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Uncoupling of electrotonic synapses by calcium

(invertebrate giant neurons/gap junction/calcium ionophore/mitochondria/metabolic inhibitors)

G. BAUX, M. SIMONNEAU, L. TAUC, AND J. P. SEGUNDO*

Laboratoire de Neurobiologie Cellulaire, Centre National de la Recherche Scientifique, 91190 Gif-sur-Yvette, France

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ABSTRACT **The degree of axo-axonal synaptic coupling between nerve cells in the buccal ganglion of *Navanax* was investigated in relation to intracellular ionic calcium. Increasing intracellular Ca^{2+} by injection of Ca^{2+}, injection of Na^+, or application of ionophore X-537A produced uncoupling after at least 90 min, if metabolic inhibitor was present in the medium. Subsequent removal of the metabolic inhibitor reestablished the coupling in less than 30 min. Injected Sr^{2+} also mimicked the uncoupling action of Ca^{2+}. The presence of a metabolic inhibitor alone had no effect on the coupling. These results lead to the following conclusions: (*i*) Uncoupling is due to an increased free Ca^{2+} concentration at the junctions. (*ii*) The liberation of endogenous sequestered Ca^{2+} is not sufficient to produce uncoupling except if an excess Ca^{2+} had been previously sequestered. The electrical synapses in the buccal ganglion of *Navanax* thus appear to be affected by Ca^{2+} in a similar way as gap junctions studied in non-neural tissues.**

Gap junctions appear to provide a special form of communication between cells of a wide variety of excitable and nonexcitable tissues and are found in almost all animals studied (1–3). Similar ultrastructural features have been described in all materials studied (4, 5), and specific proteins have been purified from isolated membrane preparations rich in gap junctions (6–8). This kind of junction is characterized by low-resistance pathways or "electronic couplings" between the cells. Such couplings constitute an important feature in some neuronal circuits—for example, in the mesencephalic nucleus of the fifth nerve and in the lateral vestibular nucleus in the rat and between dendrites of the inferior olive in the cat (9–12).

The consequences of intracellular injection of calcium in *Chironomus* salivary gland (13) and mammalian heart (14) suggested that electrotonic coupling is dependent on the intracellular concentration of this divalent cation. Calcium injection into nerve cells connected by gap junctions has so far not been performed, probably because of technical problems and because cell geometry makes direct access to the junction difficult. By using the air pressure technique and a suitable experimental preparation, we have been able to examine whether an increase in intracellular calcium concentration is correlated with junctional uncoupling in the neurons of the opisthobranch mollusc *Navanax*.

Two large (about 600 μm), three medium (400 μm), and five smaller (250 μm) neurons known to be electrically connected have been identified in *Navanax* buccal ganglia (figure 1 in ref. 15). The present report shows that these electrotonic synapses are uncoupled by intracellular injection of Ca^{2+}, Sr^{2+}, or Na^+ as well as by ionophore X-537A treatment and that this happens only if the isolated ganglia are perfused with cyanide.

Preliminary reports of this work have appeared (16, 17).

MATERIALS AND METHODS

Experiments were performed at room temperature (20–22°) on 65 adult *N. inermis* obtained from R. Fay (Pacific Biomarine, Venice, CA) and held in instantaneous artificial seawater (HW Meeressaltz) at 17°. Isolated buccal ganglia were dissected and perfused with artificial seawater (ASW; NaCl, 460 mM; KCl, 10 mM; $CaCl_2$, 11 mM; $MgCl_2$, 25 mM; $MgSO_4$, 28 mM; Tris-HCl, 10 mM).

Connective tissue around the cells was entirely removed with fine forceps for easier microelectrode penetration. The potential changes in the directly polarized neuron (V_1) and in the coupled cell (V_2) were recorded by the usual techniques. Recording and polarization were performed via separate intracellular microelectrodes. Most experiments were done on the same pair of a large and a medium cell, chosen because of their relatively high (about 0.4) V_2/V_1 ratio or "coupling coefficient." However, the results were identical regardless of the initial coupling coefficient.

Each type of experiment was repeated at least five times. Preliminary experiments were done using the two electrically coupled identified interneurons found in the *Aplysia* buccal ganglion (18, 19), but quantitative results could not be obtained because of the low coupling coefficient (0.1). Intracellular injections of solutions of $CaCl_2$ (0.2 M) or $SrCl_2$ (0.2 M) in distilled water were performed by using an air pressure system similar to that used previously (20). The injected volumes were of the order of 1% of the soma volume and corresponded to a 2 mM Ca^{2+} or Sr^{2+} load.

Control injections of approximately the same volume of distilled water were without effect on the coupling coefficient. Iontophoretic Na^+ injections were made with a 100 nA current, through the membrane, between the NaCl-filled microelectrode and ground.

X-537A ionophore was dissolved in 100% ethanol at a concentration of 1.5 mg/ml before each use and was diluted to a 1% final concentration of ethanol. Control perfusions of 1% ethanol had no effect on coupling ratio. Neuraminidase (*Clostridium perfringens* neuraminidase, Sigma, grade VI) was injected in 200 mM KCl by the same air pressure device.

RESULTS

As is the case with other metabolic inhibitors, cyanide suppresses mitochondrial sequestration of intracellular calcium (47) and blocks membrane active transport mechanisms. These effects tend to raise the intracellular Ca^{2+} concentration (21); in fact, cyanide perfusion has been shown to uncouple cells in salivary glands (1, 13). In the *Navanax* buccal ganglion, perfusion with sodium cyanide (5 mM) in ASW for many hours did not cause uncoupling. At most it only induced a transient hyperpolari-

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Abbreviation: ASW, artificial seawater.

* Fellow of the Fogarty Center of the National Institutes of Health on leave from the Department of Anatomy, University of California, Los Angeles, CA.

zation of the cells (5–10 mV) and a small decrease in resistance (see Fig. 3) similar to known actions of other metabolic inhibitors (22) and tentatively attributed by those authors to an increased K^+ conductance. The two electrically coupled identified interneurons found in the *Aplysia* buccal ganglion (18, 19) were also not affected by sodium cyanide.

The most straightforward method for increasing intracellular concentration of calcium is to introduce it into the cell. When injected into *Chironomus* salivary gland cells (1, 13) and mammalian heart cells (14), Ca^{2+} alone uncoupled cells. But in *Navanax*, intracellular air pressure injection of Ca^{2+} was not sufficient by itself to induce uncoupling. In our experiments, Ca^{2+} injection into neurons was effective only when the preparation was being continuously perfused with cyanide/ASW. However, after injection, 1.5–3 hr were necessary to obtain an uncoupling of these electrotonic synapses (Fig. 1). After decoupling had been produced in this manner, perfusion with cyanide-free ASW led to an electrical recoupling that started almost immediately and was nearly at 90% of the control level in about 20 min. On reexposure to cyanide/ASW, cells again uncoupled, but with a considerably shorter latency (<10 min). By removing and adding the cyanide in the perfusion fluid, the coupling-uncoupling procedure could be repeated several times.

Sr^{2+} replaces Ca^{2+} in a large number of biological processes—for example, transmitter release in neuromuscular junctions and squid giant synapses (23, 24). Air pressure injections of Sr^{2+} had the same effect as those of Ca^{2+} in about the same concentrations, in the presence of cyanide. Furthermore, increases of intracellular Na^+ concentration have been found to lead to an increment in Ca^{2+} influx (25). Iontophoretic injections of Na^+ (Fig. 2) also produced uncoupling, provided that the preparation was perfused with cyanide.

To induce uncoupling by intracellular Na^+, it was necessary to inject this ion by passing the current between the intracellular microelectrode and the ground. Injection of Na^+ between two intracellular electrodes was ineffective. This was perhaps due to the fact that, in the first case, the 100-nA current passing across the membrane depolarized continuously the cell close to zero potential and thus facilitated the entry of Ca^{2+}.

We estimate that our Na^+ injection increased the intracellular Na^+ by 40 mM which would produce a change in E_{Na} of 35 mV. Taking the change in E_{Na} produced by the injection and the effect of depolarization of the cell together, the plot of Mullins (26) allows us to predict that the Ca^{2+} influx increased about 10-fold. Depolarization to the same levels by iontophoresis of K^+ did not uncouple. The depolarization itself is not sufficient to induce uncoupling as has been shown also with *Chironomus* salivary gland cells (27).

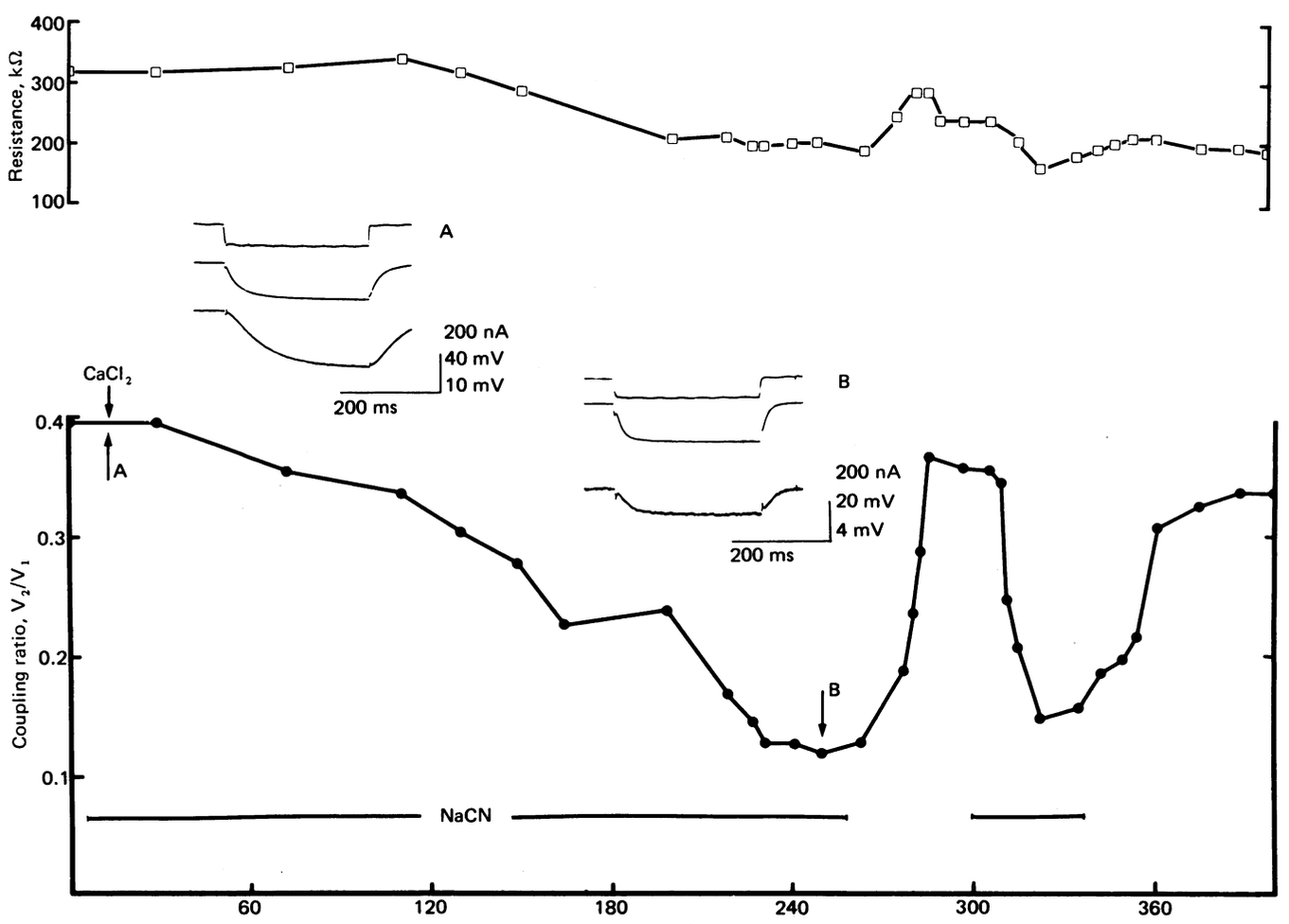

FIG. 1. Uncoupling after intracellular injection of $CaCl_2$ in a preparation bathed in ASW with NaCN (5 mM), showing reversibility of effects. Current was injected into cell 1 (referred to as "presynaptic") and records were obtained from it as well as from cell 2. The electrical coupling ratio V_2/V_1 between cells 1 and 2 and the presynaptic resistance were monitored and are plotted as function of time. $CaCl_2$ (200 mM) was pressure-injected into the presynaptic cell at the indicated time. Injection volume was in the range of 1% of the soma volume, corresponding to a 2 mM Ca^{2+} load. (*Insets*) Simultaneous records of injected current hyperpolarizing pulse (top trace) and of transmembrane potential in cells 1 (middle trace) and 2 (bottom trace) at times A and B indicated in lower graph. The pre- and postsynaptic cells were G-R and M-R, respectively, by the classification in ref. 15.

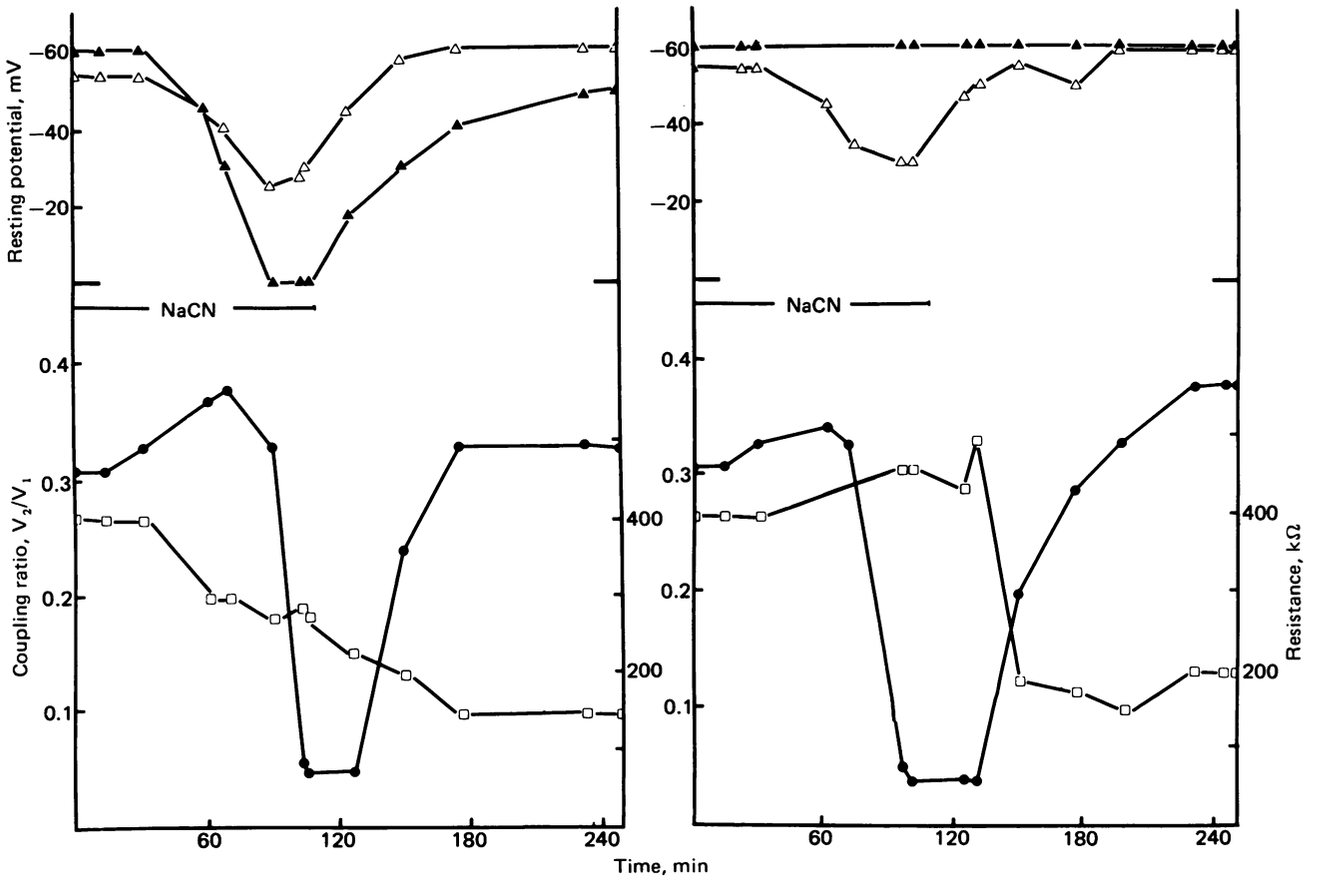

FIG. 2. Uncoupling after transmembrane Na^+ injection in a preparation bathed in ASW with NaCN (5 mM), showing partial reversibility of effects and independence from resting potential. Transmembrane Na^+ injection was performed with a 100-nA current throughout the whole experiment and started at zero time. The Na^+ injection was stopped periodically during each measure of resting potentials and of coupling ratio. (*Left*) Membrane potential of cell 1 was allowed to vary. (*Right*) Membrane potential of cell 1 clamped at −60 mV during each measurement. ▲, Resting potential of cell 1; △, resting potential of cell 2; ●, coupling ratio, V_2/V_1; □, cell 1 membrane resistance. The presynaptic cell resting potential (*Left*) reversed from 0 to −50 mV upon removal of the cyanide solution. This reversal can be accounted for on the basis that the Na^+/K^+ metabolic pump was blocked with cyanide and started to pump out Na^+ after cyanide removal. The pre- and postsynaptic cells were G-R and M-R, respectively, by the classification in ref. 15.

An increment of 30% in the osmotic pressure of the perfusion medium, a treatment known to increase internal calcium concentration in some systems (28), did not uncouple even the cyanide-treated preparations.

Ionophores, such as X-537A, have been used in various systems to increase intracellular calcium concentration (29). However, the effects of X-537A are not restricted to Ca^{2+}; other cations such as Na^+ (30) are also involved. Addition of X-537A (25 μM) to the cyanide/ASW for 20 min only at the beginning of the experiment produced uncoupling in 1.0–3 hr. This uncoupling was also reversed by switching to cyanide-free ASW (Fig. 3). Prolonged application of X-537A resulted in large and irreversible depolarization. Experiments in which *Aplysia* cells were loaded with ^{42}K showed that after short application, the ionophore can be washed out as indicated by the return of ^{42}K outflow to previous levels (R. T. Kado and L. Tauc, unpublished data). The ionophore A-23187, more specific for divalent cations, did not show any effect, possibly because in these cells (as in some other systems) it does not increase the calcium transport (31).

Perfusion of the preparation with up to 50 mM KCl, which depolarized both the pre- and postsynaptic cells (by −20 to −30 mV) and notably increased their conductance, had little effect on the coupling ratio. The action of the increased concentration of KCl was observed only during short periods because contraction in the preparation pulled out the microelectrodes.

High-gain recordings showed that, except for transient changes in synaptic activity when ionic concentrations were changed, the NaCN depressed most synaptic potentials.

If Ca^{2+} is responsible for the uncoupling, one might expect that it does this through induced conformational changes of the molecular components of the gap junction (32). The sites, in the mitochondrial membrane, that trap calcium have been shown to be sialoglycoproteins (33), and it was suggested that similar sialoglycoproteins participate in calcium entry into the nerve terminal (34). A possible participation of sialic acids in the present situation was examined by the intracellular injection of neuraminidase, an enzyme known to be transported with axonal flow and to cleave exclusively glycosidically bound sialic acid. If applied intracellularly, this enzyme blocks the transmission on chemical synapses (35). Simultaneous injection of neuraminidase into both coupled cells in *Navanax* did not have any action on the electrical synapses even after 6–7 hr, nor did it impair the uncoupling action of X-537A.

DISCUSSION

The uncoupling observed in cyanide-treated cells after Ca^{2+}, Sr^{2+}, or Na^+ injections or after X-537A application may be attributed to an increase of intracellular calcium concentration. The negative results obtained with intrasomatic injection of Ca^{2+} only or cyanide treatment only do not disprove this hypothesis. Indeed, because the gap junctions are probably remote from the cell body, each treatment in isolation could have led

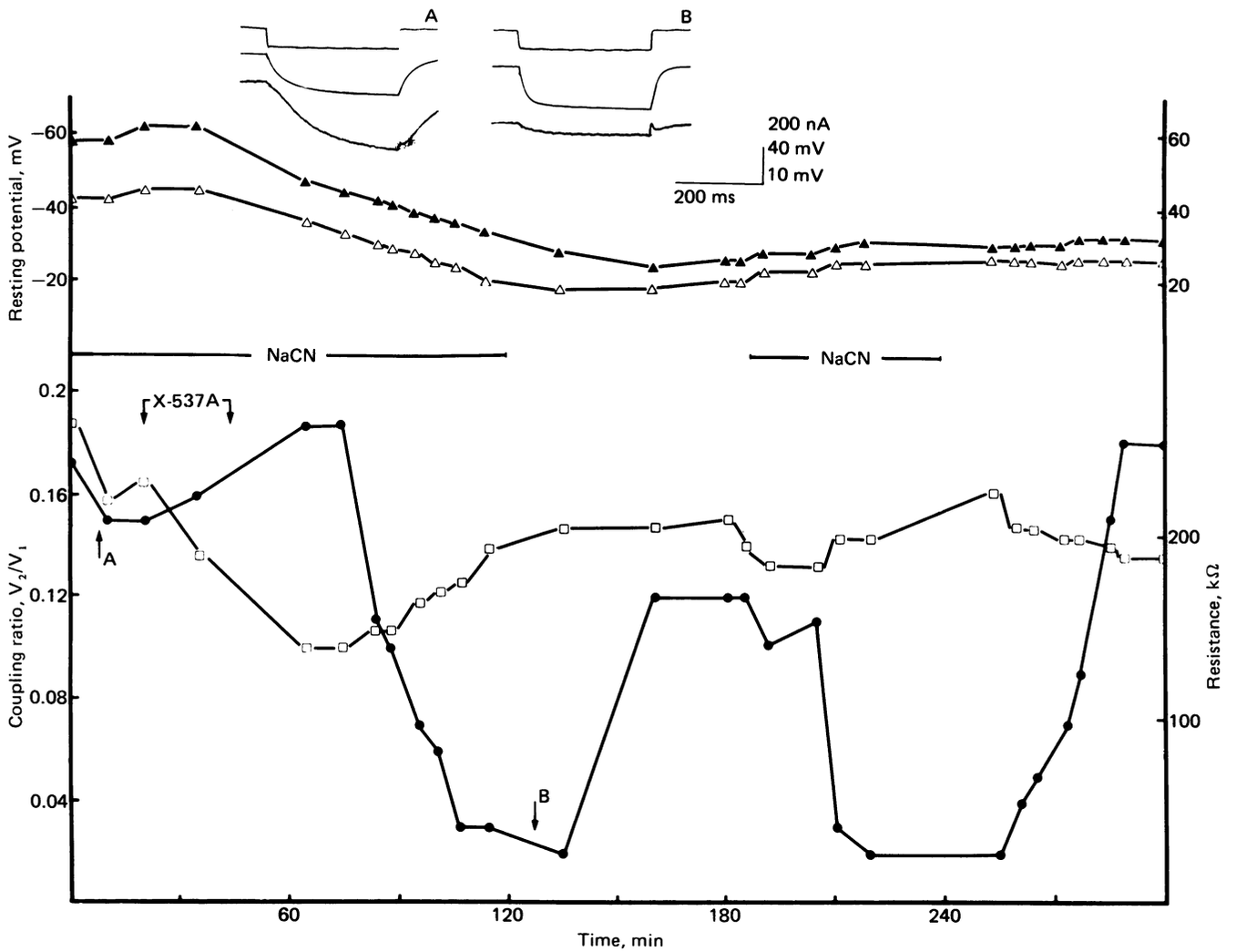

FIG. 3. Uncoupling produced by ionophore X-537A (25 μM) in a preparation bathed with NaCN (5 mM), showing reversibility of effects. The ionophore was dissolved in ethanol to a final ethanol concentration of 1%. The solution was added to the perfusion fluid during the indicated 20 min. ▲, Resting potential of cell 1; △, resting potential of cell 2; ●, coupling ratio V_2/V_1; □, cell 1 membrane resistance. The pre- and postsynaptic cells were G-L and C-LD, respectively, by the classification in ref. 15. (*Insets*) As in Fig. 1.

to an insufficient calcium increase at the critical sites near the junction. In order for enough unsequestered calcium to remain close to the synaptic region, for uncoupling, it was necessary to combine cyanide/ASW with the manipulations that increase intracellular calcium. The observed delay of hours was long compared to that, of minutes, required for uncoupling in mammalian heart muscle; the delay may be explained only in part by the cellular geometry. In *Navanax* neurons the calcium needs to be transported or to diffuse from the somatic injection site to the axonal junction sites, a distance estimated to be about 1 mm (from sections of cells pressure-injected with horseradish peroxidase into the soma). In cardiac muscle or salivary gland cells, the injection is in the immediate proximity of the gap junctions.

In view of the long time required to uncouple *Navanax* electrical synapses when somatic Ca^{2+} is increased in cyanide/ASW, we propose that a cytoplasmic compartment or compartments sequestering the Ca^{2+} delays the arrival of Ca^{2+} at the junction in sufficient quantity to produce uncoupling. Ca^{2+} loading induced by our treatments is certainly distributed between cyanide-sensitive and -insensitive compartments. The cyanide-insensitive compartment may be represented by various axoplasmic components such as calcium-binding proteins and organelles (36, 37). The cyanide sensitive compartments are possibly represented by mitochondria (38, 39) and by ATP-dependent storage systems (40, 41). The mitochondrial Ca^{2+} sequestration is blocked rapidly by cyanide (13) whereas the ATP-dependent storage system is inoperative only when the neuron is more or less depleted of its ATP, and this ATP synthesis is blocked by the metabolic inhibitor. This process requires a long time—several hours in the squid giant axon (37). The necessity of the presence of cyanide in the bath medium and the long time needed in *Navanax* neurons for Ca^{2+} to uncouple the electrical synapse can be explained by the sequestration of the injected Ca^{2+} in the ATP-sensitive pool; with time, the ATP is depleted (1–3 hr) and the Ca^{2+} can diffuse toward the junction where, when a sufficient concentration of Ca^{2+} is reached, the junction uncouples. When, at this time, the cyanide is removed, Ca^{2+} is captured by the cyanide-sensitive pool, the Ca^{2+} concentration close to the junction decreases, and the electrical synapse recouples. But this excess Ca^{2+} now appears to be taken up mainly by the mitochondria rather than the ATP-dependent pool (36). The time of this recoupling would be limited by the time necessary for the cyanide to be removed from the cell (about 20 min). Subsequent addition of cyanide will force the mitochondria to release the sequestered Ca^{2+}, increasing the concentration of Ca^{2+} in the vicinity of the junction and thus producing a new uncoupling. The uncou-

pling/coupling cycle can be reproduced several times by adding or removing the cyanide from the bath. We think that this interpretation explains in a satisfactory way the slow time course of the action of injected Ca^{2+} and the more rapid uncoupling and subsequent recoupling with cyanide once the first uncoupling is obtained. The explanation is tentative because the identification of specific Ca^{2+} sequestration compartments was beyond the scope of this work. Further experimentation is needed to prove or disprove our hypothesis.

X-537A experiments have the same time course of uncoupling. This may indicate that this ionophore does not penetrate into the neuropile and induces mainly an increase of Ca^{2+} at the somatic level. It is also possible that the time necessary to uncouple corresponds to the building up of a sufficient Ca^{2+} concentration at the level of the electrical synapse. It is also possible that, in addition, the X-537A penetrates the cell membrane and interferes with the internal stores of Ca^{2+} (31). In the *Aplysia* buccal ganglion, cholinergic synaptic transmission between identified pre- and postsynaptic cells is blocked by X-537A at the same concentration and in the same range of time (unpublished data).

Experiments with increased concentration of extracellular KCl as well as the analysis of the resistance values and coupling ratios at different times after the initial increase in Ca^{2+} (Figs. 1–3) rule out the possibility that in this case the uncoupling is due to decreased resistance of the pre- and postsynaptic cells (42). This is particularly clear in Fig. 2: after the removal of cyanide, the coupling coefficient increased drastically with no such a change in the resistance (*left*) or even with a decrease of resistance (*right*).

Meech and Thomas (43) have recently demonstrated that a decrease of cytoplasmic pH can be induced by intracellular injection of Ca^{2+}. In the amphybian embryo, intracellular pH rather than intracellular Ca^{2+} seems to control junctional permeability (44). But in snail neurons, pH changes induced by intracellular Ca^{2+} injection appear to have a rapid recovery (range, 10 min) (43). In contrast with the squid axon preparation (45), the H^+ efflux from snail neurons is insensitive to metabolic inhibitors (46). One would thus expect that, if the uncoupling in *Navanax* were due to a pH change, the uncoupling would be a transient phenomenon, but no recovery from uncoupling has been observed even after prolonged exposure to cyanide (several hours). However, direct experimental data concerning the cytoplasmic pH at a long time after injection so far are not available.

In conclusion, even if, in cases such as central neurons in which complex geometry limits the functional examination of electrotonic coupling/decoupling mechanisms, electrical synapses appear to be similar to other gap junctions as far as Ca^{2+} is concerned.

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