Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette

(rat cerebellum/repetitive stimulation/spreading depression/anoxia/potassium)

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ABSTRACT Changes in extracellular Ca\(^{2+}\) concentration were directly measured in the rat cerebellum, using an ion-selective micropipette. Extracellular K\(^+\) was measured simultaneously with a second ion-selective micropipette. The potential reference barrels of the ion electrodes also provided fast field and slow potentials. During repetitive stimulation of the parallel fiber–Purkinje cell cerebellar circuit, extracellular Ca\(^{2+}\) fell to about 80% of baseline concentration. During the spreading depression of Leão, extracellular Ca\(^{2+}\) fell to about 10% of baseline; decreases of this magnitude also occurred during terminal anoxia. In all cases extracellular K\(^+\) increased substantially. These results show that extracellular Ca\(^{2+}\) is modulated during neuronal activity in the central nervous system and that under some conditions the Ca\(^{2+}\) change can be extreme. Given the well-established and antagonistic effects of reduced extracellular Ca\(^{2+}\) on axonal excitability and synaptic transmission, these results suggest that Ca\(^{2+}\) modulation in the brain cell microenvironment may be a significant parameter in the behavior of neuronal ensembles.

Changes in extracellular calcium ([Ca\(^{2+}\)]\(_o\)) have two established effects on neuronal elements. A decrease in [Ca\(^{2+}\)]\(_o\) increases axonal excitability (1), through influence on membrane properties. Decreased [Ca\(^{2+}\)]\(_o\), however, diminishes chemically mediated synaptic transmission (2). In this case a reduction in [Ca\(^{2+}\)]\(_o\) leads to a decreased influx in Ca\(^{2+}\) across the membrane of the presynaptic terminal (3) and consequent diminution in transmitter release due to deprivation of essential Ca\(^{2+}\) in the transmitter release mechanism (2). In increases in [Ca\(^{2+}\)]\(_o\), reverse the effects described in axon and synapse.

The above results have been established on single cell preparations, predominantly the giant axon and synapse of the squid and the neuromuscular junction. The findings suggest that, were changes in [Ca\(^{2+}\)]\(_o\), to occur in the central nervous system, they could profoundly affect the operation of neuronal ensembles through the antagonistic influence of changes in [Ca\(^{2+}\)]\(_o\) on axons and synapses. Other possibilities for the influence of Ca\(^{2+}\) on neuronal ensembles have also been discussed (4). Thus, one may speculate that dynamic modulation of [Ca\(^{2+}\)]\(_o\), may be as important in the extracellular microenvironment as the recently established intrinsic variations in [K\(^+\)]\(_o\), which have led to the discussion of the role of [K\(^+\)]\(_o\) in presynaptic inhibition (5), epileptic seizures (6), and the spreading depression of Leão (7–10). Both Ca\(^{2+}\) and K\(^+\) have the capacity to influence neuronal ensembles by virtue of their low concentrations, relative to the major extracellular ions, Na\(^+\) and Cl\(^-\). The low concentration of the minority ions means that small absolute changes produce large concentration shifts.

Our experiments were designed to look for transient, intrinsic changes in [Ca\(^{2+}\)]\(_o\) within the extracellular microenvironment of the central nervous system. Such observations have previously been impossible owing to lack of techniques with adequate temporal and spatial resolution, combined with discrimination against other ionic species. The recent development by Oehme et al. (11) of a fast-responding, ion-selective micropipette (ISM) based on a neutral carrier ion exchanger, highly selective for Ca\(^{2+}\) (12), has enabled us to directly and accurately monitor transient changes in [Ca\(^{2+}\)]\(_o\).

The experiments were made on the cerebellum of the rat, because the neuronal circuitry is known in detail (13) and the electrical responses of the mammalian cerebellum are probably better characterized than those of any other brain region (14). This permits defined neuronal circuits to be activated and any ion concentration changes to be related to this activity. In addition, the rat cerebellum has the unusual property of being relatively susceptible (15) to the spreading depression of Leão (16, 17). Since large shifts in [Na\(^+\)]\(_o\), (18), [K\(^+\)]\(_o\), (7–10), and [Cl\(^-\)]\(_o\), (9) are now known to be associated with spreading depression, this phenomenon provides a useful model to seek possible [Ca\(^{2+}\)]\(_o\) variations.

Our measurements demonstrate that modulation of [Ca\(^{2+}\)]\(_o\), indeed occur during neuronal activity, spreading depression, and terminal anoxia. Furthermore, we show that [Ca\(^{2+}\)]\(_o\), consistently falls during these events, in contrast to the K\(^+\)\(_o\), which rises. These results suggest that modulation of [Ca\(^{2+}\)]\(_o\), within neuronal ensembles of the brain may be important.

MATERIALS AND METHODS

In addition to the Ca\(^{2+}\)-ISM, a K\(^+\)-ISM was used in these experiments in order to compare the two ions directly. Both ISMs were fabricated as double-barreled micropipettes, one side containing a liquid ion exchanger and the other, reference barrel, a solution of 150 mM NaCl (19, 20). Ca\(^{2+}\)-ISMs contained the new neutral carrier (11, 12) ion exchanger and were backfilled with 100 mM CaCl\(_2\). K\(^+\)-ISMs contained Corning 477317 ion exchanger and were backfilled with 100 mM KCl. The electrical outputs of the ISMs were processed to yield ion and reference signals (9) (Fig. 1). Ion and slow potential signals were photographed from an oscilloscope. The tip diameters of the ISMs were 2–3 μm. A Ca\(^{2+}\)- and a K\(^+\)-ISM were glued together with rapid epoxy so that the inter-tip spacing was 50 μm or less.

Ca\(^{2+}\)-ISMs were calibrated in CaCl\(_2\) solutions and K\(^+\)-ISMs in KC\(_2\) solutions. All calibrating solutions contained 150 mM NaCl to simulate the ionic strength of the extracellular microenvironment and to allow for Na\(^+\) interference in the K\(^+\)-ISM. The selectivity of the Ca\(^{2+}\)-ISM for Ca\(^{2+}\) over K\(^+\) and Na\(^+\) is better than 600:1 and 1000:1, respectively (11). Thus, no

Abbreviations: [X]\(_o\), extracellular concentration of X; ISM, ion-selective micropipette.

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Fig. 1. Ion-selective micropipette (ISM) recording system and basic neuronal circuitry. Double-barreled micropipettes contain ion exchangers for Ca\textsuperscript{2+} or K\textsuperscript{+} in the tips. ISMs are glued together with inter-tip spacing of 50 \mu m or less. Output potentials are buffered with ultra-high impedance buffer amplifiers (1×) and the reference signal is subtracted from the ion-exchanger barrel signal to yield the true ion signal. To evoke ionic changes, the cerebellar surface was stimulated with a local surface electrode (Loc), which excited a beam of parallel fibers (axons of granule cells) and synaptically activated Purkinje cell dendrites.

correction is necessary for changes in interference due to shifts in the monovalent ions when using the Ca\textsuperscript{2+}-ISM.

Albino rats were anesthetized with thiopental (70 mg/kg) or ketamine (90 mg/kg), and the posterior cerebellum was exposed and superfused with warm Ringer's solution. The cerebellum was activated with a local surface bipolar electrode (14) and recordings were made just beneath the pial surface (Fig. 1).

RESULTS
The unstimulated base line values for [Ca\textsuperscript{2+}]\textsubscript{0} in the rat cerebellum were typically 1.0–1.2 mM; occasionally they would be as low as 0.6 mM. [K\textsuperscript{+}]\textsubscript{0} base line values were typically about 3.0 mM, as seen in other mammalian brain regions (6–8); values occasionally were higher but did not exceed 5.0 mM.

Typical cerebellar field potentials were evoked when the surface was stimulated with the local electrode and recordings were made lateral to the stimulating position, i.e., within the beam of excited parallel fibers (Fig. 1) (14). Recordings were made about 50 \mu m underneath the pial surface and a fast negative wave, corresponding to the presynaptic parallel fiber volley (14), was detected on the reference barrels of the two ISMs (Fig. 2, inserts). This fast component was followed by a slower, negative wave predominantly representing the postsynaptic activation of the Purkinje cell dendrites (14). Single stimuli did not produce measurable changes in [Ca\textsuperscript{2+}]

\textsubscript{0} and rarely produced visible K\textsuperscript{+}-signals. When the cerebellum was stimulated at 5 Hz or more, detectable ion changes were usually seen. Fig. 2 shows ion changes evoked with 20 Hz stimulation. A fall in [Ca\textsuperscript{2+}]

\textsubscript{0} is clearly seen, accompanied by a rise in [K\textsuperscript{+}]

\textsubscript{0}. The [Ca\textsuperscript{2+}]

\textsubscript{0} change was only detectable in the vicinity of maximum negative field potentials, while the [K\textsuperscript{+}]

\textsubscript{0} increases had a greater spatial extent. This differential distribution of Ca\textsuperscript{2+} and K\textsuperscript{+} has also been seen in the cat cerebellum under similar conditions (unpublished observations) and in the presence of aminopyridine (21). The relative localization of the [Ca\textsuperscript{2+}]

\textsubscript{0} change may be related to its association with synaptic transmission.

A typical maximum decrease in [Ca\textsuperscript{2+}]

\textsubscript{0} did not exceed one quarter of the resting level; under similar conditions [K\textsuperscript{+}]

\textsubscript{0} rose to between two and three times the resting level and demonstrated an undershoot on stimulus cessation (22). Small undershoots in [Ca\textsuperscript{2+}]

\textsubscript{0} were seen in some experiments. Negative-going slow potential shifts were measured on the reference barrels of the ISMs during stimulation (Fig. 2).

The slow rising and falling phases of the ion signals indicate that each stimulus evokes a small ion shift which lasts for 100 ms or more. Repetitive stimulation allows the summation of these signals to the point where they are detectable. Thus, small, but consistent, falls in [Ca\textsuperscript{2+}]

\textsubscript{0} are evoked by activation of the elementary cerebellar neuronal circuit. Much larger ion changes were seen, however, during spreading depression.

Spreading depression was induced by a brief burst of strong local stimulation. It showed (Fig. 3) the well-known characteristics of this phenomenon: a large, slow, negative wave (17), depression of evoked activity (16, 17), and a large rise in [K\textsuperscript{+}]

\textsubscript{0} (7–10). In addition, we can now report a dramatic fall in [Ca\textsuperscript{2+}]

\textsubscript{0} by almost a factor of 10 in concentration. In 18 spreading depressions measured in 10 rats, the peak negative potential shift, measured on the reference barrel of the ISM, was 29 ± 4 mV (mean ± SD), the [K\textsuperscript{+}]

\textsubscript{0} change was 0.8 ± 0.2 log\textsubscript{10} units, and the [Ca\textsuperscript{2+}]

\textsubscript{0} change was −0.7 ± 0.2 log\textsubscript{10} units.

These findings led us to inquire what would be the maximum attainable ionic shift in the extracellular microenvironment.

An indication of this was provided by inducing circulatory arrest and terminal anoxia by administering an overdose of barbiturate. The resulting ion changes were of an order of magnitude comparable to those occurring during spreading depression, although final magnitudes were higher (Fig. 4). This confirms the often-noted similarity between anoxia and spreading depression (17). Similar ion shifts were seen in
anesthetized animals in which terminal anoxia was induced by injection of d-tubocurarine to paralyze the lungs. The onset of the ion change was more gradual in the latter case, owing to the longer duration of blood circulation.

None of our experiments showed significant changes in [Ca\textsuperscript{2+}]\textsubscript{0} prior to a spreading depression. Allowing for the response times of the ISMs and electrode separation, we estimate that we could detect time differences of one second or more. During circulatory arrest, detectable [K\textsuperscript{+}]\textsubscript{0} changes preceded [Ca\textsuperscript{2+}]\textsubscript{0} changes (Fig. 4).

**DISCUSSION**

Our experiments yield several new findings. We have shown that it is now possible to detect the modulation of [Ca\textsuperscript{2+}] in the extracellular microenvironment, using the new Ca\textsuperscript{2+}-selective micropipette. In all our experiments the dominant feature was that [Ca\textsuperscript{2+}] fell during perturbation of the resting state of the neuronal ensemble. The [Ca\textsuperscript{2+}] changes associated with normal neuronal activity are obviously quite small, although it is likely that the actual transients adjacent to cellular membranes in the extracellular microenvironment, undisturbed by the electrode, are more pronounced than those measured. The decrease in [Ca\textsuperscript{2+}]\textsubscript{0} may result from entry of Ca\textsuperscript{2+} into presynaptic terminals (2, 3) or could be associated with Ca\textsuperscript{2+} currents in axons (23), somata (24, 25), or dendrites (26, 27). The inward Ca\textsuperscript{2+} current may be accompanied by an outward K\textsuperscript{+} current (24, 25), which might account for at least some of the associated [K\textsuperscript{+}] increase detected in these experiments. It is also possible that some Ca\textsuperscript{2+} becomes membrane bound during stimulation (28) and is consequently no longer registered by the ISM.

Our results reveal a net decrease in [Ca\textsuperscript{2+}]\textsubscript{0} during repetitive stimulation; earlier experiments by Adey and coworkers (4, 29) showed an increase in [45Ca\textsuperscript{2+}]\textsubscript{0} during repetitive stimulation of cerebral cortex that had been pre-loaded with the radiotracer. These two sets of results are not incompatible. One may assume that during stimulation Ca\textsuperscript{2+} moves from extracellular to intracellular compartments, due to membrane permeability changes that allow the ion to move passively down its electrochemical gradient. The arrival of the Ca\textsuperscript{2+} inside the cell will stimulate enhanced active Ca\textsuperscript{2+} extrusion (25) from the intracellular pool. One can assume, in the experiments of Adey et al., that at the time of stimulation the greater part of the [45Ca\textsuperscript{2+}] had been taken up by cells. It then follows that an increase in [45Ca\textsuperscript{2+}] would be measured, even though our experiments indicate that there would actually be a net decrease in [Ca\textsuperscript{2+}]\textsubscript{0} because passive influx exceeds active extrusion. The fact that small overshoots in [Ca\textsuperscript{2+}] are sometimes resolved following stimulus cessation in our experiments supports the idea that active extrusion mechanisms for [Ca\textsuperscript{2+}] do exist in the cerebellum.

The decrease in [Ca\textsuperscript{2+}]\textsubscript{0} during spreading depression is surprisingly large. These changes in [Ca\textsuperscript{2+}]\textsubscript{0} together with the recently demonstrated decreases in [Na\textsuperscript{+}]\textsubscript{0} (18) and [Cl\textsuperscript{-}]\textsubscript{0} (9) and the established increase in [K\textsuperscript{+}]\textsubscript{0} (7–10), indicate that the hypothesis of Grafiest (30) explaining spreading depression in terms of [K\textsuperscript{+}] increases alone, or that of Van Harreveld (31) giving primary emphasis to glutamate-mediated Na\textsuperscript{+} movement, now must be elaborated to account for other ion change. We propose that the most parsimonious description of the ion flows during spreading depression is that all extracellular ions move towards their intracellular concentrations. This suggests a transient, nonspecific increase in membrane permeability of a large population of cellular elements, which may be neurons, glia, or both. The mechanism controlling such a permeability change remains to be discovered.

Two other facts emerge concerning spreading depression. First, although application of a high concentration of Ca\textsuperscript{2+} to

**FIG. 3.** [Ca\textsuperscript{2+}]\textsubscript{0}, [K\textsuperscript{+}]\textsubscript{0}, and potential changes during spreading depression. Spreading depression was evoked by strong stimulation at 20 Hz for 2 s (arrow). Field potentials were tested every 5 s prior to and during spreading depression. The upper record shows the characteristic large [K\textsuperscript{+}] increase. The second record shows the large [Ca\textsuperscript{2+}] decrease. The third record shows superimposed slow potential shifts recorded from the reference barrels of ISM; the lower trace corresponds to the K\textsuperscript{+}-ISM reference and relates to the right-hand calibration. The lowest record shows field potentials evoked at times indicated and recorded on the Ca\textsuperscript{2+}-ISM reference barrel. Note loss of all evoked responses during spreading depression and accentuation of potentials during recovery phase, probably due to increased tissue impedance. ISM tip separation was 25 μm.

**FIG. 4.** Ion changes and slow potentials during terminal anoxia. Anoxia follows circulatory arrest induced by overdose of pentobarbital (arrow). Note large size of ion changes and that [K\textsuperscript{+}] begins to rise before [Ca\textsuperscript{2+}] begins to fall. The potential signals from the two reference barrels are superimposed. The lower signal is from the Ca\textsuperscript{2+}-ISM reference barrel and relates to the right-hand potential calibration. ISM tip separation was 50 μm.
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This ensembles. however, spectro communication. malfunction that the homeostatic changes in [K\(^+\)]\(_0\) rises to 120 mV depolarization. This work providing this work. This work was supported by Deutsche Forschungsgemeinschaft Grant Br 242/12, the Alexander von Humboldt Foundation, and U.S. Public Health Service Research Grant NS-13740-01 from the National Institute of Neurological and Communicative Disorders.

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