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Zinc modulation of a transient potassium current and histochemical localization of the metal in neurons of the suprachiasmatic nucleus

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ABSTRACT **The effect of Zn^{2+} on a voltage-dependent, transient potassium current (I_A) in acutely dissociated neurons from the suprachiasmatic nucleus was studied with the whole-cell patch-clamp technique. At micromolar concentrations, Zn^{2+} markedly potentiated I_A activated from a holding potential of −60 mV, which is the resting potential of these neurons. This potentiation occurred at a Zn^{2+} concentration as low as 2 μM and increased with higher Zn^{2+} concentrations. The Zn^{2+} action appears to arise from a shift in the steady-state inactivation of I_A to more positive voltages. At 30 μM, Zn^{2+} shifted the half-inactivation voltage by +20 mV (from −80 mV to −60 mV), and 200 μM Zn^{2+} shifted this voltage by +45 mV (from −80 mV to −35 mV). Histochemically, we have also observed Zn^{2+} staining throughout the suprachiasmatic nucleus; the staining is particularly intense in the ventrolateral region of the nucleus, which receives the major fiber inputs. Our findings suggest that Zn^{2+}, presumably synaptically released, may modulate the electrical activity of suprachiasmatic nucleus neurons through I_A. Because vesicular Zn^{2+} is fairly widespread in the central nervous system, it is conceivable that this kind of Zn^{2+} modulation on I_A, and possibly on other voltage-activated currents, exists elsewhere in the brain.**

The transition metal Zn^{2+} is widely distributed in the central nervous system (1). There are two major pools. Enzymatic Zn^{2+}, constituting as much as 85–95% of the total, is bound tightly to many Zn^{2+}-containing enzymes (see, for example, ref. 2) and is involved in maintaining protein conformation as well as participating in catalytic function. Vesicular Zn^{2+}, constituting the remainder, is sequestered in presynaptic vesicles of Zn^{2+}-containing neurons. This latter pool is histochemically reactive and can be selectively stained by procedures such as the selenium-silver method (3, 4). Two functional roles of vesicular Zn^{2+} have been postulated (for review, see ref. 1): stabilizing the primary secretory substances in the synaptic vesicle and, after release, acting as a neuromodulator on pre- or postsynaptic targets. Indeed, at micromolar concentrations, Zn^{2+} has been observed to interact with excitatory and inhibitory amino acid receptors (5, 6) in the hippocampus, where Zn^{2+} has also been histochemically localized to synapses (1, 7, 8) and found to be released upon synaptic activation (9, 10). These actions are thought to have a physiological function in this brain region (11).

Even though Zn^{2+} has also been reported to influence the gating kinetics of neuronal voltage-activated ion channels (12–15), the effects so far observed require millimolar or higher Zn^{2+} concentrations and are, therefore, unlikely to be physiologically relevant. Here we report, however, that Zn^{2+} even at micromolar concentrations potentiates a voltage-dependent, transient potassium current (I_A) in acutely dissociated neurons from the rat suprachiasmatic nucleus (SCN). We have also observed Zn^{2+} staining in the SCN using the selenium-silver method, suggesting that this modulation might operate in the SCN under physiological conditions.

MATERIALS AND METHODS

Preparation of Dissociated SCN Neurons. Adult rats (Sprague–Dawley) were sacrificed by decapitation, and the brain was removed from the skull. Coronal slices (500–1000 μm) were sectioned with a mechanical tissue chopper. The SCN, identified as a pair of dark-colored, ellipsoidal nuclei lateral to the third ventricle, was dissected out from a hypothalamic slice and incubated in minimal essential medium (MEM, GIBCO)/0.25% trypsin (Sigma, type XIII) for 60–90 min. The nuclei were then washed in MEM and dissociated into individual neurons by trituration with a fire-polished Pasteur pipette, and the cells were plated onto coverslips coated with Con-A (Sigma). The neurons usually attached onto the glass within 20 min and were maintained in MEM medium at room temperature. During enzyme digestion and storage 95% O_2/5% CO_2 was continuously blown over the culture medium.

Whole-Cell Recording. Whole-cell, voltage-clamp recordings (16) were made with patch electrodes containing 110 mM KCl, 10 mM K-Hepes, 10 mM K_2EGTA, 1 mM $CaCl_2$, pH 7.3. The recordings were low-pass-filtered at 2.5 kHz (8-pole). During recordings, the perfusing bath solution contained 110 mM choline chloride, 30 mM tetraethylammonium chloride, 5 mM KCl, 3 mM $MgCl_2$, 10 mM tetramethylammonium-Hepes, 10 mM glucose, pH 7.4. The liquid-junction potential was ≈7 mV and was not corrected. In some experiments, 2 mM Ca^{2+} and 1 mM Mg^{2+}, instead of 3 mM Mg^{2+}, were used. $ZnCl_2$ was added to this base solution as necessary, except at 200 μM and 1000 μM, where equimolar $ZnCl_2$ was substituted for $MgCl_2$. The Zn^{2+}-containing solution was admitted by means of a 1-mm-diameter capillary positioned within a millimeter from the recorded cell. Solution change around the cell was complete within a few seconds. All experiments were done at room temperature (20–22°C).

Histochemical Localization of Zn^{2+}. The selenium-silver method (3, 4) was used for staining Zn^{2+} histochemically. Briefly, adult rats weighing 240–380 g were injected i.p. with 0.5% sodium selenite (Na_2SeO_3) solution to a dose of 20 mg Na_2SeO_3 per kg of body weight and then were sacrificed by decapitation 1 hr later. The brains were fixed in phosphate buffer/3% (vol/vol) glutaraldehyde for 30 min and then were quickly embedded in Tissue-Tek (Miles) and frozen. Coronal sections (20 μm) were cut and thawed onto slides precoated with 0.1% gelatin and then stored at −80°C until the stain-development procedure was carried out. The developer solution consisted of a mixture of 25% (wt/vol) gum arabic (30 ml), citrate buffer (5 ml), hydroquinone (7.5 ml), and silver lactate (7.5 ml). Stain development occurred in the dark and at room temperature for 2–3 hr and was stopped by thoroughly washing the slides with distilled water. The sections

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Abbreviations: I_A, voltage-dependent, transient potassium current; SCN, suprachiasmatic nucleus.

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were preserved and coverslipped in 50% (vol/vol) glycerol in phosphate buffer solution.

Two control experiments were done: (*i*) A group of rats received no injection to show that the developing procedure did not, on its own, produce false staining. (*ii*) A group of rats was first given an i.p. injection of 10% diethyldithiocarbamate at a dose of 1000 mg of diethyldithiocarbamate per kg of body weight and, after 1 hr, received the injection of sodium selenite as described above. This control was to demonstrate the specificity of selenium-silver staining for diethyldithiocarbamate-chelatable transition metals (3).

RESULTS AND DISCUSSION

The SCN, which is the primary circadian oscillator in the mammalian brain (17), was dissected out from a rat hypothalamic slice and enzymatically dissociated into individual cells. The recorded cells were randomly selected, but the Zn^{2+} modulation reported here was observed in every cell. Fig. 1 *a–f* shows the membrane current recorded from a dissociated SCN neuron and activated by a voltage pulse to different depolarized levels from a holding potential of −60 mV, which is the resting potential of the SCN neurons (18–21). Sodium and calcium currents were eliminated by substituting choline for Na^+ and substituting Mg^{2+} for Ca^{2+}. In some experiments, 2 mM Ca^{2+} and 1 mM Mg^{2+}, instead of 3 mM Mg^{2+}, were used, but the results (data not shown) were the same as described below. Tetraethylammonium (30 mM) was also present externally to remove most of the delayed rectifier potassium current. In the absence of Zn^{2+}, the voltage pulse activated only a small, mostly sustained outward current (lower traces in each panel, taken before application and after removal of Zn^{2+}, respectively). The sustained component probably represented the residual delayed rectifier current because at higher tetraethylammonium concentrations it became smaller, although not completely absent. With 50 μM Zn^{2+}, the transient component of the outward current became much more prominent (Fig. 1 *a–f*, upper trace), whereas the sustained component was unchanged (see, for example, Fig. 1*g*). The transient current activated within a few milliseconds after the start of the voltage pulse and inactivated with a time constant of ≈20 msec at voltages between +10 and +60 mV. It was obviously not blocked by the 30 mM tetraethylammonium present externally, but 5 mM external 4-aminopyridine did completely inhibit it (data not shown). This current is similar to the so-called I_A current in terms of its kinetics and pharmacology (refs. 22 and 23; for review, see ref. 24). The effect of Zn^{2+} was rapid and reversible, as the current completely recovered after Zn^{2+} washout. Fig. 1*h* shows the current–voltage relation of the transient current before, during, and after Zn^{2+}. The presence of 50 μM Zn^{2+} increased the current to ≈3-fold in this experiment. In other experiments, however, the increase was considerably larger because the I_A was virtually nonexistent in the absence of Zn^{2+} (see, for example, Fig. 2).

This I_A in SCN neurons is very sensitive to Zn^{2+}. As shown in Fig. 2*a*, the potentiating effect of this ion occurs at a concentration as low as 2 μM, and it increases with higher Zn^{2+} concentrations. At 200 μM Zn^{2+} or higher, however, the potentiation begins to decrease. This decrease appears to result from the emergence of another Zn^{2+} effect at higher concentrations—namely, a slowing of the activation rate of the current (Fig. 2 *b* and *c*). This slowing has also been observed in other neuronal voltage-activated ion channels at millimolar or higher Zn^{2+} concentrations (refs. 12–14; but see ref. 25 for skeletal muscle). The physiological significance of this latter effect is unclear because of the relatively high Zn^{2+}

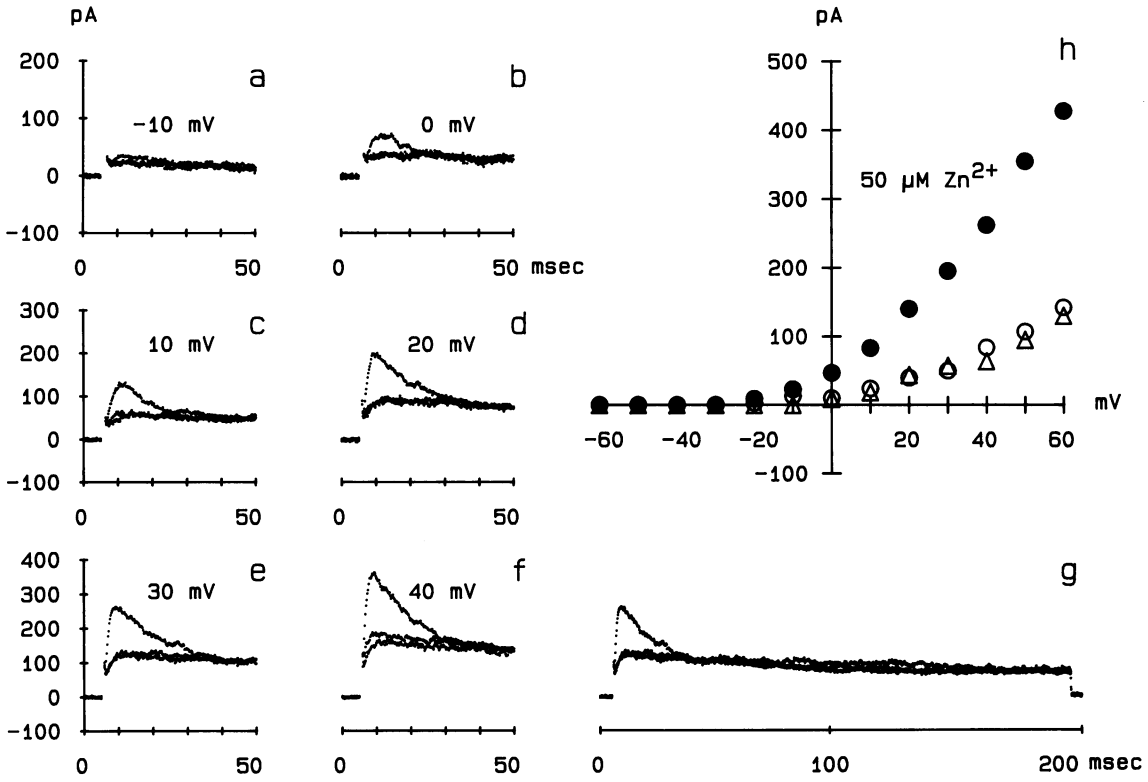

FIG. 1. I_A current potentiated by Zn^{2+} in a dissociated SCN neuron. (*a–f*) Outward potassium currents activated by a voltage pulse from a holding potential of −60 mV to different depolarized levels, as indicated. Lower traces, before and after Zn^{2+}; upper trace, average from two current traces with 50 μM Zn^{2+} in bath solution. (*g*) Same traces as in *e* but on a longer time base to show the sustained component more clearly. (*h*) Current–voltage relation of I_A before (○), during (●), and after (△) Zn^{2+}. Current amplitudes were determined by subtracting the late sustained current amplitude from the transient peak current amplitude. Sodium and calcium currents were eliminated by substituting choline for Na^+ and substituting Mg^{2+} for Ca^{2+}.

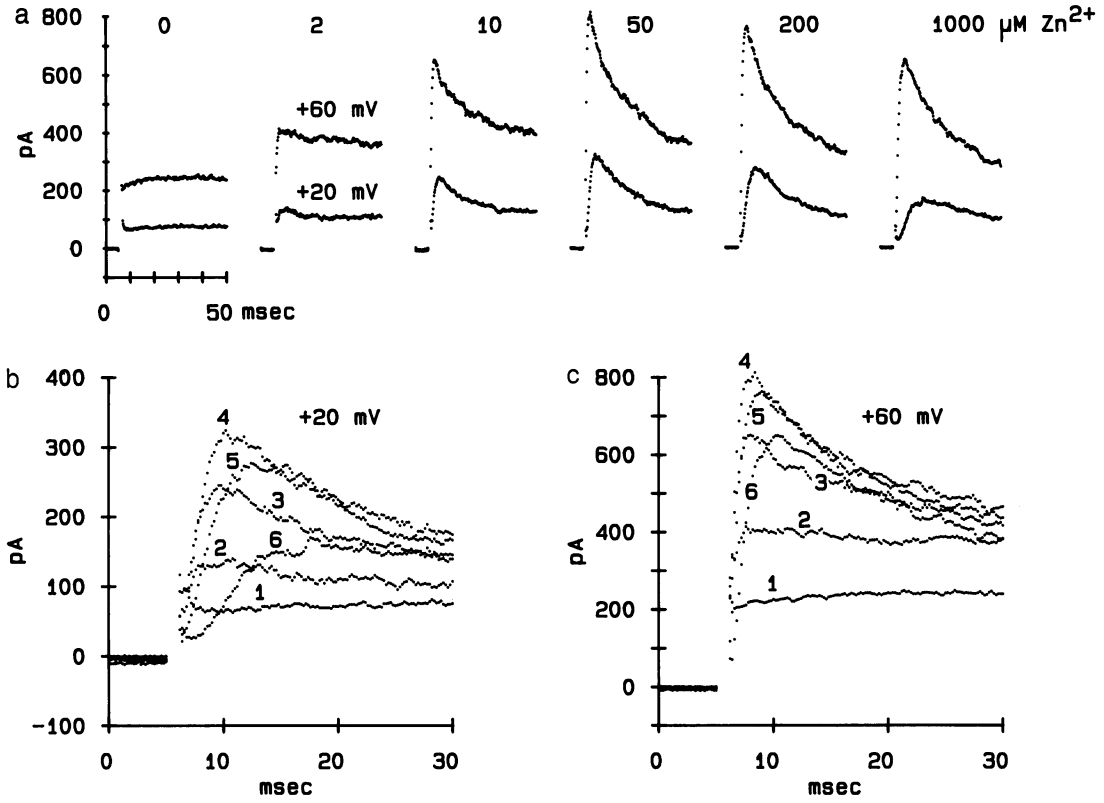

FIG. 2. Potentiation of I_A at different Zn^{2+} concentrations. (*a*) Outward current evoked by a depolarizing pulse from −60 mV to +20 mV (bottom traces) or +60 mV (upper traces) at different Zn^{2+} concentrations, as indicated above traces. Otherwise the conditions are the same as for Fig. 1. Note that the current amplitude started to decrease at Zn^{2+} concentrations >200 μM. (*b*) Traces from *a* at +20 mV superimposed. (*c*) Traces from *a* at +60 mV superimposed. In both *b* and *c*, traces 1–6 correspond to Zn^{2+} concentrations of 0, 2, 10, 50, 200, and 1000 μM, respectively. Note that the activation rate of the current decreased with increased Zn^{2+} concentrations. Similar results were obtained from two other cells.

concentration required to produce it. The inactivation rate of the current, on the other hand, was roughly unchanged even at 1 mM Zn^{2+}. As for potentiation of I_A at low Zn^{2+} concentrations, this effect appears to result from a shift in the steady-state inactivation of the current to more positive voltages. Fig. 3 shows the steady-state inactivation relation for this current at several Zn^{2+} concentrations (see Fig. 3 legend for experimental protocol; data were averaged from several experiments). In the absence of Zn^{2+} (filled circles), the relation can be fitted with the Boltzmann curve, $I/I_{max} = 1/\{1 + \exp[(V - V_{1/2})/V_h]\}$, with $V_{1/2} = -80$ mV and $V_h = 5.5$ mV (where V_h is a slope constant and $V_{1/2}$ is half-inactivation voltage). At Zn^{2+} concentrations of 30 μM (open circles) and 200 μM (filled triangles), the $V_{1/2}$ value became shifted by 20 mV and 45 mV, respectively, to more positive voltages. The V_h value might also change slightly due to Zn^{2+}; for simplicity, however, the curve-fits in the figure were all made with a V_h value of 5.5 mV. The shift in steady-state inactivation induced by Zn^{2+} was near-saturating at 200 μM because increasing the concentration to 1 mM had little additional effect (open triangles). We also examined the activation curve of this current at the same concentrations of Zn^{2+}. At 200 μM Zn^{2+}, for example, the foot of this curve shifted positively by ≈20 mV, but over most of its range no consistent change was evident (data not shown).

The effect of Zn^{2+} on steady-state inactivation can also be reproduced by Cd^{2+}, another transition IIB metal, although 3-fold higher concentrations are required. Other divalent cations such as Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} had little or no observable effect at up to 200 μM concentration. Ca^{2+} at up to a physiological concentration of 2 mM also had no significant effect. These observations suggest specific binding sites on the channel protein for transition IIB metals in mediating this shift. Experiments from pH titrations and chemical modifications of specific residues further suggest that the target for Zn^{2+} may be one or more histidine residues on the channel protein (R.-C.H. and K.-W.Y., unpublished work).

We have used the selenium-silver histochemical method (3, 4) to look for chelatable Zn^{2+} in the rat SCN. Indeed, the presence of Zn^{2+} was confirmed, being particularly intense in the ventrolateral region of the nucleus, which receives the major fiber inputs (17, 26) (Fig. 4*A*). Under high magnification, the staining was often found to form punctate spots surrounding neuronal cell bodies instead of being inside them, suggesting Zn^{2+} localization in presynaptic terminals. This hypothesis is consistent with the vesicular localization of chelatable Zn^{2+} elsewhere in the brain (1). The Zn^{2+} staining appears specific because no labeling was seen in control rats that did not receive sodium selenite injection (n = 2; Fig. 4*B*) or that received selenite injection only after preinjection of the Zn^{2+}-chelating agent diethyldithiocarbamate (n = 2; Fig. 4*C*). It has been suggested that Zn^{2+} is generally associated with excitatory synapses (1, 8), as is so for the glutamatergic (27) mossy fiber terminals in the hippocampus. Although at least one of the input fiber pathways (the retino-hypothalamic pathway) to the SCN is thought to be glutamatergic (17), at present we have no information on the neurotransmitter nature of the presumptive Zn^{2+}-containing fibers in the SCN.

SCN neurons have a resting membrane potential of ≈−60 mV (18–21). At this potential or more positive levels, the I_A is largely inactivated in the absence of extracellular Zn^{2+}. At 30 μM Zn^{2+} removes about half of this inactivation at this voltage, whereas 200 μM completely removes it. Even though the local extracellular Zn^{2+} concentration on activation of the putative Zn^{2+}-containing synapses in the SCN is

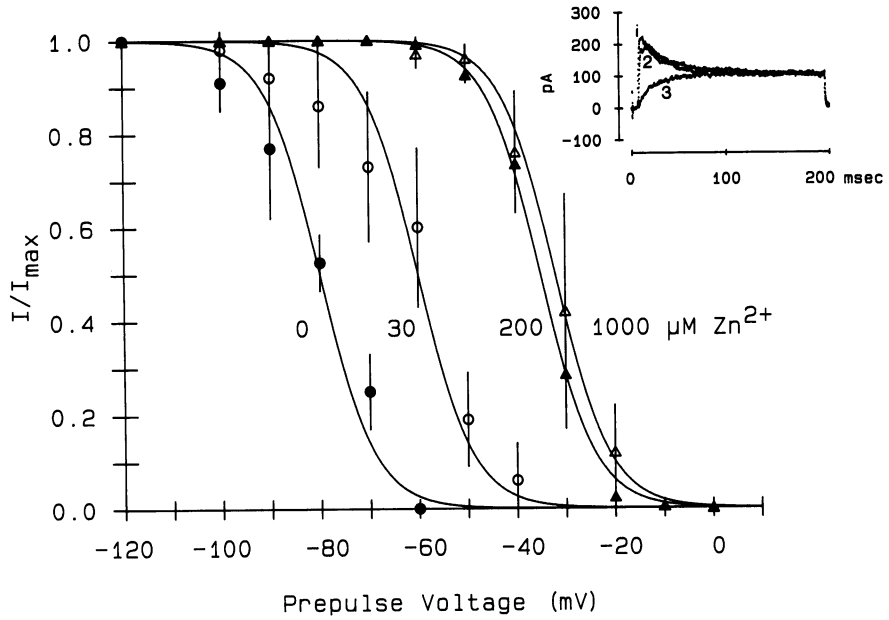

FIG. 3. Shift of the steady-state inactivation of I_A by Zn^{2+} to more positive voltages in a dose-dependent manner. The steady-state inactivation relation was obtained with a three-pulse protocol as follows. A conditioning pulse (prepulse) "stepped" the potential from −60 mV to a value between −120 mV and +10 mV for 2 sec, followed by a second pulse that returned the membrane potential to −60 mV for 5 msec. A third (test) pulse stepped to a fixed voltage (+30 mV or +60 mV) was then applied to activate the I_A and assay how much of this current had been inactivated by the prepulse. Degree of inactivation was indicated by test-current amplitude divided by its maximum value (I/I_{max}). Data points represent averages from several experiments (see below); vertical bars indicate SDs. The curves are drawn according to the Boltzmann curve (see text). In the absence of Zn^{2+} (●; n = 8), half-activation potential ($V_{1/2}$) and slope constant (V_h) are −80 mV and 5.5 mV, respectively. For Zn^{2+} concentrations of 30 μM (○; n = 4), 200 μM (▲; n = 4), and 1000 μM (△; n = 5), the $V_{1/2}$ values are −60 mV, −35 mV, and −32 mV, respectively; V_h stays at 5.5 mV in all cases (see text). (*Inset*) Illustration, as an example, of the similarity of the I_A activated at +30 mV from a holding potential of −100 mV without Zn^{2+} (trace 1) to that from a holding potential of −60 mV with 80 μM Zn^{2+} (trace 2). Note that the activation rate of I_A is slightly slowed by Zn^{2+}. I_A is virtually absent when activated from a holding potential of −60 mV without Zn^{2+} (trace 3).

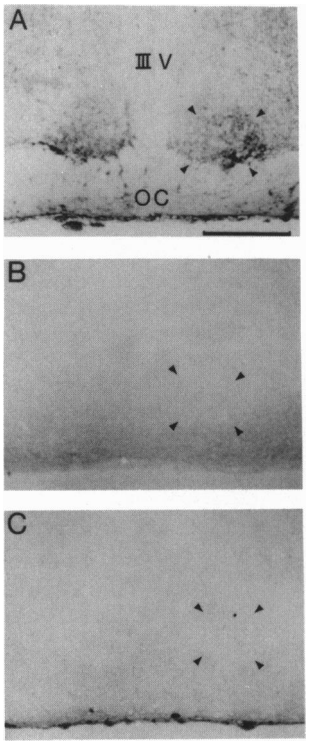

FIG. 4. Histochemical localization of Zn^{2+} in rat SCN. (*A*) Zn^{2+} staining (dark regions) by the selenium-silver method in a coronal brain section containing the SCN. The SCN is outlined by arrowheads. The animal was injected i.p. with sodium selenite. (*B*) Control animal with no selenite injection. (*C*) Control animal with selenite injection but after preinjection of the Zn^{2+}-chelating agent diethyldithiocarbamate. OC, optic chiasm; III V, third ventricle. (Bar = 0.5 mm.)

unknown, this value has been estimated in the hippocampus (9) to possibly reach 300 μM with high-potassium stimulation. Even levels considerably lower than this, however, would still produce the I_A modulation we are studying. Thus, conceivably this modulation operates in physiological conditions. Although I_A currents are generally thought to be involved in the setting of action potential duration and firing frequency in neurons (for review, see ref. 24), the exact physiological role of the present I_A modulation by Zn^{2+} in SCN neurons remains to be examined. In the *in situ* situation, Zn^{2+} may be confined to near-synaptic regions, which would make dissociated cells a less-than-ideal preparation for study because these cells often have lost their processes. Thus, closer examination of the physiological function of the Zn^{2+} modulation is preferably done in a hypothalamic slice.

In any case, the results described here suggest a modulation of voltage-gated ion channels by Zn^{2+} at concentrations low enough to probably be of physiological relevance. Whether or not similar modulations exist elsewhere in the central nervous system because of the fairly wide distribution of vesicular Zn^{2+} in this tissue (1) remains an interesting question.

Note. After we had completed this work and submitted an abstract to the Biophysical Society (1993), we became aware that Harrison *et al.* (28) had obtained an apparently similar result in hippocampal neurons. Recently, Belluzzi *et al.*[§] have also reported similar findings in cerebellar granule cells.

[§]Belluzzi, O., Bardoni, R. & Magherini, P. C., Thirty-second Congress of the International Union of Physiological Sciences, August 1–6, 1993, Glasgow, Scotland (abstr.).

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