Blockade of voltage-dependent and Ca^{2+}-dependent K^+current components by internal Ba^{2+} in molluscan pacemaker neurons

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Summary. Internal Ba^{2+} blocks both the Ca-dependent component, as well as the voltage-dependent component of the K^+ current in molluscan pacemaker neurons.

Since the discovery that an increase in intracellular Ca^{2+} can activate changes in membrane permeability to K^+ ions in red blood cells some 10 years ago[1,2], Ca^{2+} mediated K^+ permeability changes have been found in a variety of excitable and nonexcitable cells[3]. Recent evidence suggests that these changes may play a role in the repolarization of some action potentials[4,5] in the control of long lasting after-potentials[5-7] in pacemaker potentials[8] and in sensory receptor potentials[9,10]. The mechanism by which a change in intracellular Ca^{2+} can increase membrane K^+ permeability is still not well understood. In molluscan neurons where Ca-dependent K^+ currents have been extensively studied, it has been shown that a small increase in intracellular Ca^{2+} can produce a sizeable increase in K^+ current[8,11]. There is also evidence that the Ca-dependent K^+ current is not caused by a simple neutralization of negative charges on the internal surface of the membrane because large changes in either internal Mg^{2+} or H^+ ions do not have the same effect[12]. It has been suggested that Ca^{2+} binds with some membrane site that controls the movement of K^+ ions through the membrane[3]. It is not clear, however, whether Ca^{2+} opens new K^+ channels or exerts its effect specifically on voltage-dependent K^+ channels in excitable membranes. Sr^{2+}, Ba^{2+} and several other divalent cations have been reported to be effective in increasing K^+ permeability when injected into molluscan neurons[3]. There is evidence, however, that Ba^{2+} ions interfere with the movement of K^+ ions through voltage-dependent K^+ channels in some cells[13,14]. To investigate possible differences in Ca-dependent and voltage-dependent K^+ currents, we have compared their behavior when Ba^{2+} ions are injected into molluscan neurons. We report here that Ba^{2+} ions block Ca-dependent, as well as voltage-dependent K^+ currents in these cells.

The abdominal ganglion of *Aplysia californica* was dissected to expose the pacemaker neuron R-15[15]. The cell was impaled with up to four microelectrodes for recording membrane potential, passing current and injection of Ca^{2+} and Sr^{2+} or Ba^{2+} ions. The recording and current electrodes were filled with 3 M KCl and the injection electrodes were filled with equal concentrations (0.5 M) of KCl and $CaCl_2$, $BaCl_2$ or $SrCl_2$. All ions were electrophoretically injected in the voltage clamp mode so that there was no change in net flow of current across the membrane during injection. R-15 has no true resting potential. The range of the 'pacemaker potential' is between about −70 mV and −30 mV. All cells were held at −50 mV to simplify comparison. At this potential a small (circa 5–15 nA) inward current had to be supplied. The cell was bathed in artificial sea water (ASW) which contained (in mM/l) 477 NaCl, 10 KCl, 10 $CaCl_2$, 55 $MgCl_2$ and 15 tris-HCl at pH 7.8 and was maintained at a constant temperature of 16 °C. Substitution of Mg^{2+} or Ba^{2+} for Ca^{2+} in ASW solutions

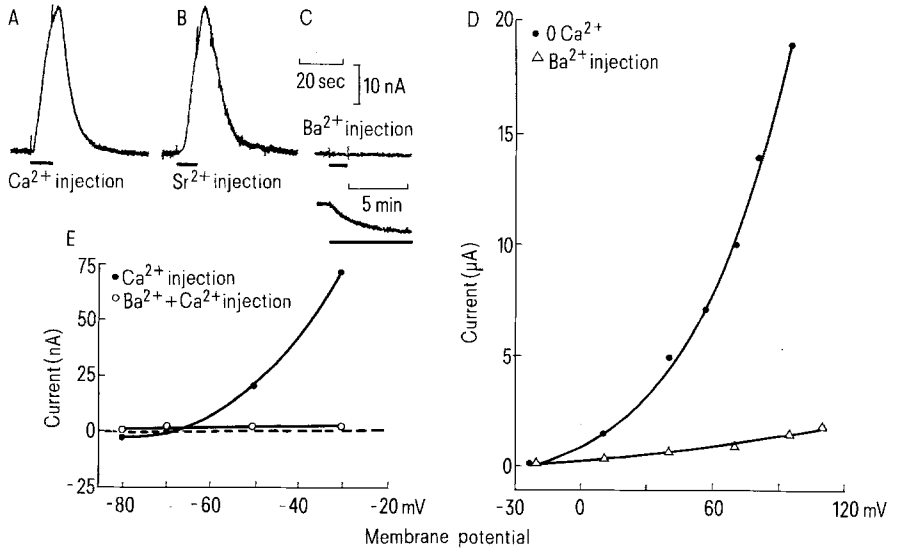

Fig. 1. Effects of intracellular Ba^{2+} ions on Ca-dependent and voltage-dependent K^+ currents in the *Aplysia* pacemaker neuron R-15. A: Outward K^+ current activated by iontophoretic Ca^{2+} injection (3 μC) under voltage-clamp conditions. B: Outward K^+ current activated by Sr^{2+} injection (6.0 μC). C: Ba^{2+} injection (3 μC) does not activate the K^+ current (above) but a prolonged Ba^{2+} injection (120 μC) leads to an apparent inward current (below). Note the different time scale. The holding potential in A–C was −50 mV; the bars indicate the period of Ca^{2+}, Sr^{2+} or Ba^{2+} injection. D: Peak values of *voltage-dependent outward K^+ currents* in zero Ca^{2+} solution activated by 200 msec voltage steps to different membrane potentials, before (circles) and after Ba^{2+} injection (triangles, 240 μC) (holding potential −50 mV). E: Peak values of *Ca-dependent K^+ current* activated by iontophoretic Ca^{2+} injections versus membrane potential (≅ holding potential) before (●) and after injection of Ba^{2+} ions (○, 240 μC) into the cell. Same cell as in D. Note the difference in current scales in D and E.

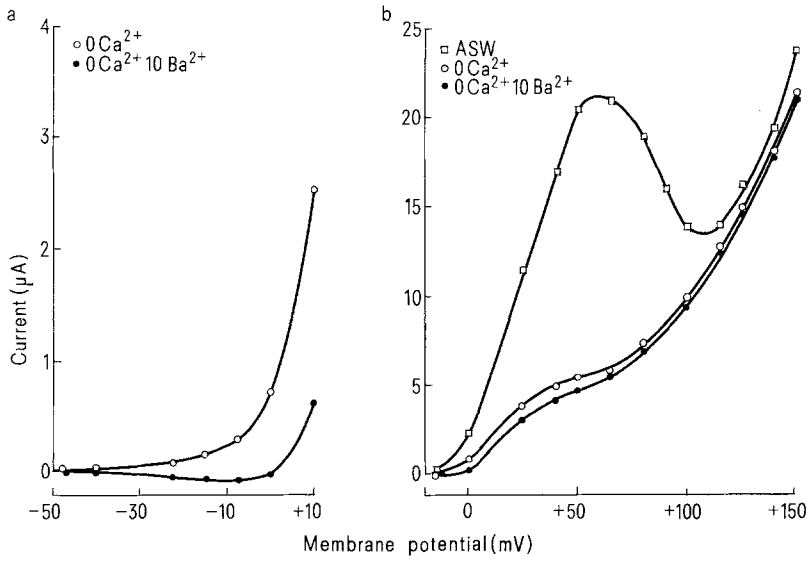

Fig. 2. Effect of extracellular Ba^{2+} ions. A: Current-voltage plot in zero Ca^{2+} solution (○) and in zero Ca^{2+}, 10 mM Ba^{2+} solution (●). The addition of Ba^{2+} ions induces an inward current which is most obvious in the voltage range between −40 and 0 mV. B: Current-voltage plot from the same cell at more positive membrane potentials to show the N-shaped relationship (□) in normal ASW. The N-shape is greatly diminished in zero Ca^{2+} solution (○) and is not restored by the addition of 10 mM Ba^{2+} (●). The experimental points in A and B represent measurements of the peak current produced by a 200 msec voltage step to various potentials from a holding potential of −50 mV.

was done on an equimolar basis so that divalent ion concentration remained constant.

Injection of Ca^{2+} activates a current carried by K^+ ions whose amplitude depends on the magnitude of the charge passed through the electrode and the membrane holding potential. At a holding potential of −50 mV this current was always outward (figure 1, A). An injection of Sr^{2+} ions had similar effect (figure 1, B). In contrast, Ba^{2+} injection, for the same duration, had no apparent effect on membrane current (figure 1, C) whereas longer Ba^{2+} injection produced a small, slow inward current (figure 1, C). This inward current is associated with a decrease in membrane conductance suggesting that an increase in intracellular Ba^{2+} may reduce membrane K^+ permeability.

To study the effects of Ba^{2+} ions on different components of the K^+ current, Ba^{2+} ions were injected into the cell prior to all tests. There are, however, 2 complicating factors in attempting to analyze separately the effects of Ba^{2+} on the Ca-dependent and voltage-dependent components of the K^+ current. 1. Ba^{2+} ions are removed from the cytoplasm, but about 10 times more slowly than Ca^{2+} ions (unpublished observations, M.V. Thomas and A.L.F. Gorman). To circumvent this problem, prolonged and repeated injection of Ba^{2+} ions were used and all tests were done either during or shortly after Ba^{2+} injection. 2. At positive membrane potentials in normal ASW voltage-dependent Ca^{2+} channels, as well as K^+ channels are activated and the resulting increase in intracellular Ca^{2+} can activate the Ca-dependent K^+ current[18]. This problem can be partially circumvented. To study the voltage-dependent K^+ current, we have used brief (200 msec) depolarizing steps to various positive potential levels in the absence of external Ca^{2+} which reduces substantially any increase in internal Ca^{2+} through the voltage-dependent Ca^{2+} channel in these cells[16]. The magnitude of this current becomes appreciable at potentials more positive than about −20 mV (figure 1, D). The Ca-dependent K^+ current was studied by using repeated, identical injection of Ca^{2+} ions at different holding potentials more negative than about −20 mV. Results from these 2 types of experiments are not directly comparable because the 2 components of the K^+ current have to be studied at different voltage ranges, nonetheless, they provide a means of investigating the effects of internal Ba^{2+} on the 2 components separately.

The effects of internal Ba^{2+} on the voltage-dependent and on the Ca-dependent K^+ currents are shown in figure 1, D and E. The closed circles in figure 1, D, show a plot of the total outward current activated by brief membrane depolarizations in zero Ca^{2+} ASW. This current is carried primarily by K^+ ions. Injection of Ba^{2+} ions into the cell reduced substantially the voltage-dependent K^+ current at all potential levels (open triangles, figure 1, D). The closed circles in figure 1, E, show a plot of the Ca-dependent K^+ current activated by Ca^{2+} injections in the same cell at different holding potentials. The magnitude and polarity of this current depends on the holding potential and has a reversal potential of about −70 mV. This is close to the average K^+ equilibrium potential for these cells estimated with K^+ sensitive electrodes[17]. Injection of Ba^{2+} ions also reduced this current at all potential levels (open circles) without effecting the reversal potential.

These findings may help to explain many of the effects of *external* Ba^{2+} on excitable cells. The increase in amplitude and duration of action potential in *external* Ba^{2+} in several tissues has been related to the ability of Ba^{2+} ions to carry charge through the voltage-dependent Ca^{2+} channels and to its inhibitory effect on K^+ channels[18-20]. Figure 2, A and B, shows an experiment in which external Ca^{2+} was replaced by an equal quantity of either Ba^{2+} or Mg^{2+}. Mg^{2+} was used because it does not penetrate through voltage-dependent Ca^{2+} channels and, therefore, is a suitable replacement for a zero Ca^{2+} ASW solution. The curve drawn through the open circles in figure 2, A, was taken under conditions where external Ca^{2+} was replaced by Mg^{2+} and shows the voltage-dependent K^+ current activated by brief depolarizing steps to positive membrane potentials. When Ba^{2+} is used in place of Mg^{2+} (closed circles, figure 2, A) the voltage-dependent K^+ current is reduced and an inward current develops between about −40 mV and 0 mV. A likely explanation is that this inward current is carried by Ba^{2+} ions through the Ca^{2+} channel[18]. The curve drawn through the open squares in figure 2, B, shows the N-shaped current-voltage relation at very positive membrane potentials in the same neuron which characterizes these cells in normal external Ca^{2+}. The local minimum of net outward current between about +90 mV and +110 mV has been related to the reduction of the Ca-dependent component of the total K^+ current as the membrane potential approaches the Ca^{2+} equilibrium potential. The net inward flux of Ca^{2+} ions and, therefore, the Ca^{2+}-dependent K^+ current, is sharply reduced in this region. Replacement of Ca^{2+} with Mg^{2+} (open circles) removes a

substantial fraction of the total outward current between −20 mV and +100 mV which has been identified as a Ca-dependent K^+ current[21,22]. Substitution of Ba^{2+} for external Ca^{2+} did not restore the Ca-dependent component and further reduced the total outward current at all potential levels (closed circles, figure 2, B).

Our results demonstrate that internal Ba^{2+} blocks the Ca-dependent, as well as the voltage-dependent K^+ current. Out findings agree with recent results from squid axon[23,24] where internal perfusion of Ba^{2+} ions blocks the voltage-dependent K^+ current and with a variety of results from muscle fibres[13,14,18]. Our finding that internal Ba^{2+} blocks the Ca-dependent K^+ current is not consistent with previous reports from other molluscan neurons[3]. We have no explanation for this conflict in results except for the possibility that cells differ in their response to Ba^{2+} ions. The present results with the effects of external Ba^{2+}, as well as previous findings with other nerve cells[19,20,22,25] are, however, more consistent with our Ba^{2+} injection results than with the possibility that internal Ba^{2+} activates a Ca-dependent K^+ current. We have no information about the absolute internal Ba^{2+} concentration necessary to block K^+ channels. We find, however, that the Ba^{2+} block occurs at internal concentrations that do not alter substantially the Ca-dependent K^+ current reversal potential and, therefore, at concentrations that presumably do not alter the intracellular K-concentration. Unless internal Ba^{2+} exchanges for some other cation (e.g. Na^+ or Mg^{2+}) the concentration necessary to block K channels must be small. It has been suggested that Ba^{2+} ions can enter but not pass through the K^+ channel because of steric factors[13,14]. Our finding that *both* components of the K^+ current are blocked by internal Ba^{2+} suggests that the 2 components of the K^+ current may represent different aspects of the same K^+ channel.

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Pigment aggregation by melatonin in the retinal pigment epithelium and choroid of guinea-pigs, *Cavia porcellus*

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Summary. The treatment of pigmented guinea-pigs with melatonin aggregates pigmented cells in the retinal pigment epithelium and choroid of the eye. This suggests that melatonin may regulate eye pigmentation in vertebrates.

Melatonin (N-acetyl-5-methoxytryptamine), a derivative of the putative neurotransmitter, serotonin, was isolated and identified from the bovine pineal gland[2,3]. Subsequently, melatonin was found in the pineal gland of all the animals studied[4,5]. Pineal melatonin is secreted into the circulation with a high activity at night and a low or undetectable activity in the daytime[6-10], and is suggested to have an antigonadal function[11-13]. In addition to the well-established antigonadal function of melatonin, in the lower vertebrates such as the frog, melatonin is the best melanophore aggregating agent known[3]. However, melatonin appears to have little effect on mammalian pigmentation[14]. Recently, melatonin has been identified in the nervous tissue, retina, Harderian gland and intestine of chicks and rats[15-18]. The function of melatonin in the above tissues remains to be elucidated. In this report, the effect of melatonin on the retinal pigment epithelium of the guinea-pig was investigated, and melatonin was found to have a potent pigment aggregating action on the retinal pigment epithelium and choroid of the eye.

Materials and methods. Male and female pigmented (coloured) guinea-pigs *Cavia porcellus* (200–450 g), obtained from the animal house, Faculty of Medicine, University of Hong Kong, were used. All experiments were performed under 300–400 lux illumination and a temperature of 23–26 °C. The animals were anaesthetized with 60 mg/kg sodium pentobarbital (50 mg/ml, Sigma). The corneas of the 2 eyes were removed and the lens were taken out. A pair of blunt forceps was used to remove as much vitrous humor as possible. Operated animals were placed in a prone position. Melatonin solution (1 ng/ml, 10 ng/ml, 100 ng/ml and 1 µg/ml in 0.9% saline) was perfused into one of the operated eye cups while the other eye cup was perfused with carrier, both at a rate of 1.5 ml/min. There were at least one left eye cup and one right eye cup perfused by melatonin solution in each group. After 20 min of melatonin or carrier perfusion (20 min was used because it takes 20–90 min for the completion of physiological color changes in lower vertebrates), the eyes were removed and fixed in Bouin's solution. The fixed materials were embedded in paraffin, sectioned at 6 µm, stained with hematoxylin and eosin and observed under light microscope. The above experiment was repeated with N-acetylserotonin (1 µg/ml, Sigma) and 5-hydroxytryptamin (1 µg/ml, Sigma).

In other experiments, guinea-pigs were anaesthetized and 50 µl of melatonin solution (2 µg/ml, 20 µg/ml, 200 µg/ml and 2 mg/ml) were injected into the posterior chamber of