III (Hassler and Muhs-Clement 1964). Just after the cell impalement, steady hyperpolarizing currents were injected to stabilize the cell. The recording of electrical activity, however, was done without steady hyperpolarizing currents. In 60 cases, the recorded neurons were verified to be layer III pyramidal neurons with intracellular staining using methods described previously (Nishimura et al. 1996).

Voltage responses were evoked by current injections (5-ms or 0.5-~1-s pulses) through the microelectrode using an active bridge (MEZ 8201, Nihonkohden, Tokyo or Axoclamp 2B, Axon Instrument, Foster City, CA) or a discontinuous current clamp (Axoclamp 2B, Axon Instrument). Data were recorded on a multichannel videocassette recorder with pulse code modulation (sampling rate; DC ~44 KHz in 2 channels, Neurocorder, Neuro Data Instrument, New York, NY). The recorded data were analyzed by a personal computer after off-line digitization (McLab 4, Analog Digital Instrument, Castle Hill, Australia). Spike height was measured from the baseline (the resting membrane potential). Input resistance (R\textsubscript{i}) was calculated from a linear portion of current-voltage relations obtained by plotting steady-state subthreshold voltage responses against injected currents.

To block voltage-dependent Ca\textsuperscript{2+} channels, we employed the following procedures: application of CdCl\textsubscript{2} (400 µM) in HEPES-buffered solution or NiCl\textsubscript{2} (100 µM); Ca\textsuperscript{2+}-free solutions in which equimolar (2 mM) Co\textsuperscript{2+} (Co/Ca substitution) or Mn\textsuperscript{2+} (Mn/Ca substitution) replaced Ca\textsuperscript{2+} in NaH\textsubscript{2}PO\textsubscript{4}-free ACSF; Ca\textsuperscript{2+}-free solution containing EGTA (0.5 mM) and raised Mg\textsuperscript{2+} (5 mM) (Ca-0). When employing the Ca\textsuperscript{2+}-channels blockade, electrical properties were examined ~20 min, a time judged sufficient to substantially block voltage-gated Ca\textsuperscript{2+}-channels (see Fig. 2). Intracellular application of EGTA or application of ampin in the ACSF was used to block Ca\textsuperscript{2+}-mediated K+ currents. In initial experiments, EGTA was injected iontophotically, but in most experiments, EGTA entered the neuron by diffusion from the intracellular recording electrodes. Apamin (SIGMA, 300 nM) and tetrodotoxin (TTX, SIGMA, 1 µM) were applied in the perfusate. In some experiments, TTX (170 µM) and apamin (10 µM) were applied in a droplet in the recording chamber away from the slice in the case of TTX or on the slice surface near the recording electrode in the case of apamin. Tetraethylammonium chloride (TEA, 20 mM) was applied by substitution for equimolar NaCl. When the voltage responses were compared before and after application of the chemicals, the membrane potential was maintained at the control value by DC-current injections (~0.3 to +0.2 nA). Data were analyzed statistically by Student’s t-test.

**RESULTS**

**General properties**

Intracellular recordings were obtained from 137 layer III pyramidal neurons from the sensorimotor cortex of 53 cats. Layer III was estimated to lie ~700 µm below the pial surface. Sixty of these neurons were verified to be layer III pyramidal neurons by intracellular staining. The mean values of resting membrane potential (RP), spike height, and input resistance (R\textsubscript{i}) of the sampled 137 neurons were ~73.1 ± 10.6 mV, 87.3 ± 14.9 mV, and 55.0 ± 34.1 MΩ, respectively (means ± SD). Because the mean values of these parameters were not different between stained and unstained neurons, we assume that all sampled neurons were layer III pyramidal neurons.

**Firing patterns**

We examined the firing patterns of the layer III neurons evoked by 0.5-~1-s injected current pulses. We classified current-evoked firing pattern into two types: regular spiking (77.4%, n = 106) and bursting (22.6%, n = 31). Regular spiking indicates continuous repetitive firing, and bursting represents the generation of an endogenous burst of three to five action potentials. Moreover, the bursting pattern was grouped into two subtypes, burst-and-regular-spiking (71.0% in 31 layer III bursting neurons) and repetitive bursting. In the former group, an initial burst at the onset of the current pulse was followed by regular spiking. As reported previously (Nishimura et al. 1996), the mean values of RP, spike height, and R\textsubscript{i} were not significantly different between regular-spiking and bursting neurons.

The firing patterns evoked by constant current injection in layer III pyramidal neurons are shown in Fig. 1. The regular-spiking neurons exhibited spike frequency adaptation (Fig. 1A). The firing reached its steady-state firing-adapted rate in 63.6 ± 51.8 ms (n = 106) after the pulse onset. The mean value of the steady-state firing rate evoked by a current pulse of 0.7 nA was 75.0 ± 50.0 Hz (n = 106). Action potentials evoked by 5-ms current pulses in regular-spiking neurons were followed by a fast afterhyperpolarization (fAHP, Fig. 4, A and C), an ADP (Fig. 4C), and a medium AHP (mAHP, Fig. 4, A1 and B1). The regular-spiking pattern did not change to a bursting pattern if the injected current intensity was increased or when RP was changed by DC-current injections (data not shown).

The burst-and-regular-spike firing pattern of layer III neurons is represented in Fig. 1B. In these neurons, a burst was followed by a train of regular spikes (Fig. 1B3). In 18 of 22
burst-and-regular-spiking layer III neurons the burst was evoked in an all-or-none manner (Fig. 1B, 1 and 2), but 4 neurons produced regular spikes to just-suprathreshold current pulses. The average intraburst firing rate in burst-and-regular-spiking firing neurons was 157.6 ± 52.7 Hz (n = 22), which was much higher than the firing rate during regular spiking that followed the initial burst.

The other type of bursting, repetitive bursting, is shown in Fig. 1C. Some of the sampled repetitive-bursting neurons were similar to “chattering cells” reported in cat visual cortex (Gray and McCormick 1996). The burst was generated in all-or-none manner in all the repetitive bursting neurons (n = 9, see Fig. 1C, 1 and 2). The frequency of the repetitive bursts evoked by the current at the threshold was 13.9 ± 9.3 Hz (n = 9) and became faster as the current was increased. The intraburst firing rate was similar to that in the burst-and-regular-spike firing neurons.

In both subtypes of bursting neurons, the burst consisted of spikes that incompletely repolarized (Fig. 1C3), and the burst was followed by a prominent hyperpolarization as indicated by an asterisk in Fig. 1B, B3 and C3. The average value for the membrane potential of the ADP in the individual spike during the firing (see “ADP amplitude” in Fig. 1C3) was −34.1 ± 11.4 mV (n = 31) in burst neurons. In burst neurons, current pulse (5 ms) evoked a prominent ADP that sometimes reached threshold and evoked an extra spike in an all-or-none manner (Fig. 1B, 4–6). Such an ADP was seen in 22 of 31 bursting neurons and in 52 of 106 regular-spike firing neurons. The amplitude of ADP was significantly (P < 0.05) larger in burst neurons (21.9 ± 9.3 mV, n = 22) than in regular-spike-firing neurons (13.8 ± 7.7 mV, n = 52) although RPs were not different in these groups. The activation of an extra spike on the ADP was seen in 31.8% of the burst neurons examined (n = 31), but no spikes were seen on the ADP in regular-spiking neurons.

We hypothesized that the ADP plays an important role in generation of the burst firing. Thus we studied ionic mechanisms underlying the ADP. Because a contribution of voltage-dependent Ca2+ currents to the ADP was implied in past studies (Friedman and Gutnick 1987; Kobayashi et al. 1997; Wong and Prince 1981), we first examined the effects of Ca2+ channel blockade on the ADP.

**Effects of Ca2+ channel blockade on ADP**

We used a variety of divalent cations to block voltage-gated Ca2+ channels, as well as Ca2+-free solution to reduce Ca2+ influx through these channels, to ensure that the primary effects observed on the ADP were due to reduction of Ca2+ influx rather than a nonspecific effects of one of the divalent cations. To test the effectiveness of Ca2+ blocking solutions (see methods), we first verified that the Ca2+-channel-blocking agents used in this study readily block Ca2+ spikes at the concentrations employed and over the recording times employed. Ca2+ spikes were evoked by depolarizing current pulses in TTX (1 μM)- and TEA (20 mM)-containing solution. All the agents used to block Ca2+ channels abolished the Ca2+ spikes reversibly without any significant (P > 0.05) changes of Rg in ~20 min: Co-2 mM/Ca-2 mM substitution, 18.0 ± 6.6 min, n = 3; Mn-2 mM/Ca-2 mM substitution, 18.0 ± 1.7 min, n = 3 (Fig. 2A); Ca-0, 26.3 ± 14.9 min, n = 6 (Fig. 2B); Ni2+ (100 μM), 22.0 ± 3.5 min, n = 3; Cd2+ (400 μM), 20.4 ± 2.2 min, n = 5. Next, we examined the effect of these Ca2+-channel-blocking agents on the ADP that followed Na+ spikes evoked by brief current pulses.

Figure 3A shows an enhancement of the ADP and activation of an action potential on it (Fig. 3A2) in Ca2+-free solution.
containing EGTA and raised Mg\(^{2+}\) solution (Ca-0). These changes were reversible (Fig. 3A3) and without a significant change in \(R_n\) (64.5 MΩ in Fig. 3A4, 66.1 MΩ in Fig. 3A5, 64.0 MΩ in Fig. 3A6). Enhancement of the ADP was seen in eight of nine neurons, and activation of an extra spike on the ADP was seen in one of nine neurons examined in the Ca-0 solution. The ADP was increased in six of seven neurons examined after Cd\(^{2+}\) was added in the solution. In one of these seven neurons, an action potential was activated on the enhanced ADP (Fig. 3B2). Twenty-five minutes after the washout of Cd\(^{2+}\), the ADP remained still large but the extra spike on the ADP disappeared (Fig. 3B3). These changes were also observed without a significant alteration in \(R_n\) (74.6 MΩ in Fig. 3B4, 73.9 MΩ in Fig. 3B5, 74.8 MΩ in Fig. 3B6).

Co-2 mM/Ca-2 mM substitution blocked the mAHP in three neurons having a control mAHP and enhanced the ADP or evoked an extra spike on the enhanced ADP in all the neurons having a control ADP (\(n = 7\)). Mn-2 mM/Ca-2 mM substitution also blocked mAHP in one neuron. It enhanced the ADP (including extra spike activation in 2 neurons) in all the neurons having control ADP (\(n = 6\)). Application of Ni\(^{2+}\) (100 µM) to the perfusate increased the ADP or generated an extra spike on the enhanced ADP in five of eight neurons having a control ADP.

The agents used in this study to block Ca\(^{2+}\) influx increased the ADP in amplitude significantly (\(P < 0.05\)) as shown in Table 1. The \(R_n\)'s did not change significantly (\(P > 0.05\)) by using these pharmacological agents (see Table 1).

It is reported that the mAHP in layer V pyramidal neurons in sensorimotor cortex is generated by activation of Ca\(^{2+}\)-mediated K\(^+\) current, \(I_{K(Ca)}\) (Lorenzon and Foehring 1992; Schwindt et al. 1988, 1992). We hypothesized that the enhanced ADP amplitude observed after Ca\(^{2+}\) channel blockade was secondary to the blockade of \(I_{K(Ca)}\). We tested this possibility by examining the ADP after blockade of \(I_{K(Ca)}\) by intracellular Ca\(^{2+}\) chelation or by extracellular apamin.

Effects of intracellular EGTA and extracellular apamin on the ADP

Figure 4 shows the afterpotentials recorded by an electrode filled with EGTA-containing solution. After diffusion of EGTA into the neuron (40 min after impalement) the mAHP (Fig. 4A1, arrow) was blocked and the ADP increased (Fig. 4A2). This enhancement of the ADP, including the activation of an extra spike or the alteration of an mAHP to an ADP, was seen in all the neurons examined (\(n = 17\)), but the fAHP (Fig. 4A2, arrows) remained present. In 15 of the neurons having a control ADP, the ADP increased significantly (\(P < 0.05\)) without any significant (\(P > 0.05\)) changes in \(R_n\) (see Table 2). We presume that the mAHP was eliminated by preventing the rise of intracellular Ca\(^{2+}\) that normally activates \(I_{K(Ca)}\).

Application of apamin in the recording chamber in a droplet (10 µM) also blocked the mAHP (Fig. 4B). In Fig. 4C, apamin (300 nM) enhanced the ADP (Fig. 4C, downward arrow) without a significant change in \(R_n\) (control, 51.0 MΩ; after application of apamin, 47.8 MΩ). The blockade of mAHP by apamin was seen in all of four neurons, which showed a control mAHP. By apamin (300 nM), enhancement of the ADP was seen in 9 neurons without a significant (\(P > 0.05\)) change in \(R_n\) (see Table 2) and evoked an extra spike on the enhanced ADP in 2 neurons of 11 neurons having a control ADP. As in the case using EGTA-filled electrodes, the fAHP was not blocked by apamin (Fig. 4C, upward arrow). Thus \(I_{K(Ca)}\) activation normally limits ADP amplitude.

The pharmacological experiments described in the preceding text indicate that blockade of \(I_{K(Ca)}\) enhances the ADP. We then examined whether the enhanced ADP could be blocked by Ca\(^{2+}\) channel blockade. It is possible that a reduction of the ADP by Ca\(^{2+}\) channel blockade in our first set of experiments was masked by the ADP-enhancing effect of blocking \(I_{K(Ca)}\). In three neurons, we applied Ni\(^{2+}\) or Cd\(^{2+}\) in the perfusate after first eliminating the mAHP by intracellular chelation of Ca\(^{2+}\). In all three neurons examined, the amplitude of the enhanced ADP was not reduced. The addition of 100 µM Ni\(^{2+}\) after intracellular EGTA became effective in activating an extra spike on the ADP (Fig. 4A3). Effects of Ni\(^{2+}\) or Cd\(^{2+}\) on the enhanced ADP by apamin (300 nM) were also examined in five neurons. In each of these neurons, application of Ni\(^{2+}\) or Cd\(^{2+}\) could not reduce the enhanced ADP (see Fig. 4D in the case of application of Ni\(^{2+}\)).

In these experiments, all the agents used to block the influx containing Ca\(^{2+}\) spikes induced by depolarizing current pulses (A, 10 ms; B, 30 ms) in TTX (1 µM)- and TEA (20 mM)-containing solution (trace 1, control; trace 2, after application of Ca\(^{2+}\) channel blockers; trace 3, after washout of blockers). Blockade of Ca\(^{2+}\) spikes by Mn (2 mM)/Ca (2 mM) substitution (A) and Ca-0 solution (B). The resting membrane potentials were −78 mV for A and −70 mV for B. Scales are identical in A and B.

![Figure 2](http://jn.physiology.org/ by 10.220.33.4 on September 8, 2017)
of Ca\(^{2+}\) enhanced the ADP rather than blocking it. It is apparent that the inward currents other than Ca\(^{2+}\) currents generate the ADP. We hypothesized therefore that Na\(^{+}\) currents contribute to the ADP.

**Effects of TTX on the ADP**

We examined the effects of Na\(^{+}\) channel blockade on the ADP by applying TTX in a droplet near the slice (4 neurons) and by using 1 \(\mu\)M TTX-containing solution (other 3 neurons). In the former case, we put 3 \(\mu\)l of 170 \(\mu\)M TTX in the recording chamber at a distance from the slice to see the time course of the changes in the ADP as TTX gradually diffused into the slice and progressively blocked Na\(^{+}\) currents.

After TTX application, the amplitude of the ADP decreased, and the action potential evoked on the ADP disappeared (Fig. 5A2) at a time when the spike height and R\(_{n}\) (see Fig. 5A, 5 and 6; control, 104.0 M\(\Omega\); TTX, 96.0 M\(\Omega\)) did not change significantly. Corresponding records of the time derivatives (Fig. 5A, 3 and 4) of the spikes shown in Fig. 5A, 1 and 2, also did not differ greatly (d\(\theta\)/dt for initial spike: 390.0 mV/ms in Fig. 5A1; 364.3 mV/ms in Fig. 5A2). Such a reduction of the ADP was seen in all five neurons showing a control ADP without a significant change in R\(_{n}\) (see Table 2). An extra spike on the ADP was seen in two neurons, and it also disappeared after TTX application. During these changes, the rate of rise of the action potentials did not change significantly (control: 365.1 \(\pm\) 223.2 mV/ms, TTX: 355.5 \(\pm\) 221.6 mV/ms, n = 7, \(P > 0.05\)). To observe the effects of TTX on the ADP clearly, we examined the effects of TTX on the enhanced ADP due to blockade of Ca\(^{2+}\) influx in five neurons. The ADP enhanced by application of Cd\(^{2+}\) (400 \(\mu\)M, Fig. 5B1) was also reduced by TTX reversibly (see Fig. 5B, 2 and 3) without a significant change in R\(_{n}\) (see Fig. 5B, 4 and 5, 60.0 M\(\Omega\) and 64.0 M\(\Omega\)). In all five neurons showing enhancement of the ADP by the blockade of \(I_{\text{K(Ca)}}\), TTX decreased the ADP significantly (\(P < 0.05\)), but R\(_{n}\) (Table 2) and derivatives of action potentials were not changed significantly (control: 442.4 \(\pm\) 144.6 mV/ms, TTX: 440.4 \(\pm\) 140.9 mV/ms, \(P > 0.05\)).

It is reported that intracellular application of QX314 could abolish the persistent Na\(^{+}\) current (\(I_{\text{Na(p)}}\)) while the rate of rise of spikes reflecting spike Na\(^{+}\) conductance was not completely reduced (Stafstrom et al. 1985). We then examined the effects of intracellular application of QX314 using electrodes filled with 3 M KCl plus 25 mM QX314 on the ADP. Figure 5C1 shows an action potential followed by the ADP recorded just after penetrating a cortical neuron with an electrode containing QX314. In \(\sim\)2 min after a penetration, the ADP was blocked (Fig. 5C2). Such a complete blockade of ADP due to diffusion of QX314 could be observed in all of four neurons examined.

We next examined the effects of blocking Ca\(^{2+}\)-influx, \(I_{\text{K(Ca)}}\) and Na\(^{+}\) channels on the firing patterns evoked by long-lasting current pulses. If the ADP was responsible for a bursting behavior, we would not expect bursting to be abolished by Ca\(^{2+}\) channel blockade. Rather we would expect bursting to be enhanced because the Ca\(^{2+}\) channel blockade...
enhanced the ADP. Furthermore as the ADP was blocked by application of TTX, it is expected that TTX would also eliminate bursting.

**Effects of blockade of Ca²⁺ currents and I_{K(Ca)} on regular-spiking neurons**

The rate of regular spiking evoked by a depolarizing current pulse was increased by blockade of Ca²⁺ influx or I_{K(Ca)} (Co-2 mM/Ca-2 mM substitution, 7 neurons; Mn-2 mM/Ca-2 mM substitution, 5 neurons; Ca-0, 4 neurons; 100 μM Ni²⁺, 4 neurons; 400 μM Cd²⁺, 5 neurons; intracellular diffusion of EGTA, 12 neurons; apamin, 9 neurons; see Table 3). Figure 6A shows an example of the reversible effects of Ni²⁺ on regular spiking and B indicates the increment of firing rate by apamin. Concomitantly, the AHP following the repetitive firing was decreased by these pharmacological agents (Table 3). These changes in the repetitive firing were observed without a significant (P > 0.05) change in R_n (see Table 3). These results indicate that the firing rate in a regular-spiking neuron is restricted by activation of I_{K(Ca)}.

The blockade of Ca²⁺-influx or I_{K(Ca)} switched the regular-spiking pattern into a bursting pattern in ~17.9% (n = 10) of neurons examined. Figure 7A shows the alteration of the regular-spiking pattern into the repetitive bursting without a significant change in R_n (control, 52.6 MΩ; Mn/Ca substitution, 46.5 MΩ) after replacement of Ca²⁺ (2 mM) with Mn²⁺ (2 mM). In Fig. 7B, a regular-spiking pattern reversibly changed to a repetitive bursting without a significant alteration of R_n (Fig. 7B4, 60.0 MΩ; Fig. 7B5, 53.0 MΩ; Fig. 7B6, 60.0 MΩ) by recording in Ca-0 solution. Cd²⁺ (400 μM) and Ni²⁺ (100 μM) also could alter regular-spiking pattern to repetitive bursting. The blockade of I_{K(Ca)} due to intracellular Ca²⁺ chelation by EGTA-filled electrode or application of apamin induced a change of a regular-spiking pattern to a bursting pattern (data not shown). In these experiments using blocking agents for I_{K(Ca)} and Ca²⁺-influx, alteration of the regular-spiking patterns to the bursting patterns was observed without any significant changes (P > 0.05) in R_n (control, 56.5 ± 38.9 MΩ; after blocking Ca²⁺-influx and I_{K(Ca)}, 59.0 ± 26.0 MΩ, n = 10).

We attribute these effects of Ca²⁺ channel blockade to blockade of I_{K(Ca)}. Because the blockade of I_{K(Ca)} resulted in burst firing in some regular-spiking neurons and increased excitability in the rest, we suppose I_{K(Ca)} normally prevents bursting and reduces excitability in regular-spiking neurons. These results are consistent with our finding that Ca²⁺ blockade increases ADP, which we hypothesize to underlie bursting. We next examined the effects of blocking Ca²⁺-influx and/or I_{K(Ca)} on bursting neurons.

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**TABLE 2. Effects of blocking I_{K(Ca)} and Na⁺ currents on ADP**

<table>
<thead>
<tr>
<th>Agent</th>
<th>n</th>
<th>Control, mV</th>
<th>Test, mV</th>
<th>Control, MΩ</th>
<th>Test, MΩ</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA</td>
<td>15</td>
<td>14.6 ± 8.2*</td>
<td>22.4 ± 9.7*</td>
<td>39.4 ± 18.6</td>
<td>38.9 ± 18.7</td>
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<tr>
<td>Apamin</td>
<td>9</td>
<td>13.5 ± 12.3*</td>
<td>20.7 ± 10.6*</td>
<td>43.3 ± 17.9</td>
<td>42.8 ± 17.1</td>
</tr>
<tr>
<td>TTX</td>
<td>5</td>
<td>16.6 ± 9.7*</td>
<td>5.2 ± 4.8*</td>
<td>94.7 ± 39.4</td>
<td>83.3 ± 26.6</td>
</tr>
<tr>
<td>TTX⁴</td>
<td>5</td>
<td>17.1 ± 9.9*</td>
<td>3.9 ± 5.3*</td>
<td>50.1 ± 24.2</td>
<td>50.6 ± 25.0</td>
</tr>
</tbody>
</table>

EGTA, intracellular Ca²⁺ chelation by EGTA; apamin, extracellular application of apamin; TTX, application of TTX; TTX⁴, ADP and R_n before and after TTX-application in Cd²⁺-containing solution (control).

**Effects of the blockade of Ca²⁺ currents and I_{K(Ca)} on the burst**

If Ca²⁺ influx was responsible for bursting, we could expect the bursting pattern to change into a regular-spiking pattern after blockade of Ca²⁺ influx or I_{K(Ca)}, but this was not observed in any of 17 bursting neurons tested. Instead the results were consistent with our finding of an enhanced ADP

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**FIG. 5. Effects of TTX and N-(2,6-dimethylphenylcarbamoylmethyl) triethyl ammonium bromide (QX314) on the ADP.** A: a drop application of TTX (170 μM) decreased the ADP and eliminated an activation of an extraspike on the ADP (A2). Time derivatives of the spikes in A, B, C, and D, are shown in top traces in A5. The ADP was decreased by application of TTX (B2) reversibly (B3). Hyperpolarizing responses against current pulses indicated in top traces in A5 are shown in B, 4–6. C: intracellular application of QX314 (25 mM) blocked the ADP (C2). The resting potentials were ~73 mV (A), ~76 mV (B), and ~70 mV (C). Calibrations are identical in A, 1 and 2, in A, 3 and 4, in A, 5 and 6, in B, 1–5, in B, 4–6, and in C, 1 and 2. Time scale in A2 applies to A, 1–4.
In four burst-and-regular-spike firing neurons without any significant changes in $R_n$ (control, $49.5 \pm 20.5 \text{ M}\Omega$; blocker, $50.8 \pm 20.5 \text{ M}\Omega$; $n = 4$, $P > 0.05$).

In these cases, after returning to the control solution, the burst-and-regular-spike firing pattern recovered. In Fig. 8A, the burst-and-regular-spike firing pattern (Fig. 8A1) changed to the repetitive burst (Fig. 8A2) reversibly by Mn/Ca without a significant change in $R_n$ (Fig. 8A4, 70.2 MΩ; Fig. 8A5, 63.2 MΩ; Fig. 8A6, 63.2 MΩ). In the rest of 13 burst-and-regular-spike firing neurons tested, the bursting pattern was not changed by blocking agents of Ca$^{2+}$ influx or $I_{K(Ca)}$ even though a stable recording was maintained over $\sim 1$–2 h after using these agents.

In all of four repetitive bursting neurons examined, blockade of Ca$^{2+}$ influx or $I_{K(Ca)}$ did not change the bursting pattern even though the firing pattern was examined over $\sim 1$–2 h after an application of the blockers, a time far in excess that needed to block Ca$^{2+}$ spikes by these agents. Figure 8B shows increased intraburst firing and bursting rates after recording in Ca-0 solution (Fig. 8B2), but the bursting pattern did not convert into a regular-spike-firing pattern even in a long-period recording in this solution (90 min). Both of these rates decreased after returning to Ca$^{2+}$-containing solution (Fig. 8B3). These changes occurred without a significant change in $R_n$ (see Fig. 8B, 4–6; control, 44.0 MΩ; Ca-0, 42.9 MΩ; washout, 38.1 MΩ).

The effects of Ca$^{2+}$ influx or $I_{K(Ca)}$ blockade on bursting were consistent with the effects on the ADP. In contrast to Ca$^{2+}$-influx blockade, we found that TTX or QX314 reduced the ADP, and we next examined whether TTX or QX314 blocked the bursting pattern.

**Effects of TTX on the burst**

In two of four bursting neurons tested, TTX application eliminated the burst. Figure 9A shows the change of the repetitive burst to the regular-spike-firing pattern without a significant change in $R_n$ (Fig. 9A3, 104.0 MΩ; Fig. 9A4, 96.0 MΩ). In all of four bursting neurons tested after Cd$^{2+}$ application (2 burst-and-single-spike firing neurons and 2 repetitive bursting neurons) TTX application induced regular-spiking pattern reversibly without a significant change in $R_n$ (Fig. 9B4, 60.0 MΩ; Fig. 9B5, 64.0 MΩ; Fig. 9B6, 62.4 MΩ) as shown in Fig. 9B.

The elimination of burst firing by TTX could be observed without significant ($P > 0.05$) changes in $R_n$ (control, $56.5 \pm 30.5 \text{ M}\Omega$; and TTX, $57.2 \pm 31.0 \text{ M}\Omega$; $n = 6$).

Intracellular application of QX314 (25 mM) also changed the repetitive burst into a regular-spike firing as shown in Fig. 9C. Figure 9C, 3 and 4, shows records, in a faster sweep, of action potentials indicated in Fig. 9C, 1 and 2 (---), respectively. Coincident with a blockade of ADP by TTX or QX314, Na$^+$-channel blockade eliminated the burst firing.

**Discussion**

In this study we obtained the following results: the ADP was enhanced rather than blocked by Ca$^{2+}$-channel blockade in layer III cortical neurons; Ca$^{2+}$ channel blockade or $I_{K(Ca)}$ blockade induced burst firing in regular-spiking layer III neurons; burst firing in layer III neurons was enhanced rather than blocked by Ca$^{2+}$ channel blockade; TTX or QX314, which minimally altered the spike, blocked the ADP and burst firing in layer III neurons. Each of the pharmacological result described in the preceding text was obtained in at least one neuron verified to be a layer III pyramidal neuron by intracellular staining. Thus we suppose our results are representative of cat layer III pyramidal neurons. These results are mainly based on pharmacological experiments employing agents to block Ca$^{2+}$ influx, which change composition and concentrations of extracellular divalent cations. Therefore such procedures induce alterations of resting membrane properties including input resistances probably due to surface potential changes (Hille 1991). We certified that the changes in ADP or firing pattern are not merely due to a change in input resistance by

![Figure 6](https://i.imgur.com/3Q5Q5Q.png)

**FIG. 6**. Effects of blockade of Ca$^{2+}$-influx and $I_{K(Ca)}$ on the regular-spiking patterns. A: Ni$^{2+}$ (100 μM) increased the firing rate induced by 0.33-nA current pulse (A2) reversibly (A3). B: apamin (300 nM) increased the firing rate evoked by a current pulse of 0.4 nA. The resting membrane potentials were -65 mV in both neurons. Calibrations in B2 apply to A and B.
In this study we examined whether the ADP in layer III cortical neurons is related to Ca\(^{2+}\)-currents by using external Ca\(^{2+}\)-free solutions, Cd\(^{2+}\) and Ni\(^{2+}\). In all cases, however, these agents did not block the ADP but rather enhanced ADP amplitude. These results are not likely due to insufficiency of blocking Ca\(^{2+}\) influx by pharmacological agents used in this study because of the following reasons. These agents sufficiently blocked Ca\(^{2+}\) spikes (Fig. 2) and AHP following a spike. Their effects on the ADP and the bursting pattern were examined after a sufficient time for Ca\(^{2+}\) spikes to be blocked had passed (sometimes 2 h). We found that the enhancement of the ADP in the Ca\(^{2+}\)-free solutions was due to the blockade of \(I_{K(Ca)}\) because an application of apamin or intracellular EGTA diffusion, agents that eliminate \(I_{K(Ca)}\) (Hugues et al. 1982; Knrjevic et al. 1978), also enhanced the ADP (Fig. 4, A2 and C2). The enhancement of the ADP after intracellular Ca\(^{2+}\) chelation by EGTA might be due to the removal of Ca\(^{2+}\) dependent inactivation of Ca\(^{2+}\) conductance (Eckert and Tillotson 1981). This is, however, unlikely the case for our results because enhanced ADP by EGTA diffusion could be neither reduced nor blocked by blocking Ca\(^{2+}\) influx (Fig. 4A3).

Thus it is likely that primary effects of the blockade of Ca\(^{2+}\) influx in our preparation was the blocking \(I_{K(Ca)}\), which secondarily enhanced the ADP. That the ADP was not reduced by the Ca\(^{2+}\)-channel blockers might be simply because the AHP (i.e., \(I_{K(Ca)}\)) was more sensitive to reduction of Ca\(^{2+}\) influx, but our results reject this idea. Even after \(I_{K(Ca)}\) was blocked by apamin or by intracellular Ca\(^{2+}\) chelation, Cd\(^{2+}\) or Ni\(^{2+}\) could neither block nor reduce the ADP but rather further increased the consistency in input resistances before and after application of chemical agents. We discuss here the ionic mechanisms underlying the ADP and burst firing in cat sensorimotor cortical neurons.

Many authors have pointed out the importance of the ADP following a spike in the generation of the burst (Azouz et al. 1996; Friedman and Gutnick 1987, 1989; Jensen et al. 1996; Kandel and Spencer 1961; McCormick et al. 1985; Silva-Barrat et al. 1992; Wong and Prince 1978, 1981). In our experiments, the bursting neurons are characterized by activation of an extra spike on the ADP. Neurons with an ADP sufficiently large to reach threshold generate extra spikes on the ADP following a preceding spike, and this causes the burst. AHPs also followed the spike, and the outward current generating the AHP opposes the inward current generating the ADP. We proposed that these neurons having an inward ADP current large enough to overcome the outward AHP current generate a large enough ADP to evoke extra spikes and the burst firing.

In many central neurons, including sensorimotor cortical neurons, the mAHP following a spike is mainly generated by the activation of \(I_{K(Ca)}\) (Lorenzon and Foehring 1992; Schwindt et al. 1988, 1992). The mechanism underlying the ADP is controversial: 1) Ca\(^{2+}\)-dependent mechanisms including the activation of the voltage-dependent Ca\(^{2+}\) channels (Friedman and Gutnick 1987; Kobayashi et al. 1997; Wong and Prince 1978, 1981), Ca\(^{2+}\)-activated cation currents (Haj-Dahmane and Andrade 1997), Ca\(^{2+}\)-dependent Cl\(^{-}\) currents (Higashi et al. 1993), and electrogenic Ca\(^{2+}\) extrusion (Friedman et al. 1992); 2) Na\(^{+}\)-dependent mechanisms (Azouz et al. 1996; Brumberg et al. 2000; Guatetto et al. 1996; Hoehn et al. 1993); and 3) the somatic spread of dendritic excitation (Mainen and Sejnowski 1996).
and influx of Ca\(^{2+}\) in the same voltage range as in the control solution and the same intracellular EGTA (Friedman et al. 1992). The ADP due to electrogenic Ca\(^{2+}\) extrusion generates the ADP because it is reported that this mechanism of somatic spread of dendritic excitation (Mainen and Sejnowski 1996). However, in our study TTX abolished the ADP without a significant change in fast Na\(^{+}\)-spikes amplitude. Thus the ADP is unlikely to be caused solely by the somatic spread of dendritic excitation although we cannot exclude these mechanisms completely.

Considering the pharmacological experiments done in this study, the generation of the burst is unlikely due to influx of Ca\(^{2+}\). The alteration of the burst-and-regular-spike firing pattern into repetitive bursting by Ca\(^{2+}\)-channel blockade (Fig. 8) is likely due to the blockade of \(I_{\text{Na}}\) and the enhancement of the ADP. This idea is supported by the results, which showed a similar alteration of the firing patterns by the blockade of \(I_{\text{Na}}\) with intracellular diffusion of EGTA or amap application. The switch of the regular-spiking pattern into the bursting pattern by Ca\(^{2+}\)-channel blockade and \(I_{\text{Na}}\) blockade shown in Fig. 7 also supports this idea. Such a change of regular spiking into the bursting by blockade of \(I_{\text{Na}}\) or mAHP was reported in several neurons (Azouz et al. 1996; Deisz 1996; Deisz and Prince 1987; De Waele et al. 1993; Schwindt et al. 1988). Thus the main role of Ca\(^{2+}\) influx in neocortical neurons seems to be the suppression or limitation of burst firing by activation of \(I_{\text{Na}}\).

Coincident with the reduction of the ADP, the bursting pattern induced in normal solution or Cd\(^{2+}\)-containing solution could be blocked by low concentration of TTX or intra-cellular injection of QX314 (Fig. 9). Based on these results, we propose that \(I_{\text{Na}}\) plays a major role in generation of the bursting pattern normally. The contribution of \(I_{\text{Na}}\) to the generation of burst firing was reported in the rat neocortical neurons (Franceschetti et al. 1995; Guatteo et al. 1996), the hippocampal pyramidal neurons (Azouz et al. 1996), the medial vestibular neurons (De Waele et al. 1993), and the visual

**FIG. 9**. Effects of TTX and QX314 on the bursting firing. **A1**: the repetitive bursting firing in control. **A2**: the regular-spike firing after TTX application. **A3** and **A4**: hyperpolarizing responses on the currents in **A3**. **B1**: the repetitive burst induced in Cd\(^{2+}\)-containing solution. **B2**: a regular-spike firing induced by application of TTX in the Cd\(^{2+}\)-containing solution. **B3**: the repetitive burst induced after washout of TTX. **B4–6**: voltage responses on currents in **B3**. **C1**: repetitive burst recorded in control solution. **C2**: regular-spike firing recorded after intracellular application of QX314 (25 mM). **C3** and **C4**: action potentials of ADP following the last spike in each burst are shown in a faster sweep. The resting potentials are ~73 mV (A), ~76 mV (B) and ~70 mV (C). Voltage calibrations are identical in A, 1 and 2 and B, 1–3, in A, 3 and 4, in B, 4–6 and in C, 1–4. Time scales are identical in A, 1 and 2, in A, 3 and 4, in B, 1–3, in B, 4–6, in C, 1 and 2 and in C, 3 and 4.

the amplitude of ADP enough to evoke extra spikes (Fig. 4, A3 and D3).

Neither Ca\(^{2+}\)-activated cation currents nor Ca\(^{2+}\)-dependent Cl\(^{-}\) currents seem to play an important role in generation of the ADP in cat layer III cortical neurons because these Ca\(^{2+}\)-dependent currents would have been reduced or blocked by Ca\(^{2+}\) channel blockade or by EGTA injection. Neither do our results with EGTA support the possibility that electrogenic Ca\(^{2+}\) extrusion generates the ADP because it is reported that the ADP due to electrogenic Ca\(^{2+}\) extrusion was sensitive to intracellular EGTA (Friedman et al. 1992).

In contrast to the ineffectiveness of Ca\(^{2+}\)-channel blockers at layer III cortical pyramidal neurons, application of TTX or injection of QX314 decreased or blocked the ADP (see Fig. 5) at a time when the amplitude of the action potential was not decreased. The fact that the ADP was blocked without a significant change in spike height is particularly important. This means that voltage-gated Ca\(^{2+}\) channels (or any other voltage-gated channel activities during the spike) experienced the same voltage range as in the control solution and the same influx of Ca\(^{2+}\) occurred. The slower rise time of the spike in TTX may have allowed slightly more Ca\(^{2+}\) channels to have been activated than in control.

The blockade of the ADP by TTX was also reported in the rat cortical pyramidal neurons (Guatteo et al. 1996), the hippocampal CA1 pyramidal neurons (Azouz et al. 1996), and the ferret visual cortical neurons (Brumberg et al. 2000). Layer V cortical neurons have two functionally different Na\(^{+}\) currents, the transient Na\(^{+}\) current (\(I_{\text{NaT}}\)) underlying the spike upstroke and a persistent Na\(^{+}\) current (\(I_{\text{NaP}}\)) that is activated below spike threshold (Brown et al. 1994). Either or both currents could generate the ADP: \(I_{\text{NaP}}\) because it is activated at the membrane potential of the ADP and does not inactivate or \(I_{\text{NaT}}\) due to rapid recovery from partial inactivation at the membrane potential of ADP. Because the membrane potential of the ADP during the firing (~34.1 ± 11.4 mV) in burst neurons was more depolarized than needed for complete inactivation of \(I_{\text{NaP}}\) in rat neocortical neurons (Huguenard al. 1988), it is more likely that \(I_{\text{NaP}}\) generates the ADP, and although the membrane potential of the ADP following the last spike in each burst (~34 mV) was more depolarized than the threshold of a spike evoked from resting potential (~55 mV). The ability of the ADP to reach this membrane potential without triggering another spike is likely due to inactivation of \(I_{\text{NaT}}\). \(I_{\text{NaT}}\) inactivation would be favored by the initiation of the high-rate preceding spikes with their progressively shallower AHPs and the slow rise of the membrane potential envelope during the burst.

A computer simulation study has indicated that dendritic fast Na\(^{+}\) channels can cause the generation of an ADP by the mechanism of somatic spread of dendritic excitation (Mainen and Sejnowski 1996). However, in our study TTX abolished the ADP without a significant change in fast Na\(^{+}\)-spikes amplitude. Thus the ADP is unlikely to be caused solely by the somatic spread of dendritic excitation although we cannot exclude these mechanisms completely.

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cortical neurons (Montoro et al. 1988). Recent studies by Brumberg et al. revealed the dependence of ADP and bursts on Na\(^+\) currents but not Ca\(^{2+}\) currents (Brumberg et al. 2000). And they also indicated that neither blocking of Ca\(^{2+}\) influx nor the intracellular chelation of free Ca\(^{2+}\) inhibited the generation of bursts, but that pharmacologically blocking Na\(^+\) currents with TTX or QX314 inhibited the burst (Brumberg et al. 2000). The present study also supports these results in cat layer III cortical neurons and especially demonstrates that the mechanisms postulated by Brumberg et al. (2000) are also true in the cat sensorimotor layer III neurons. They also reported that incidence of bursts is higher in in vitro preparation bathed in extracellular solution with a physiological concentration of Ca\(^{2+}\) (1 mM) to compare with that in the conventionally used extracellular solution ([Ca\(_o\)] = 2 mM) (Brumberg et al. 2000). The present study is also consistent with it as the incidence of bursts was increased in Ca-0 solution in our experiments.

As the bursting was blocked by TTX in this study, the TTX-insensitive Na\(^+\) current that was reported to generate burst firing in guinea pig neocortical neurons (Deizs 1996) is unlikely to be related to burst generation in cat layer III cortical neurons. Although the present results do not allow us to completely exclude the contribution of the other mechanisms except an activation of I\(_{NaP}\), we conclude that the activation of I\(_{NaP}\) may be one of the major contributors to the generation of the ADP and the burst.

In cat layer III pyramidal neurons, depolarizing inputs activate both I\(_{NaT}\) and I\(_{NaP}\). The depolarization induced by these currents initiate the Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels, which in turn activate I\(_{CaL}\). In the neurons in which I\(_{NaP}\) surpasses I\(_{CaL}\), the large ADP develops and the burst is induced. During intraburst firing the further influx of Ca\(^{2+}\) induces an enhancement of AHP leading to produce the larger AHP and terminate the burst. On the other hand, mechanisms to cease the burst are complicated in the burst firing induced by I\(_{CaL}\) blockade. Several mechanisms are available to contribute to repolarize the burst after blockade of I\(_{CaL}\): voltage-gated I\(_{K}\) (e.g., M current), apamin insensitive I\(_{CaL}\) (McCormick and Pape 1990), electrogenic Na/K pump (Angstadt and Friesen 1991), Na\(^+\)-dependent K\(^+\) current (Schwindt et al. 1989). Further investigation is needed to reveal the mechanisms of burst termination after I\(_{CaL}\) blockade.

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