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Properties of the slow inward current induced by pentylenetetrazol in Helix neurons

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The slow inward current (SIC) induced by 50 mM pentylenetetrazol was studied on central neurons of *Helix pomatia* in view of its ionic dependence and sensitivity to channel blockers in the presence of 30 mM TEA. A single electrode voltage clamp was used to measure currents evoked by ramp pulses and voltage steps. Sodium withdrawal had variable effects on this current while TTX had no influence on it. Inorganic and organic calcium channel blockers, on the contrary, always produced a partial or total block of the SIC. It is concluded that the slow inward current is mediated by Ca channels impaired by PTZ. Various ions — Na, Ca, and even Tris — may participate in it in variable proportions.

INTRODUCTION

In a previous paper[5] the effects of pentylenetetrazol (PTZ) on the electric membrane parameters, action potentials, inward and outward currents were analyzed using the metacerebral giant cell (MCC) of *Helix pomatia*. At the same time paroxysmal depolarization shifts (PDSs) elicited by the drug were also recorded. The most obvious effect of PTZ, the depression of I_A and I_K currents, although proved to be a corollary of the convulsive actions, failed to provide an explanation for the PDSs, all the more because it did not modify substantially the inward Na and Ca currents, contributing to the spike potential. Our attention was, therefore, directed to the origin of those long-lasting depolarization plateaus which underlie the PDSs and which bear normal or partially inactivated spike potentials on their crest. Our aim was to establish: (i) what kind(s) of channels is/are used by this slow, scarcely inactivating current, and (ii) what kind(s) of ions carry it.

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MATERIALS AND METHODS

The experiments were carried out on different neurons of the central ganglion of the snail, *Helix pomatia* L. To prepare the cells, the ganglionic mass was dissected from the animal and was fixed to the bottom of an organ bath covered with Sylgard. After peeling off the connective tissue sheaths, the neurons were sought under binocular magnification (22 ×). The giant metacerebral cell (MCC) and several neurons in the right parietal

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ganglion were used primarily, together with unidentified ones. Neurons were identified by position and activity; those in the right parietal ganglion were numbered according to Kerkut et al.[12]. A standard mechanical manipulator was used to penetrate the neurons with the microelectrode.

The preparation was continuously superfused with normal or modified Helix physiological solution. The normal solution contained (in mM): NaCl 80, KCl 4, $CaCl_2$ 7, $MgCl_2$ 5, Tris Cl 5; the pH was 7.4. Sodium-free solution was prepared by equimolar substitution of NaCl with Tris–HCl. Pentylenetetrazol (PTZ) was used at a concentration of 50 mM and 30 mM tetraethylammonium (TEA)-Br was always added to depress the slow potassium current. $NiCl_2$, $MnCl_2$ and $CoCl_2$ at concentrations of 8, 10 or 15 mM as inorganic, verapamil and diltiazem at 1 mM as organic Ca channel blockers, as well as 10 μM tetrodotoxin (TTX) were used. Agents were dissolved without osmotic balance. All experiments were performed at room temperature (22–25 °C).

Voltage clamp recordings were carried out using a single channel voltage clamp amplifier built according to the design of Merickel[14] and Wilson and Goldner[20]. Glass microelectrodes, filled with 1 M potassium citrate, had a resistance of 2–7 MΩ. Cells in voltage clamp mode were depolarized using slow ramp and square wave command pulses (starting from the holding level, typically −40 to −50 mV). The steepness of the ramp (dV/dt) was 20 mV/sec, the maximal depolarization extended to +30 mV. Voltage and current records were visualized and photographed from the screen of a Tektronix storage oscilloscope. Current-voltage curves were drawn using an X-Y plotter. The sample-and-hold frequency was about 5 kHz with voltage measurement in 50% of duty cycle and current injection in another 50% (this was taken in correction). The maximum injection possible with this device was 400 nA. Time constant of voltage stabilization was 2 msec.

RESULTS

Induction of the slow inward current

Since PDSs (at least the spontaneous ones) are generally introduced by a spike potential, it was supposed that the slow inward current (SIC) underlying them is mediated via voltage-dependent ionic channels. This was previously demonstrated by Gola[7]. The upper left curve in Fig. 1 demonstrates the I–V characteristics of an untreated MCC neuron, as recorded during a voltage ramp. The hysteresis on the downstroke of depolarization can be ascribed to a slowly activating outward potassium current. Hyperpolarization activated only some leakage current. On superfusion of 50 mM PTZ (upper mid curve in Fig. 1), an inward current appeared (sometimes together with a moderate depolarization), which attained an apparent maximum at −20 mV. Then this went over into an outward current with decreased final amplitude and without hysteresis. At the beginning of the depolarization, spike artifacts from poorly clamped regions of the cell appeared.

Then 30 mM TEA-Br was added to the Ringer to depress most of the outward potassium currents which may exert a masking effect on the SIC. No outward current appeared on depolarization under these conditions, and the negative slope resistance (NSR) region of the I–V curve showed the SIC in its total amplitude (upper right curve). It began to activate at −35 mV, became maximal at −20 mV and showed a reversal at +20 mV membrane potential. This state of the neuron, when recorded in current clamp mode, is already strongly convulsive: PDSs follow each other at irregular intervals. The spike potentials, initiating the PDS or riding on its crest were widened due to the presence of PTZ and TEA.

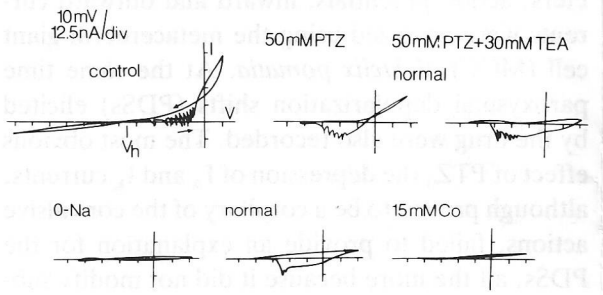

Fig. 1. Membrane currents of a neuron (MCC) exposed to PTZ alone and with TEA (the concentrations indicated are the same for all figures throughout). Effects of sodium withdrawal and 15 mM $CoCl_2$. Normal refers to complete Ringer with no agents except PTZ and TEA. Calibration top left. Notations for Figs. 1–6 are the same. Figures consisting of more than one column are understood to be line-consecutive.

The effect of sodium withdrawal

Since it was plausible that at least part of the SIC is carried by sodium ions, the effect of sodium withdrawal was tested. Omission of sodium led, at the neuron shown in Fig. 1, to a complete but reversible disappearance of the SIC. In this case leakage current was minimal.

The role of sodium ions as charge carriers proved to be rather variable. Fig. 2 shows neuron RPa3 where, despite the presence of TEA, the SIC on the I–V curve was followed by an outward one. In Na-free Ringer the SIC was reduced only at lower depolarizations, up to 0 mV. Beyond that level it remained strong and its maximum was shifted from 0 to +20 mV. It was supposed that besides sodium ions, there could be other participants in this current. These are most probably calcium ions, but Tris ions (replacing Na in the Ringer) could also penetrate through channels which had lost their selectivity under the influence of PTZ. In another experiment (Fig. 5), when superfusion of PTZ and TEA resulted in a 2-humped inward current, omission of sodium abolished only the first less positive maximum and had little effect on the second one (which appeared at higher depolarizations). In 1 case (Fig. 3, MCC), when withdrawal of sodium ions had no effect, the SIC was completely abolished by 8 mM $NiCl_2$. This represents, however, a minority of cases.

Since participation of sodium ions in the SIC seemed in most cases indisputable, it could not be excluded that part of it is conveyed through tetrodotoxin (TTX) sensitive 'fast' sodium channels. But, as can be seen in Fig. 4, 10 μM TTX failed to modify the I–V characteristics even after 28 min

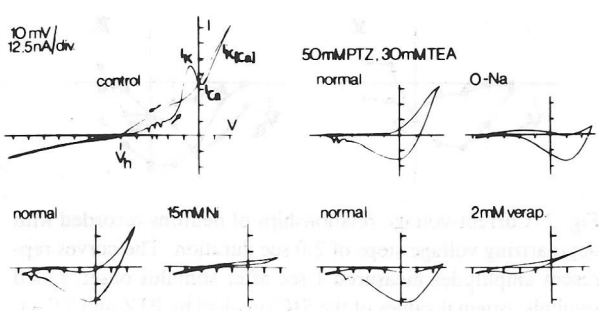

Fig. 2. Membrane currents of a neuron (RPa 3) exposed to PTZ and TEA. Effects of sodium withdrawal, 15 mM Ni^{2+} and 2 mM verapamil. Notations of the current components are usual.

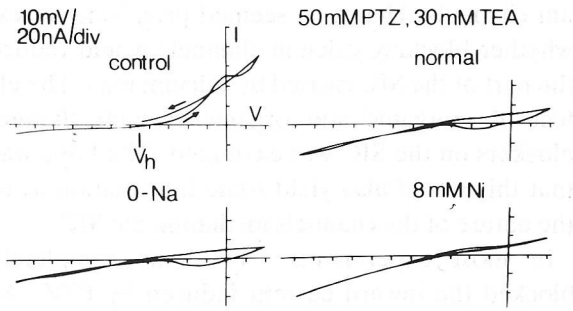

Fig. 3. Membrane currents of a neuron (MCC), exposed to PTZ and TEA. Effect of 8 mM $NiCl_2$ and sodium withdrawal.

of application. On the same cell (an MCC), withdrawal of sodium depressed the SIC to a small fraction of the original. There are some observations[15] confirming that sodium channels in Helix neuron membrane are TTX resistant.

The experiments detailed above yielded some evidence for the role of sodium ions in the SIC. However, it also became obvious that this role is not exclusive, and that other ions, like calcium, may also participate. Experiments with calcium-deficient solutions would have been necessary to prove this, but as under such conditions the behaviour of the membrane is disturbed, the problem required a different approach.

The effects of calcium channel blockers

Calcium ions penetrate the membrane via calci-

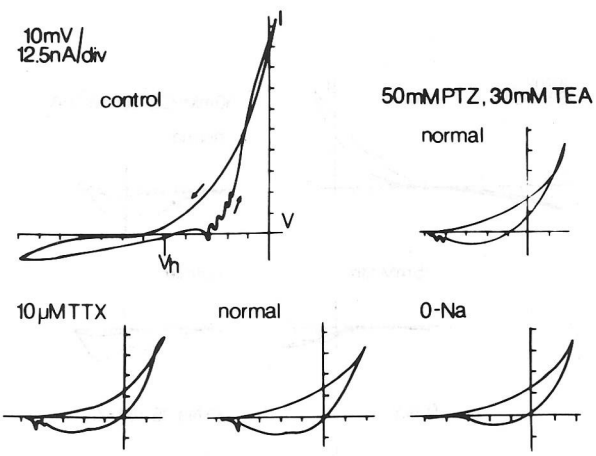

Fig. 4. Membrane currents of a neuron (MCC) exposed to PTZ and TEA. Effects of 10 μM tetrodotoxin (TTX) and sodium withdrawal. Normal refers to complete Ringer with no agents except PTZ and TEA.

um channels. Hence, it seemed promising to test whether blocking calcium channels would reduce the part of the SIC carried by calcium ions. The effect of inorganic and organic calcium channel blockers on the SIC was examined. The hope was that this would also yield some information as to the nature of the channels mediating the SIC.

In most experiments, Ni^{2+} ions completely blocked the inward current induced by PTZ. As seen in Fig. 2, 15 mM $NiCl_2$ completely abolished the SIC. Later it turned out that Ni^{2+} was also effective in 8 mM, as seen in Fig. 3. This effect was reversible except for a few cases.

In the neuron shown in Fig. 2, the slow inward current was followed by an outward current not blocked by TEA in the applied concentration. On the addition of 15 mM Ni^{2+}, not only the SIC but also the outward current disappeared. This suggests that the SIC had a strong Ca-dependent component and the outward current was a consecutive Ca-dependent K current. The components of the outward current could also be distinguished on the I–V curve of the untreated neuron (first curve in Fig. 2, see marks).

With cobalt and manganese ions (both at a concentration of 15 mM), the block of the SIC was as complete as with nickel (Figs. 1 and 5). In Fig. 5 there was a component of the SIC which was Na-dependent and was not blocked by Mn alone.

Organic calcium antagonists were effective in far smaller concentrations, although they did not produce a total block. This is in accordance with observations on naturally existing inward currents of Helix[18]. One mM diltiazem and verapamil depressed the SIC by about 50% (Fig. 6, RPa 77). Verapamil in 2 mM exerted a stronger effect (Fig. 2, last curve) but this was not a total block either. It took about 30 min for the depression caused by these drugs to develop and it was always more pro-

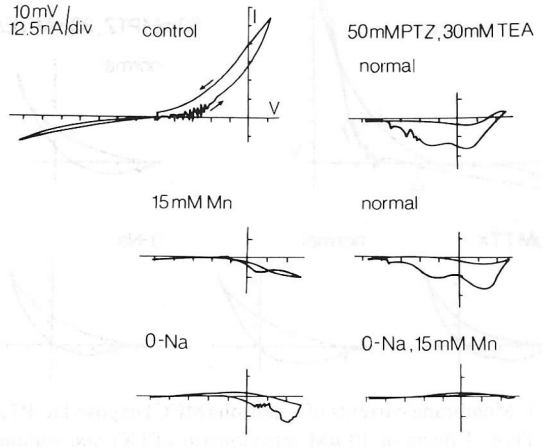

Fig. 5. Membrane currents of a neuron (MCC) exposed to PTZ and TEA. Effects of sodium withdrawal and $MnCl_2$.

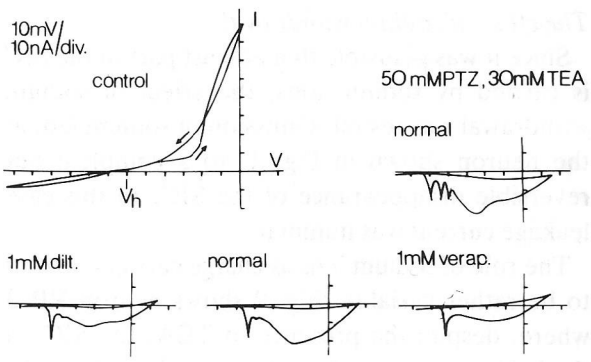

Fig. 6. Membrane currents of a neuron (RPa 77) exposed to PTZ and TEA. Effect of 1 mM diltiazem and 1 mM verapamil.

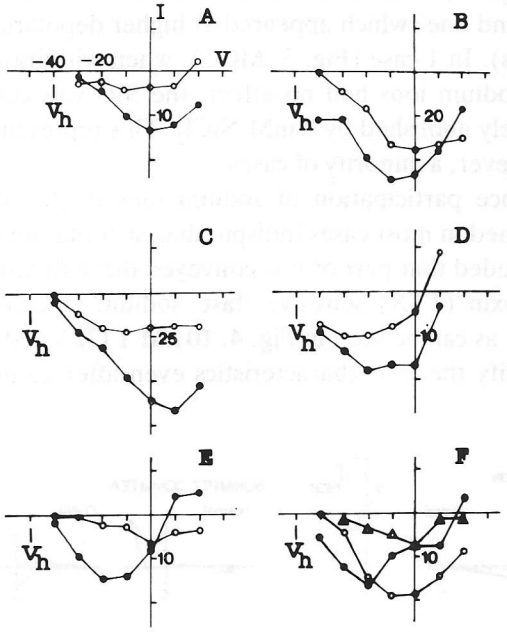

Fig. 7. Current-voltage relationships of neurons recorded with depolarizing voltage steps of 2.0 sec duration. The curves represent amplitudes measured 1 sec after stimulus onset. Filled symbols: original values of the SIC (evoked by PTZ and TEA). Empty symbols: SICs modified by sodium withdrawal (A, B), verapamil (C), diltiazem (D), Ni^{2+} ions (E) and Mn^{2+} ions (F; circle: 8 mM, triangle: 15 mM Mn^{2+}).

nounced at higher depolarizations. The effect was reversible, albeit slowly.

In our experiments, the SIC was also evoked by application of depolarizing steps lasting 2.0 sec. The amplitude of the SIC was measured at 1 sec from the onset of the pulse and I–V curves were plotted from them by hand.

Omission of sodium ions diminished the SIC at lower depolarizations in every case, and sometimes over the entire voltage range (Fig. 7A). In some experiments, however, Na-free Ringer resulted, at larger depolarizations, in a stronger SIC than with normal Ringer (Fig. 7B). In the former case, the reversal potential of the SIC was more negative than with full sodium; in the latter case it was farther in the positive direction.

Verapamil, 1 mM, exerted essentially the same effect as seen with ramp pulses. It depressed the SIC over the entire voltage range as diltiazem did (Fig. 7C and D).

Inorganic Ca channel blockers showed an effect which also depended on membrane potential. At lower depolarizations, a strong depression was invariably found (Fig. 7E and F). In the positive potential range, however, the blocking effect seemed to disappear and even some enhancement of the SIC was seen. The potential at which depression ceased depended on the concentration of the blocking ion, as shown for Mn^{2+} in Fig. 7F. It was supposed that the SIC was not really enhanced but further unmasked when a Ca-dependent (and TEA-resistant) potassium current disappeared due to Ca entry being blocked.

DISCUSSION

Bursting pacemaker neurons (BPN) of Aplysia and Otala[1–3,6] have become widely accepted models of the convulsive state in molluscs. One of the first findings reported was that bursting pacemaker activity (BPA) relies upon a slow voltage-insensitive sodium conductance, resistant to TTX. Voltage clamp analysis revealed that moderate depolarizations provoked a slow, hardly inactivating inward current (SIC), present in the I–V characteristic as a negative slope resistance region (NSR)[7,19]. Its occurrence proved to be the sine qua non of the bursting pacemaker activity (BPA), whether this was a natural activity pattern or provoked by convulsive agents. In view of its ionic basis, it was supposed by Smith et al.[15] that it is carried by sodium ions, since withdrawal of sodium stopped BPA while high external calcium concentration enhanced it. Eckert and Lux[4] were the first to analyze the ionic mechanism of SIC in Helix. They concluded that SIC is carried by calcium ions. This was confirmed by the findings of Hagiwara and Byerly[10], who found that blockers of the calcium channel abolish SIC whereas Ba ions, which penetrate calcium channels easily, augment the SIC considerably. Participation of a calcium component in SIC in Aplysia was reported by Johnston[11]. Using a metallochrome dye, Arsenazo III, he showed that during bursts, the intracellular calcium concentration increased rapidly and this was prevented, together with the bursts, by calcium channel blockers. It was, however, noteworthy that SIC began to activate at membrane potentials of –65 to –55 mV (holding potential was –70 mV), while calcium increase could not be detected at membrane potential values below –45 mV. It was also observed that omission of sodium ions abolished SIC.

According to Gorman et al.[9], lack of sodium ions activates a large outward current, which counterbalances SIC, thus creating an apparent sodium dependence. In our experiments, after complete block of outward currents sodium deficiency was able to abolish or at least to reduce SIC, with some exceptions for positive potentials. Here it seems interesting that according to Swandulla and Lux[17], injection of calcium ions into the neuron gives rise to a non-specific cationic conductance, resulting in BPA.

None of the studied neurons are bursting pacemakers and under normal conditions neither SIC nor cyclically varying potassium conductance is displayed by them. On the effect of PTZ, however, they appear and this results in a membrane behavior comparable to that of the BPNs. This, of course, may not mean their full identity is revealed.

On application of 50 mM PTZ its actions can be followed on the I–V characteristics taken by use of ramp pulses: a well pronounced depression of the outward currents and appearance of the NSR re-

gion indicating the SIC. Addition of 30 mM TEA to the perfusion fluid usually eliminated all the active outward currents and made the SIC more pronounced by unmasking it.

The analysis of the ionic dependence of the SIC did not prove to be easier than it was at the BPNs. Omission of the sodium ions led to complete disappearance of the SIC at some neurons (Fig. 1), to marked depression by others (Figs. 2, 4 and 5), or was quite ineffective (Fig. 3). It is noteworthy that SIC was reliably TTX resistant (Fig. 4), in agreement with other reports.

Depolarizing with long voltage pulses gave a somewhat different picture. Withdrawal of sodium ions (Fig. 7A and B) resulted in marked depression of the SIC from 10 to 50 mV depolarization but, as in Fig. 7B, at or over 50 mV depolarization SIC showed only moderate or even no voltage dependence and kept the amplitude near the maximum. The I–V curves taken with and without sodium showed an intersection in such cases.

The remaining current under conditions of Na deficiency could be carried by calcium ions. This probably holds for the experiment shown in Fig. 3, in which $NiCl_2$ completely abolished the SIC, not influenced by lack of sodium. Essentially the same was done by $MnCl_2$ although, as seen in Fig. 5, sodium deficiency depressed the first hump of the SIC severely.

As to the ionic basis of SIC, one is led to conclude that both sodium and calcium ions participate in varying proportions. In the case of 2-humped SICs, the first hump seemed to rely on the sodium current and the second one on the calcium current. Sodium withdrawal caused a greater depression in the first maximum (Fig. 5), while calcium channel blockers had this effect on the second one. Although MCCs represent quite a homogeneous population, and the large neurons studied, RPa 2, 3, 5 and 77 do not vary greatly, considerable differences may occur in the ionic basis of their SICs evoked by PTZ. Some predominance of the calcium component could be distinguished on autumnal specimens. Thus, SICs displayed a wide spectrum from pure sodium to pure calcium dependence through more transitional forms. This spectrum showed no relation to individual neurons and the most important properties of the SIC were the same throughout.

In any case, in order to answer the question: what is carrying current after removal of sodium ions and blockage of the calcium channels?, one must inevitably take the Tris ion into account, replacing sodium in sodium-deficient Ringer. Its radius is not sufficiently large to prevent penetration through channels which had lost selectively among cations, due to the action of PTZ.

The identification of channels transmitting the SIC poses a difficult problem. As shown by us and others[13], SIC is TTX resistant. Among channel blockers, only Ca channel blockers were active on it, being organic or inorganic[18,22]. In contrast to Na deficiency, which was sometimes without effect, calcium channel blockers always had some action on the SIC and this ranged from light depression to complete blockage. This may suggest that calcium channels play the main role in transmitting cations of the SIC. In cases when both sodium withdrawal and calcium channel block alone are able to reduce the SIC, one is led to conclude that sodium ions penetrate at least partly via calcium channels (Figs. 1 and 3). Since TTX fails to block slow Na channels[13], the possibility remains that sodium ions are carried through some kind of channel.

It may be assumed that in the membrane, impaired by the drugs applied, cationic channels lose their strict specificity and capability to cause inactivation. They can be aligned into a continuous spectrum ranging from mostly sodium-conducting to mostly calcium-conducting ones with some intermediate range of channels transmitting both ions to more or less the same extent. They, however, retain 2 common features: cation specificity and voltage dependence. All these are favorable for SIC if the membrane becomes depolarized.

An alternative explanation of the SIC induced by PTZ may be some kind of unspecific cation conductance observed by Swandulla and Lux[17]. This conductance is activated by Ca, either entering the neuron or released intracellularly by PTZ[16]. The crucial role of Ca in epileptogenesis is indisputable. Based on our observations on the effect of Ca channel blockers, one cannot avoid the conclusion that Ca channels and the current transmitted by them must be taken into account when explaining abnormal neuronal discharges.

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