L-Type Ca\(^{2+}\) Channel Blockers Attenuate Electrical Changes and Ca\(^{2+}\) Rise Induced by Oxygen/Glucose Deprivation in Cortical Neurons

Antonio Pisani, MD; Paolo Calabresi, MD; Alessandro Tozzi; Vincenza D’Angelo; Giorgio Bernardi, MD

**Background and Purpose**—Experimental evidence supports a major role of increased intracellular calcium [Ca\(^{2+}\)] in the induction of neuronal damage during cerebral ischemia. However, the source of Ca\(^{2+}\) rise has not been fully elucidated.

To clarify further the role and the origin of Ca\(^{2+}\) in cerebral ischemia, we have studied the effects of various pharmacological agents in an in vitro model of oxygen (O\(_2\))/glucose deprivation.

**Methods**—Pyramidal cortical neurons were intracellularly recorded from a slice preparation. Electrophysiological recordings and microfluorometric measurements of [Ca\(^{2+}\)] were performed simultaneously in slices perfused with a glucose-free physiological medium equilibrated with a 95% N\(_2\)/5% CO\(_2\) gas mixture.

**Results**—Eight to twelve minutes of O\(_2\)/glucose deprivation induced an initial membrane hyperpolarization, followed by a delayed, large but reversible membrane depolarization. The depolarization phase was accompanied by a transient increase in [Ca\(^{2+}\)] levels. When O\(_2\)/glucose deprivation exceeded 13 to 15 minutes, both membrane depolarization and [Ca\(^{2+}\)] rise became irreversible. The dihydropyridines nifedipine and nimodipine significantly reduced either the membrane depolarization or the [Ca\(^{2+}\)] accumulation. In contrast, tetrodotoxin had no effect on either of these parameters. Likewise, antagonists of ionotropic and group I and II metabotropic glutamate receptors failed to reduce the depolarization of the cell membrane and the [Ca\(^{2+}\)] accumulation. Finally, dantrolene, blocker of intracellular Ca\(^{2+}\) release, did not reduce both electrical and [Ca\(^{2+}\)] changes caused by O\(_2\)/glucose depletion.

**Conclusions**—This work supports a role of L-type Ca\(^{2+}\) channels both in the electrical and ionic changes occurring during the early phases of O\(_2\)/glucose deprivation. (Stroke. 1998;29:196-202.)

Key Words: calcium channels ■ electrophysiology ■ fura 2 ■ glucose ■ ischemia ■ oxygen

Ischemic stroke remains a common cause of severe neurological disability. In the recent past, an intensive effort has been directed at clarifying the pathophysiological mechanisms underlying neuronal degeneration during ischemic injury. Several studies have shown that the acute changes in neuronal electrical properties induced by either anoxia, aglycemia, or a combination of both, are extremely variable, depending on the brain area analyzed.\(^1\) For example, nigral dopaminergic and CA1 and CA3 hippocampal neurons hyperpolarize in response to anoxia,\(^2,3\) whereas brain stem and striatal neurons depolarize.\(^4,5\) During anoxia, cortical neurons exhibit an early hyperpolarization followed by a delayed membrane depolarization.\(^6,7\) The differential responses observed during energy deprivation are thought to underlie the diverse vulnerability of neuronal subtypes to energy metabolism failure.\(^1\) Compelling evidence led to the hypothesis that calcium [Ca\(^{2+}\)] overload is involved in neuronal injury occurring in cerebral hypoxia or ischemia.\(^8-10\) Mechanisms responsible for the elevation of [Ca\(^{2+}\)], during ischemia include opening of transmitter- or voltage-gated channels, mobilization from intracellular pools, inhibition of ion pumps, imbalance of sodium/Ca\(^{2+}\) exchanger.\(^11\) However, despite these advances in the understanding of the ischemia-related events, the origin of Ca\(^{2+}\) influx has not been defined precisely.

Cortical neurons are among the most vulnerable cell subtypes in the brain if exposed to energy deprivation.\(^8\) This susceptibility is believed to derive from a high metabolic demand and poor energy storage capability.\(^15\) Electrophysiological studies performed on rat and human neocortical slices demonstrated that neocortical cells maintain membrane potential for relatively long periods of oxygen or glucose deprivation alone.\(^6,7\) However, when these neurons were deprived of both oxygen and glucose, membrane potential changes were much faster, suggesting that anaerobic glycolysis has a crucial importance in the maintenance of ionic gradients across the cell membrane in these cells.\(^6\) By means of a combined approach of

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electrophysiological recordings and microfluorometric measurements of Ca\(^{2+}\) concentrations, we have recently shown that in cortical neurons the time-course of the anoxia-induced membrane potential changes closely resembles the data by Jiang and Haddad.\(^6\) Additionally, we have reported that [Ca\(^{2+}\)]i elevations occur simultaneously with electrical changes.\(^1\)

Therefore, in an attempt to analyze further the source of [Ca\(^{2+}\)]i, increase during O\(_2\)/glucose deprivation, we examined simultaneously both the electrophysiological alterations and the [Ca\(^{2+}\)]i changes, with the Ca\(^{2+}\)-sensitive dye fura 2, in a cortical slice preparation. We then tested the efficacy of various pharmacological agents in counteracting these changes and found that membrane depolarization triggers an increase in [Ca\(^{2+}\)]i, which is mainly dependent on the opening of voltage-gated L-type Ca\(^{2+}\) channels.

Materials and Methods

Seventy-two male Wistar rats (age, postnatal day 18 to 24) were used for the experiments. Preparation and maintenance of the slices have been described\(^5\) previously and followed the institutional guidelines. Briefly, animals were killed under ether anesthesia by cervical dislocation, the brain was removed, and coronal slices (200–µm thick), containing cortex and striatum, were cut with a vibratome. Slices were gassed with a 95% N\(_2\)/5% CO\(_2\) gas mixture. Glucose was replaced with a 2 mol/L KCl solution. After cell impalement, cells were loaded to 4%, Sigma), and 100 mmol/L KCl, while the shank was backfilled with both fura 2 and biocytin by injecting, through the recording electrode, a solution of 2 mmol/L fura 2 (Molecular Probes, The Netherlands), biocytin (2 to 4%, Sigma), and 100 mmol/L KCl, while the shank was backfilled with a 2 mol/L KCl solution. After cell impalement, cells were loaded with both fura 2 and biocytin by injecting, through the recording electrode, 0.1 to 0.5 nA negative current for 10 to 15 minutes. This loading procedure interfered neither with electrical nor with optical recordings. After the experiment, each biocytin-stained slice was fixed in paraformaldehyde (in 0.1 mmol/L phosphate buffer at pH 7.4) overnight and then processed according to standardized protocols.\(^14\)

An Axoclamp 2A amplifier (Axon Instruments) was used for electrophysiology. Traces were displayed on an oscilloscope and stored on a digital system. The recording chamber was mounted on the stage of an upright microscope (Axiostar FS, Zeiss), equipped with a 60X water immersion objective (Olympus). Epi-illumination was provided by a 75-W Xenon lamp. Excitation light passed through a shutter and was filtered at 340 and 380 nm. Emission light was filtered by a long-pass barrier filter (500 nm to 530 nm) and detected by a CCD camera (Photonic Science, UK). Images were stored and analyzed off-line (IonVision, ImproVision, UK). The background fluorescence was measured in a part of the slice with no fura 2–filled neurons and subtracted from the signal to obtain the basal fluorescence level (F). Changes in [Ca\(^{2+}\)]i are expressed in terms of ∆F/F, where ∆F is the normalized change in fluorescence and F is the background-subtracted basal fluorescence. The ∆F/F value can be interpreted as changes in [Ca\(^{2+}\)]i.\(^15,17\) Changes in mitochondrial NADH levels during energy deprivation are known to interfere with fura 2 signals.\(^25\) Therefore, some undyed slices were exposed to O\(_2\)/glucose deprivation, and the resulting raw fluorescence was detected, averaged, and subtracted from the control signal obtained in dyed slices. In most of the experiments, the intensity of excitation light was small; thus the fluorescence signals resulting from photo-bleaching of the dye were negligible. Values in the text and in the table are expressed as mean±SEM. Student’s t test was used to compare the means. The experiments performed to check the possible effect of the pharmacological agents (nimodipine, D-APV, CNQX, MCPG, BAY K 8644, and dantrolene) on both the membrane potential changes and the elevation of the [Ca\(^{2+}\)]i of the recorded cells, were first statistically analyzed using an ANOVA test. The result from this test has indicated a large and significant difference between groups (P<.001) so that we proceeded with the Student’s t test to compare the means of each group referred, one by one, to the control.

Results

Electrophysiological and Morphological Features of Cortical Cells

Data were obtained from electrophysiologically and visually identified cortical pyramidal neurons. Pyramidal cortical neurons showed a resting membrane potential of −73±6 mV. As previously described\(^15\) during depolarizing current pulses, two types of pyramidal cells were observed: “regular-spiking” and “bursting” neurons. In both of these types of pyramidal neurons, spikes were followed by a pronounced afterhyperpolarization. As described above (see Materials and Methods section) electrodes were filled with both fura 2 and biocytin. This loading procedure allowed an immediate visualization of the recorded cell (Fig 1A), due to fluorescence emission of the Ca\(^{2+}\)-sensitive dye fura 2. In addition, subsequent processing of the loaded slices permitted morphological analysis. Pyramidal (IV layer) cells had a large soma (28 to 33 µm) and an extensive dendritic tree densely studded with spines (Fig 1B and 1C).

Effects of O\(_2\)/Glucose Deprivation on Cortical Pyramidal Neurons

Perfusion with an ischemic solution for short periods induced small membrane potential changes in cortical neurons. In fact, 2 to 6 minutes of O\(_2\)/glucose deprivation caused either no change or a small, not significant hyperpolarization of the recorded neurons (−5±3 mV, P>0.1, n=118). Likewise, these changes in membrane potential were not coupled with significantly relevant [Ca\(^{2+}\)]i changes (98±0.7 expressed as percentage of control, in terms of ∆F/F, see Materials and Methods section; P>0.1, n=118). Longer periods of omission of O\(_2\) and glucose (8 to 12 min) caused an early membrane hyperpolarization followed by a delayed, large depolarization of the cell membrane (48±12 mV, P<0.005, n=96). This membrane depolarization was associated with a transient, delayed increase in [Ca\(^{2+}\)]i (54±9%, P<0.001). Fig 2 shows that an 8-minute perfusion with a solution lacking both O\(_2\) and glucose, produced a late, significant increase in [Ca\(^{2+}\)]i (Fig 2A), which was fully reversible on washout. Simultaneously, a delayed, large membrane depolarization occurred (Fig 2B),
followed, after the washout, by a pronounced afterhyperpedalization. When O$_2$/glucose depletion exceeded 13 to 15 minutes, both the electrical changes and the increase in [Ca$^{2+}$], were irreversible (n=18, not shown).

**Role of L-Type Ca$^{2+}$ Channels in In Vitro Ischemia**

To address the possible involvement of L-type voltage-dependent Ca$^{2+}$ channels in the early events that follow the onset of O$_2$/glucose deprivation, we tested the effects of nifedipine, a dihydropyridine-derivative, on the electrical and [Ca$^{2+}$]i changes caused in this experimental condition. The incubation with nifedipine (10 to 15 min), before starting perfusion with the O$_2$/glucose-deprived medium, did not affect the intrinsic membrane properties of the recorded cells (resting membrane potential, input resistance, action potential discharge, not shown). Fig 3 shows that in the presence of a saturating concentration of nifedipine (20 μmol/L), both the [Ca$^{2+}$], rise and the membrane depolarization caused by 10 minutes of O$_2$/glucose deprivation (Fig 3A), were significantly reduced (Fig 3B, 17±3% of control, P<.001 and 21.7±1.7%, P<.003, respectively, n=18). After washout of the drug, the membrane response and [Ca$^{2+}$], rise induced by O$_2$/glucose depletion returned to the control values (Fig 3C). Both the changes in membrane potential and [Ca$^{2+}$], were reduced by nifedipine in a dose-dependent manner (Fig 4). The EC$_{50}$ value for the effect on membrane potential changes was 1.826 μmol/L, whereas the EC$_{50}$ for the inhibition of [Ca$^{2+}$], increase was 3.288 μmol/L.

In addition, we tested the action of nimodipine on this in vitro model of ischemia. A maximal concentration of nimo-
erate the effects of O2/glucose deprivation, but it significantly increased the peak amplitude and delayed the recovery time of 


Figure 4. Dose-response curve of the effects of nifedipine on both membrane potential and [Ca2+] during exposure to O2/glucose deprivation. Each point represents the average of at least four single experiments (± SEM). Normalized data points were fitted according to the formula \( y = m_0 \left( \frac{m_0}{m_0 + m_1} \right) \), where \( m_0 \) defines the EC50 values: the EC50 value for the effect on membrane potential changes (ΔVm) was 1.826 μmol/L, whereas the EC50 for the inhibition of [Ca2+] increase (ΔF/F) was 3.286 μmol/L.

dipine (20 μmol/L) significantly reduced both the [Ca2+] elevation (Fig 5, 18±2% of control, \( P<.003, n=7 \)), and the concurrent depolarization of the cell membrane (Fig 5, 22±3.1%, \( P<.001 \)). Finally, we tested the effect of BAY K 8644 (3 μmol/L), an agonist of L-type Ca2+ channels. BAY K 8644, per se, produced significant changes neither in the passive properties of the recorded cells, nor in the resting [Ca2+]. Interestingly, 3 μmol/L BAY K 8644 did not accelerate the effects of O2/glucose deprivation, but it significantly increased the peak amplitude and delayed the recovery time of both the membrane depolarization and of [Ca2+], rise (Fig 5; 124±4%, \( P<.001 \), and 140±5.3%, \( P<.005 \), respectively, \( n=4 \)).

Effects of Blockade of Synaptic Transmission, Glutamate Receptor Activation and Intracellular Ca2+ Release

The blockade of synaptic transmission by 1 μmol/L TTX did not modify the alterations caused by O2/glucose depletion on cortical neurons. Fig 5 shows that neither the electrical changes (96±5%, \( P<.001 \)) nor the elevation in [Ca2+], (97±5.2%, \( P<.005 \)) were affected by TTX. To analyze the possible involvement of glutamate receptors in the induction of membrane potential changes and [Ca2+], increase caused by in vitro ischemia, we preincubated the slices in a solution containing either ionotropic or metabotropic (group I and II) glutamate receptor antagonists. Fig 5 shows that in the presence of 50 μmol/L D-APV, an antagonist of NMDA receptors, plus 10 μmol/L CNQX, an antagonist of AMPA receptors, plus 500 μmol/L MCPG, an antagonist of both group I and II metabotropic glutamate receptors, the membrane potential changes and [Ca2+] rise induced by O2/glucose deprivation were unaffected (98.9±2.1, \( P>.001 \), and 98.6±1.9, \( P<.003 \), respectively, \( n=7 \)). We finally addressed the possible role of intracellular Ca2+ stores by using dantrolene, a drug known to inhibit the intracellular release of Ca2+ by interfering with the Ca2+-induced Ca2+ release.21 Dantrolene (10–30 μmol/L) failed to significantly affect both membrane potential changes and [Ca2+], rise induced by O2/glucose depletion (Fig 5, 96.9±2.1, \( P<.001 \) and 97.9±1.9, \( P<.005 \), \( n=6 \)).

Discussion

Four main findings emerge from our current study: (1) cortical pyramidal neurons respond to combined O2/glucose deprivation with an early hyperpolarization followed by a large, delayed depolarization of the cell membrane potential; (2) a simultaneous increase in [Ca2+] occurs only in the latter phase of these electrical changes; (3) both the membrane potential changes and the elevation in [Ca2+] could be strongly attenuated by L-type Ca2+ channel blockers, and enhanced by BAY K 8644, an agonist of L-type channels. (4) Conversely, glutamate receptor antagonists, TTX or dantrolene, failed to prevent the O2/glucose deprivation-induced electrical and ionic alterations. Taken together, these data suggest that during the early phases of ischemia a major part of the acute changes of both membrane potential and [Ca2+] in cortical neurons is likely due to a net Ca2+ influx through voltage-gated L-type Ca2+ channels.

Both in vivo and in vitro experimental evidence supports the postulated role of increase in cytosolic Ca2+ in anoxic/ischemic brain damage.8–10 There is a partial agreement that most of [Ca2+], increase derives from the extracellular space.22–25 Microfluorimetric measurements of [Ca2+], in cerebellar and hippocampal slices showed that large [Ca2+], elevations induced in vitro ischemia were mostly due to Ca2+ influx from the extracellular fluid.26 However, to date, the mode of entry of Ca2+ still represents a matter of debate. Several studies showed that the antagonism of both NMDA and non-NMDA glutamate receptors attenuates Ca2+ overload in the course of energy deprivation.23,27,28 However, the efficacy of glutamate-
Oxygen/Glucose Deprivation and L-Type Calcium Channels

vic antagonists in models of focal cerebral ischemia is still controversial. In our experimental condition, the contribution of either the ionotropic or metabotropic (group I and II) glutamate receptor to the membrane potential and \([\text{Ca}^{2+}]_i\), changes appears negligible. It is noteworthy that, in most of the slice models of in vitro ischemia, glutamate receptor antagonism did not exert protective effects. In striatal and brain stem neurons, intracellularly recorded from slice preparations, the anoxia-induced membrane depolarization was not prevented by combined antagonism of NMDA and non-NMDA glutamate receptors. In a recent study, in fura 2–loaded cortical slices exposed to ischemia, the combined antagonism of NMDA and AMPA receptors slowed the rate of \([\text{Ca}^{2+}]_i\) accumulation, but not the peak concentration of \([\text{Ca}^{2+}]_i\). In dissociated hippocampal neurons, Friedman and Haddad failed to prevent the anoxia-induced \([\text{Ca}^{2+}]_i\), rise either by combined antagonism of NMDA and AMPA glutamate receptors or by TTX. Moreover, comparing the effects of glutamate- and anoxia-induced increase in \([\text{Ca}^{2+}]_i\), they found that neurons exhibited a completely different time-course in \([\text{Ca}^{2+}]_i\) accumulation, suggesting that the mechanisms underlying these two events are different. These results were obtained from dissociated neurons, a preparation with no synaptic inputs. It is possible that some of the reasons for the discrepancy between the slice data and those obtained in cultures and “in vivo,” can be attributed to the different experimental model used. Alternatively, it is possible that in our slice experiments we have evaluated only the “acute” effects of ischemia, whereas excitatory amino acids might play a major role in the “delayed neuronal death.” Our data prove that even in a slice preparation, where synaptic inputs, neurotransmitter actions and glial functions are partially preserved, the blockade of either synaptic transmission or of glutamate receptors does not prevent the temporal profile of both membrane potential and \([\text{Ca}^{2+}]_i\), changes caused by ischemia. The substantial attenuation of the modifications of both membrane potential and \([\text{Ca}^{2+}]_i\), in the presence of nifedipine and nimodipine suggests that during \(\text{O}_2/\text{glucose depletion, the raise in [Ca}^{2+}]_i\), is dependent, to a large extent, on \([\text{Ca}^{2+}]_i\) entry through L-type \([\text{Ca}^{2+}]_i\) channels. Moreover, the inability of dantrolene to prevent electrical and ionic changes induced by \(\text{O}_2/\text{glucose deprivation suggests that the release of Ca}^{2+}\) from intracellular stores does not seem to play a major role in the early events of energy depletion. These findings appear to be in accordance with fluorescence imaging studies obtained from dissociated hippocampal CA1 neurons, in which, during anoxia, most of the \([\text{Ca}^{2+}]_i\) influx occurs through cobalt-sensitive \([\text{Ca}^{2+}]_i\) channels. In hippocampal slices, Lobner and Lipton found significant attenuation of \(^{45}\text{Ca}^{2+}\) uptake during the second 2.5 minutes of ischemia in the presence of nifedipine. Interestingly, it has been shown that cardiac arrest is associated with an elevation in the binding of a L-type \([\text{Ca}^{2+}]_i\) channel specific ligand in cortical synaptosomes, suggesting that, during ischemia, the increase in the number of L-type channels in the cell membrane may allow an increased \([\text{Ca}^{2+}]_i\) entry. Yet, in vivo models of both focal and complete cerebral ischemia, dihydropyridines have been shown to ameliorate ischemic \([\text{Ca}^{2+}]_i\) accumulation. However, a major problem in interpreting data from in vivo studies is identifying the site at which drugs act. In fact, these in vivo studies cannot distinguish between a relaxant effect on blood vessels, thus increasing blood flow, and an action at neuronal level. Our experimental model is represented by a slice preparation that lacks blood vessels. Therefore, the present results support a neuronal effect of L-type \([\text{Ca}^{2+}]_i\) channel blockers.

Our experiments show that a minor portion of membrane potential changes (15 ± 2.1%) and \([\text{Ca}^{2+}]_i\), rise (16 ± 3.9%) observed during \(\text{O}_2/\text{glucose omission was not blocked by dihydropyridine, suggesting the presence of an additional mechanism involved in the pathophysiological changes occurring in course of ischemia. We are currently investigating the possible mechanisms subtending these minor changes occurring in cortical cells in conditions of energy depletion.

Clinical Implications

Over the past years the involvement of intracellular \([\text{Ca}^{2+}]_i\) in the ischemic cell death has become evident. This observation led to the hypothesis that the inhibition of \([\text{Ca}^{2+}]_i\) influx might prove useful in the treatment of acute stroke. Various antagonists of voltage-dependent \([\text{Ca}^{2+}]_i\) channels were investigated in a variety of stroke models with different degrees of efficacy. The overall results of several double-blind, randomized trials with nimodipine did not give the expected results. However, a recent meta-analysis of the nimodipine trials demonstrated that previous analyses had methodological flaws, the major of which was that patients were randomized up to 48 hours after stroke. In the meta-analysis it emerged that patients who had nimodipine within 12 hours after acute stroke had a beneficial outcome, supporting the idea that an early therapeutic intervention with L-type channel blockers might be useful. Another group of drugs recently developed, aimed at counteracting \([\text{Ca}^{2+}]_i\) overload, was represented by glutamate receptor antagonists, in particular both competitive and noncompetitive NMDA antagonists. Unfortunately, most recently, clinical trials performed with a competitive NMDA antagonist were stopped, because it increased brain-related death rate.

Our experimental model precludes the possibility to investigate the involvement of blood vessels. Indeed, thrombolytic agents are currently under evaluation in clinical trials; this approach seems to be effective in the clinical outcome of acute ischemic stroke. Understanding the pathophysiology of the different stages of ischemic stroke is of crucial importance to establish the exact criteria of the so-called therapeutic window and, consequently, for drug development.

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References


A large body of evidence indicates that intracellular calcium accumulation is a critical step in the chain of events leading to ischemic neuronal death.\(^1\),\(^2\) However, the mechanisms of the increase in intracellular calcium and the calcium accumulation and anoxia-induced changes in membrane potentials have not been clearly defined. In the accompanying article, Pisani et al have used calcium-sensitive dyes and intracellular recordings in brain slices subjected to oxygen-glucose deprivation to provide additional insights into the mechanisms of calcium accumulation. By monitoring both intracellular calcium and membrane potential in morphologically identified neurons, they were able to study the effect of pharmacological agents that block selected calcium entry pathways during oxygen-glucose deprivation. The results suggest that the majority of calcium enters neurons through voltage-gated L-type channels toward the late stage of membrane depolarization. Interestingly, the contribution of glutamate receptors and intracellular calcium pool was found to be negligible. The data reaffirm the importance of voltage-gated L-type calcium channels in the mechanisms of calcium influx in neurons after energy failure.

This elegant study uses state-of-the-art approaches to address a critical issue in the neurobiology of neuronal death secondary to energy deprivation. The results are of interest, as they provide new data in support of the importance of voltage-gated calcium channels in the mechanism of cerebral ischemia. In addition, the findings reemphasize that calcium channel blockers may be a viable therapeutic modality for the treatment of stroke. Of interest is the observation that in this preparation, glutamate receptor antagonists did not block calcium entry. This finding leads to the prediction that glutamate receptor antagonists are not effective in preventing the deleterious effects of intracellular calcium accumulation during energy failure. However, this conclusion is not supported by the abundant literature demonstrating a protective effect of glutamate receptor antagonists in focal cerebral ischemia and in neuronal cultures subjected to oxygen-glucose deprivation.\(^3\),\(^4\) While this discrepancy underscores the importance of the experimental model used when studying the consequences of cerebral ischemia, it also indicates that our understanding of the role of calcium and glutamate receptors in ischemic brain injury is far from complete. Future experiments focusing on the ionic changes elicited by activation of glutamate receptors would contribute to further elucidate the relation between glutamate and calcium influx in this model.

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