

STEP REDUCTIONS IN EXTRACELLULAR Ca^{2+} ACTIVATE A TRANSIENT INWARD CURRENT IN CHICK DORSAL ROOT GANGLION CELLS

JOHN J. HABLITZ, UWE HEINEMANN, AND H. D. LUX

Department of Neurophysiology, Max Planck Institute for Psychiatry, D-8033 Planegg-Martinsried, Federal Republic of Germany

ABSTRACT We investigated whether transient step reductions in divalent cations would produce detectable changes in neuronal excitability similar to those reported in the total absence of divalent cations. Using cultured chick dorsal root ganglion cells as a model system, our results indicate that a step reduction in divalent cations induces a transient inward current. This response is mediated by a tetrodotoxin-resistant, Na^+ -permeable, cation channel that is blocked by cadmium. This, and our observation that the response is abolished by verapamil, suggests that the current passes through calcium channels. This transient inward current was estimated to be activated by decreases in extracellular calcium ($[\text{Ca}^{2+}]_o$) as small as 0.5–0.8 mM and thus represents a different response from the one previously observed when steady-state $[\text{Ca}^{2+}]_o$ levels were reduced to micromolar levels.

INTRODUCTION

Divalent cations play an important role in the regulation of a wide variety of neuronal functions. Their absence in the extracellular microenvironment causes alterations in the selectivity of calcium channels in invertebrate neurons (1), mammalian heart cells (2), and frog muscle fibers (3, 4). Responses to certain neurotransmitters are also modified (5, 6). Transient reductions in divalent cations, particularly calcium, are known to occur in the vertebrate nervous system during intense neuronal activity (7–9). Considerable evidence indicates that neurons in the mammalian cortex possess voltage-dependent calcium currents (10–12). It is possible that these currents would be altered by a reduction in the levels of extracellular calcium ($[\text{Ca}^{2+}]_o$). Since the amplitude of calcium currents is related to the level of $[\text{Ca}^{2+}]_o$, it would seem that the decreases in $[\text{Ca}^{2+}]_o$ observed during epileptiform interictal discharges would reduce inward calcium currents. However, as noted above, it has been reported that lowered $[\text{Ca}^{2+}]_o$ can have excitatory effects, due to an alteration in channel selectivity.

We now report that step decreases in $[\text{Ca}^{2+}]_o$ of a magnitude and time course similar to those typically seen during enhanced neuronal activity (7–10, 13), result in

activation of a transient inward current in avian dorsal root ganglion cells.

MATERIALS AND METHODS

Whole-cell and single-channel currents from excised outside-out membrane patches were recorded from cultured chick dorsal root ganglion cells. Cells were obtained from 10-d-old embryos and maintained in culture for 2–10 d prior to use (14). The techniques used for data recording and analysis were as described previously (15, 16). Transient step reductions in divalent cation levels were achieved by the pressure application (1–3 psi) of solutions containing known concentrations of divalent cations from single- or double-barreled micropipettes positioned at distances of 5–20 μm from the surface of the cells under study. Ion-sensitive electrodes (7) were used to determine the concentration of Ca^{2+} at the surface of the cell after use of this pressure ejection technique.

The cells were bathed in saline containing (in mM): NaCl, 140; KCl, 3; CaCl_2 , 2; MgCl_2 , 2; glucose, 10; and HEPES, 10. The pipette solution consisted of (in mM): CsCl, 120; tetraethylammonium chloride, 20; CaCl_2 , 0.25; EGTA, 5; glucose, 10; and HEPES, 10. Divalent cation-deficient solutions were made by omitting calcium and magnesium from the extracellular saline. The pH of all solutions was adjusted to 7.3–7.4 with NaOH.

RESULTS AND DISCUSSION

The results of a typical experiment are shown in Fig. 1 *A*. Pressure ejection of a solution containing 0 mM Ca^{2+} and 0 mM Mg^{2+} produced an inward current. Application of a solution identical to the extracellular bathing medium was without effect in this neuron or in any of the additional cells tested ($n > 100$). As shown in Fig. 1 *B*, the response

Dr. Hablitz's address is Section of Neurophysiology, Department of Neurology, Baylor College of Medicine, Houston, Texas 77030.

Correspondence should be addressed to Dr. Hablitz.

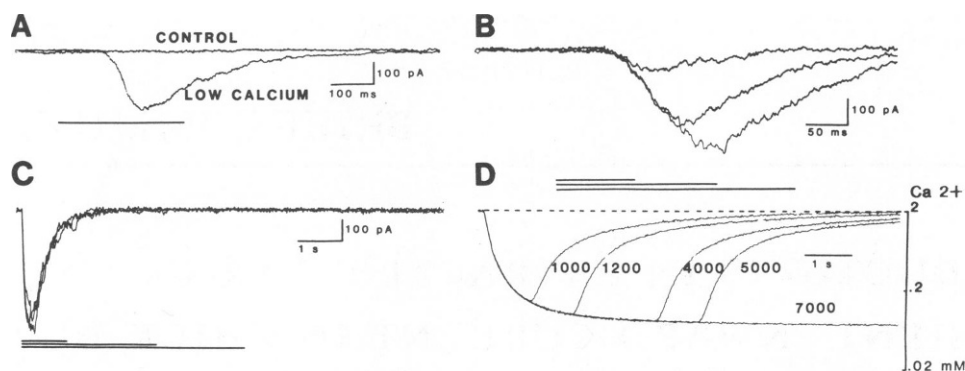


FIGURE 1 Activation of whole-cell currents in chick dorsal root ganglion cells by pressure application of divalent cation-free solutions. The duration of the application is indicated by the horizontal line in this and subsequent figures. (A) At a holding potential of -60 mV, there is no response to pressure application of the bathing medium (control). When calcium and magnesium are omitted, pressure application evokes an inward current that slowly declines (low calcium). (B) The amplitude and duration of the induced inward current increase as the pressure pulse is prolonged. (C) Long-duration pressure applications evoke an inward current that declines during maintained ejections. (D) After the record in C was taken, a calcium-sensitive microelectrode was placed at the surface of the cell. Pressure applications were made for the durations (in ms) indicated. It can be seen that the level of calcium achieved by the pressure technique did not decline in a fashion similar to the decline in ionic current observed in C.

was graded in nature, and the inward currents became larger when the duration of the pressure pulse was increased. This was presumably due to exposure of larger portions of the neuronal membrane to the cation-deficient solution and to a larger reduction of $[Ca^{2+}]_o$ (see Fig. 1 D). Variations in the amplitude and rise time of the current were seen among cells and appeared to depend on the location of the pressure pipette and the size of the neuron. After the position of the pipette had been adjusted, variations in responses in a given neuron were negligible if ejections were made at intervals of 10 s or more. Maximum currents ranged from 600 pA to 1 nA at a holding potential of -60 mV.

Inward currents decayed within seconds, even though the ejection was maintained, which suggests that the inward current was transient in nature. To determine whether this transience was due to a true time-dependent decrease in the response ("inactivation") or to a variation in the levels of extracellular divalent cations caused by the pressure technique, ion-sensitive electrodes were used to measure these levels directly. Fig. 1 C shows the response of a ganglion cell to long-duration applications of a divalent cation-free solution. The duration of the inward current did not vary while the pressure pulse was prolonged from 1 to 5 s. After these recordings were obtained, a calcium-sensitive microelectrode was positioned just outside the cell, and the pressure applications were repeated. It can be seen that the duration of the recorded decrease in $[Ca^{2+}]_o$ was directly related to the duration of the pressure pulse. The decline in the change in $[Ca^{2+}]_o$ was also slower than that of the transient current. The results of these experiments show that the pressure-application method produces stable reductions in $[Ca^{2+}]_o$ to levels of 50–60 μ M calcium (Fig. 1 D). Furthermore, inward currents were typically produced by step-like applications of divalent cation-free solutions for durations of 100–500 ms. Extrapolation from

Fig. 1 D indicates that this would produce a drop in $[Ca^{2+}]_o$ of ~ 0.5 mM. This response is therefore different from the previously reported monovalent cation permeation through calcium channels, which requires a steady-state reduction of calcium to micromolar levels and is voltage dependent (1–4).

Addition of tetrodotoxin (TTX) (1 μ g/ml) to the bathing and pressure-pipette solutions had no effect on the ability to evoke an inward current with divalent cation-deficient solutions. Application of solutions containing 4 mM Mg^{2+} and 0 mM Ca^{2+} produced responses that were qualitatively similar to those obtained with divalent-free solutions but greatly reduced in amplitude. Presumably, the decreased responsiveness observed when magnesium ions were present in the pipette was due to magnesium's calcium-antagonist properties (3, 17). Furthermore, inward currents were not observed when the calcium antagonist cadmium (1 mM) was present in the bathing and pipette solutions. The finding that inward currents could still be elicited with 0 mM Ca^{2+} and 4 mM Mg^{2+} also implies that a simple reduction in surface charge screening cannot account for the activation of the inward current. In fact, lowering the magnesium concentration alone did not elicit the response.

The voltage sensitivity of the evoked inward current was examined by applying constant-duration pressure pulses while systematically varying the holding potential between -100 and $+80$ mV. As can be seen in Fig. 2 A, response amplitude was a linear function of membrane potential over the range of -100 to -10 mV. Response reversal was never observed with use of our normal intracellular saline, in which cesium is the major cation (Fig. 2 B). Since internal cesium is relatively impermeant through calcium channels (18), even under conditions where monovalent cations are the charge carriers (17), experiments were performed in which intracellular sodium was raised to 100

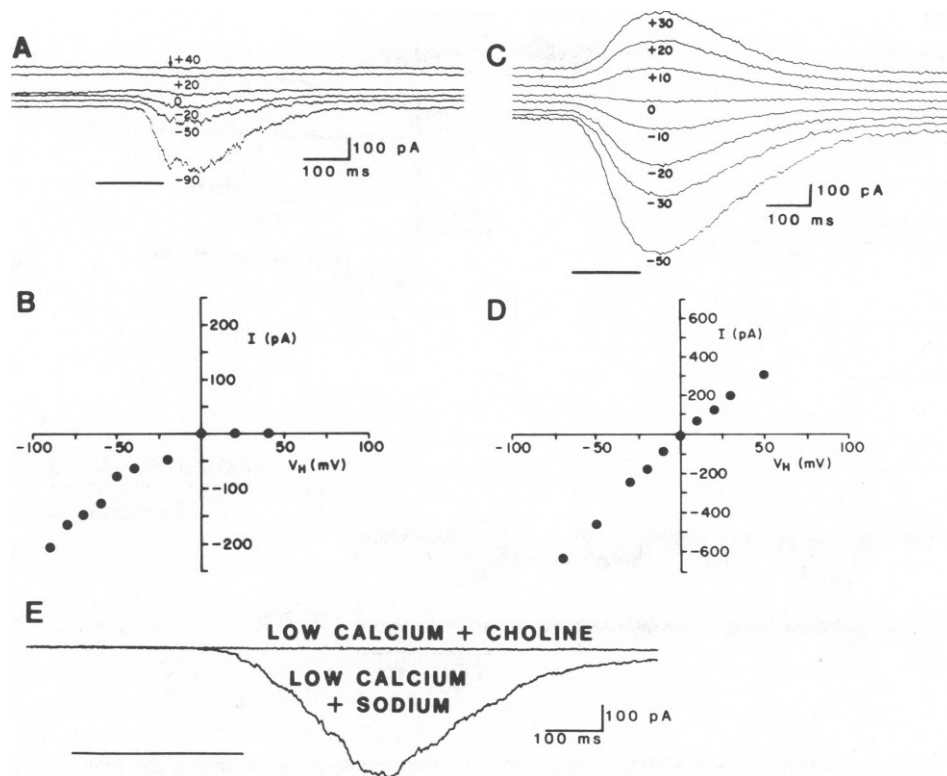


FIGURE 2 (A) Voltage dependence of the inward current. The response of a dorsal root ganglion cell to a constant-duration application of divalent cation-free saline was measured while the holding potential was systematically varied. When cesium was the major intracellular cation, the amplitude of the response declined as the membrane potential was held at less negative values. However, as shown in B, the response never reversed polarity. A similar experiment, in which 100 mM NaCl was substituted for CsCl (C), showed a clear reversal potential near 0 mV (D). (E) Ionic dependence of the inward current. Neurons were bathed in a medium in which choline chloride was substituted for NaCl on an equimolar basis. No response to divalent cation-free solutions was observed in the absence of sodium (*above*). When sodium was included in the pressure pipette (and therefore transiently replaced outside the cell), typical responses were obtained (*below*).

mM by substituting NaCl for CsCl in the recording pipette (Fig. 2 C). Under these conditions, a symmetrical response reversal occurred at 0 mV (Fig. 2 D). This is close to the reversal potential calculated from the Nernst relation. These results indicate that removal of calcium ions activates an Na^+ -permeable cation channel. This interpretation is supported by the results of the ion-substitution experiments shown in Fig. 2 E. Cultures were bathed in a saline in which NaCl was completely replaced by choline chloride. Pressure application of a divalent cation-free solution in which choline had replaced sodium failed to produce any response in the neurons tested ($n = 15$). However, responses were obtained in these same neurons when the pressure pipette contained sodium (Fig. 2 E).

Two calcium currents are known to be present in avian dorsal root ganglion cells (19). It has been shown that the organic calcium antagonist verapamil strongly depresses the traditional, slowly inactivating, high-threshold calcium current while sparing the fully inactivating calcium current, which is activated at negative membrane potentials (20). We therefore tested the effect of verapamil on the transient inward current observed here. In these experiments, drug- and divalent cation-deficient solutions were

applied from a multibarreled ejection pipette to ensure a rapid and complete exchange of the medium bathing the neuron (20). Using the ejection pipette, the induced current was again noted to be transient in nature. At a concentration of 100 μM , verapamil markedly reduced or abolished (in a reversible manner) the response to divalent cation-deficient solutions (Fig. 3 A). Depolarizing step commands given after the transient current had declined produced no inward currents (Fig. 3 B). Since no calcium chelating agents were used in these studies, it is estimated that due to contamination of the reagents used, calcium levels were in the range of 10–20 μM . The lack of voltage-activated currents is thus in keeping with previous reports that monovalent cation permeation through calcium channels is blocked by $[\text{Ca}^{2+}]_o$ levels on the order of 1 μM (2, 4, 17). These results, together with the observation that the current is TTX-resistant and blocked by cadmium, indicate that the conventional, slowly inactivating, calcium channel is most likely involved in the transient response. The present results also imply that calcium channels can assume another Na^+ -permeable state, different from that described under steady-state conditions of extremely low levels of $[\text{Ca}^{2+}]_o$.

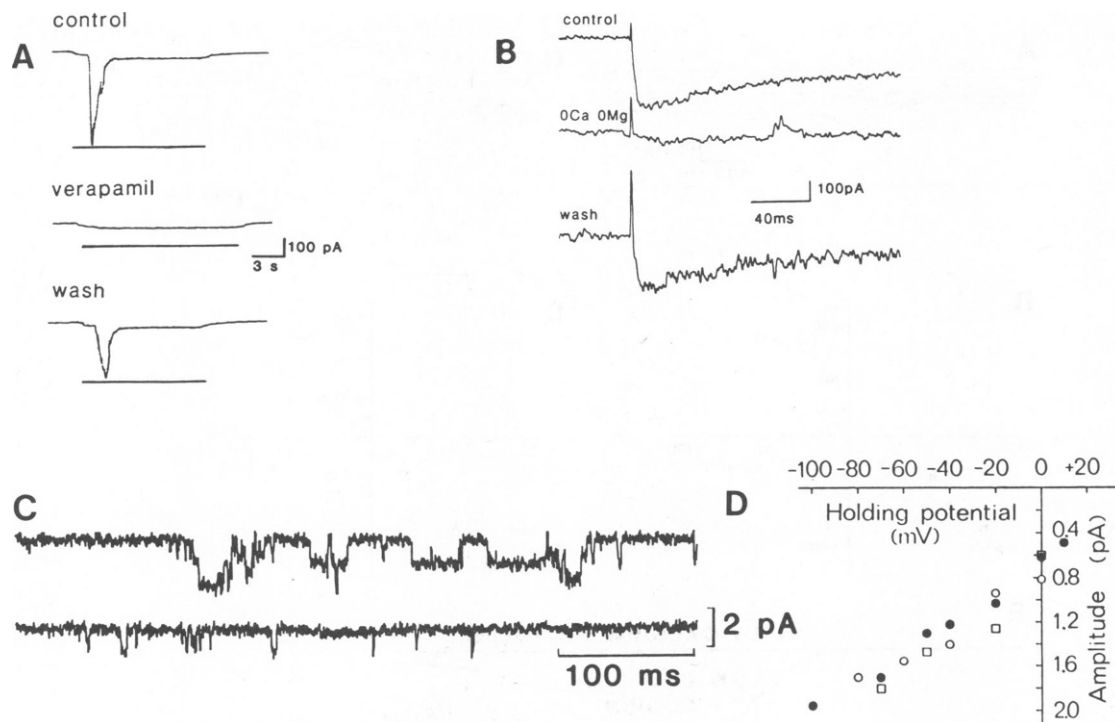


FIGURE 3 (A) Effect of verapamil on the response to application of divalent cation-free saline from a perfusion pipette. (B) Whole-cell currents in response to a voltage step of +40 mV from a holding potential of -60 mV. Middle trace was obtained after the transient inward current had declined. (C) Unitary currents from an excised outside-out patch of membrane. The patch is quiet until pressure ejection of a calcium-deficient solution produces openings of up to six channels. This activity declines over time until the patch again becomes quiescent. Lower trace is a continuation of the upper record. Holding potential was -70 mV, 1 kHz bandwidth. (D) Plot of single-channel amplitude as a function of membrane potential from three patches. Channel amplitude was determined from Gaussian fits to amplitude histograms. Single-channel conductance was 14 ± 1.5 pS.

Openings of single ionic channels could be seen in outside-out membrane patches when divalent cation-free solutions were applied. Such membrane patches displayed little or no spontaneous activity until the divalent cation concentration was altered. Pressure application of divalent cation-free solutions caused a transient burst of channel openings that declined over time (Fig. 3 C). Patches typically displayed activity from two to six channels, as judged from the superimposition of equal-amplitude current steps. This activity was weakly voltage dependent; the number of channel openings and the response duration decreased as the membrane potentials were made less negative. Channel-open time did not appear constant over the time course of the response. Long open times predominated at the onset of the response whereas shorter openings were more common at the end (see Fig. 3 C). The amplitude of single-channel currents was voltage dependent, and, in the voltage range of -80 to 0 mV, the slope conductance (Fig. 3 D) was 14 ± 1.5 pS ($n = 8$).

Although $[Ca^{2+}]_o$ declines only slightly during normal activity (13, 21), pathophysiological states such as epilepsy and spreading depression are associated with marked reductions in $[Ca^{2+}]_o$. Interictal epileptiform discharges are accompanied by decreases of 0.45 to 0.55 mM (8), and decreases of up to 1.2 mM have been recorded during

epileptic seizures (22). $[Ca^{2+}]_o$ levels decline to as low as 0.08 mM during spreading depression (23). The changes in divalent cations that activated an inward current in the present study were similar in time course and degree to those occurring during these pathophysiological states. This response, if present in central neurons, could contribute significantly to the membrane depolarization associated with these conditions and could mediate excitation of inactive neurons. Although it has been determined that the observed inward current is generated by activation of a cation-permeable ionic channel, the precise nature of the channel is uncertain. Alternative possibilities include a novel type of channel or a modified sodium or calcium channel. External calcium ions have been shown to block sodium channels in neuroblastoma cells (24). This sodium-channel blockade was maximal at hyperpolarized levels and caused a nonlinearity in the single-channel current-voltage relationship. Such a mechanism is unlikely to underlie the present results, since responses persisted in the presence of TTX, and whole-cell and single-channel current amplitudes were linearly related to membrane potential. Since voltage-dependent and calcium-activated potassium channels were blocked in the present experiments, it is unlikely that they contribute to the observed response.

The present findings in vertebrate sensory neurons indi-

cate that step decreases in extracellular divalent cation levels produce an inactivating inward current. The response observed here differs from the voltage-dependent sodium permeability that appears when $[Ca^{2+}]_o$ is reduced with calcium chelators (1–4, 17). This transient current could be elicited over a range of membrane potentials from -100 to -20 mV, with little variation in time course. Although the amplitude of the responses was smaller in solutions containing 4 mM Mg^{2+} and 0 mM Ca^{2+} , the time courses were similar. It is therefore unlikely that the current is due to a direct effect of changes in surface charge screening.

Activation of an inward current via cation reduction occurred with relatively modest reductions in $[Ca^{2+}]_o$ and was transient in nature. This current thus represents a novel response to alterations in levels of $[Ca^{2+}]_o$. It is similar in several respects to an inward current seen in response to rapid acidification of the surface membrane (25). Although the pH was not varied here, it is still possible that a common mechanism underlies these responses. Our findings suggest that modified Ca^{2+} channels are involved in the transient response. However, the possibility remains that the current involves the flow of ions through a novel type of channel. The properties of this transient current make it a likely candidate for playing a role in modifying the excitability of neuronal aggregates during pathophysiological states such as epilepsy and spreading depression.

This work was supported by grant SFB-220 from the Deutsche Forschungsgemeinschaft and by National Institutes of Health grant NS-22373.

Received for publication 10 December 1985 and in final form 22 April 1986.

REFERENCES

- Kostyuk, P. G., S. L. Mironov, and Y. M. Shuba. 1983. Two ion-selecting filters in the calcium channel of the somatic membrane of mollusk neurons. *J. Membr. Biol.* 76:83–89.
- Hess, P., and R. W. Tsien. 1984. Mechanism of ion permeation through calcium channels. *Nature (Lond.)* 309:453–456.
- Almers, W., and E. W. McCleskey. 1984. Non-selective conductance in calcium channel of frog muscle: calcium selectivity in a single-file pore. *J. Physiol. (Lond.)* 353:585–608.
- Almers, W., E. W. McCleskey, and P. T. Palade. 1984. A non-selective cation conductance in frog muscle membrane blocked by micromolar external calcium ions. *J. Physiol. (Lond.)* 353:565–583.
- Nowak, L., P. Bregestowski, P. Asher, A. Herbet, and A. Prochiantz. 1984. Magnesium gates glutamate-activated channels in mouse central neurones. *Nature (Lond.)* 307:462–465.
- Stanford, C. E., and D. I. Wallis. 1984. Generation of an unusual depolarizing response in rabbit primary afferent neurones in the absence of divalent cations. *J. Physiol. (Lond.)* 352:49–72.
- Heinemann, U., H. D. Lux, and M. J. Gutnick. 1977. Extracellular free calcium and potassium during paroxysmal activity in the cerebral cortex of the cat. *Exp. Brain Res.* 27:237–243.
- Pumain, R., I. Kurcewicz, and J. Louvel. 1983. Fast extracellular calcium transients: involvement in epileptic processes. *Science (Wash. DC)* 222:177–179.
- Nicholson, C., G. Ten Bruggencate, H. Stöckle, and R. Steinberg. 1978. Calcium and potassium changes in extracellular microenvironment of cat cerebellar cortex. *J. Neurophysiol.* 41:1026–1039.
- Schwartzkroin, P. A., and M. Slawsky. 1977. Probable calcium spikes in hippocampal neurons. *Brain Res.* 135:157–161.
- Johnston, D., J. J. Hablitz, and W. A. Wilson. 1980. Voltage clamp discloses slow inward current in hippocampal burst-firing neurones. *Nature (Lond.)* 286:391–393.
- Llinás, R., and M. Sugimori. 1980. Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. *J. Physiol. (Lond.)* 305:171–195.
- Somjen, G. G. 1980. Stimulus-evoked and seizure-related responses of extracellular calcium activity in spinal cord compared to those in cerebral cortex. *J. Neurophysiol.* 44:617–632.
- Barde, Y. A., D. Edgar, and H. Thoenen. 1980. Sensory neurons in cultures: changing requirements for survival factors during embryonic development. *Proc. Natl. Acad. Sci. USA* 77:1199–1203.
- Brown, A. M., H. Camerer, D. L. Kunze, and H. D. Lux. 1982. Similarity of unitary Ca^{2+} currents in three different species. *Nature (Lond.)* 299:156–158.
- Lux, H. D., and A. M. Brown. 1984. Single channel studies on inactivation of calcium currents. *Science (Wash. DC)* 225:432–434.
- Fukushima, Y., and S. Hagiwara. 1985. Currents carried by monovalent cations through calcium channels in mouse neoplastic B lymphocytes. *J. Physiol. (Lond.)* 358:255–284.
- Lee, K. S., and R. W. Tsien. 1984. High selectivity of calcium currents in single dialysed heart cells of the guinea-pig. *J. Physiol. (Lond.)* 354:253–272.
- Carbone, E., and H. D. Lux. 1984. A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurones. *Nature (Lond.)* 310:501–502.
- Boll, W., and H. D. Lux. 1985. Action of organic antagonists on neuronal calcium currents. *Neurosci. Lett.* 56:335–339.
- Heinemann, U., and J. Louvel. 1983. Changes in $[Ca^{2+}]_o$ and $[K^+]_o$ during repetitive electrical stimulation and during pentetrazol induced seizure activity in the sensorimotor cortex of cats. *Pflügers Arch. Eur. J. Physiol.* 398:310–317.
- Pumain, R., C. Menini, U. Heinemann, J. Louvel, and C. Silva-Barrat. 1985. Chemical synaptic transmission is not necessary for epileptic seizures to persist in the baboon *Papio papio*. *Exp. Neurol.* 89:250–258.
- Hansen, A. J., and T. Zeuthen. 1981. Extracellular ion concentrations during spreading depression and ischemia in the rat brain cortex. *Acta Physiol. Scand.* 113:437–445.
- Yamamoto, D., J. Z. Yeh, and T. Narahashi. 1984. Voltage-dependent calcium block of normal and tetramethrin-modified single sodium channels. *Biophys. J.* 45:337–344.
- Krishtal, O. A., and V. I. Pidoplichko. 1982. H^+ sensor in the nerve cell membrane. In *Physiology and Pharmacology of Epileptogenic Phenomena*. M. R. Klee, H. D. Lux, and E.-J. Speckmann, editors. Raven Press, New York. 207–212.