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Effects of Barium on Delayed Rectifier Potassium Current in Bullfrog Sympathetic Neurons Pretreated with Wortmannin

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The effect of barium (1 mM) on a delayed rectifier-type potassium current was examined in bullfrog sympathetic neurons. An M-type potassium current was eliminated by pretreatment of the cells with a microbial product, wortmannin (10 μM). An A-type potassium current was continuously inactivated by setting a holding potential at −65 mV. In treated cells ($n = 10$), the delayed rectifier at 0 mV averaged 2200 ± 107 pA in the presence of barium (1 mM) as compared to 2308 ± 110 pA in the controls, and 2085 ± 103 pA after washing out the barium. It is concluded that the delayed rectifier is insensitive to barium blockage in amphibian autonomic neurons.

Key Words: Potassium current, Delayed rectifier, M-current, Barium, Wortmannin, Sympathetic ganglia

INTRODUCTION

Wortmannin is a microbial product which has been identified as a highly potent inhibitor of phosphatidylinositol 3-kinase and myosin light chain kinase [23, 24]. We have previously shown that wortmannin, in high concentrations (e.g., 10 μM), has direct channel blocking activity on a voltage-dependent potassium current (termed M-current) in bullfrog sympathetic neurons [18]. Blockage occurred slowly and recovery from the block was even slower, implying that wortmannin either acted within the cell, or at the pore region of the channel but the rate constant for binding and unbinding is extremely slow [18]. In any case, once the cells were treated with wortmannin (10 μM) for 30-60 min, the M-current was almost non-existent for the next 30-60 min [18].

We hypothesized that the actions of barium on a delayed rectifier potassium current could be examined in sympathetic neurons pretreated with wortmannin, for the following reasons: first, barium is known as a potent M-current blocker [1, 5, 20] with an IC_{50} value of about 100 μM (Tokimasa, unpublished data); second, barium (1-2 mM) has been used widely to study the properties of a delayed rectifier in the absence of the M-current (reviewed in [4, 10, 14]); therefore, the actions of barium on delayed rectifiers have not yet been reported not only for sympathetic neurons but also for other vertebrate neurons (reviewed in [3, 11, 12, 17]). We report here that the addition of barium (1 mM) to the superfusate did not significantly reduce the amplitude of the delayed rectifier in bullfrog sympathetic neurons.

MATERIALS AND METHODS

All the experiments in the present study were carried out at 22-24℃. Statistics are expressed as mean ± SEM for the cells tested. Differences were considered to be significant if $p < 0.05$, using paired or unpaired Student's *t*-test.

After decapitation and pithing, sympathetic ganglia were removed from small (200-250 g) bullfrogs (*Rana catesbeiana*) and placed in normal Ringer's solution composed of (mM):128 NaCl, 2.4 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$ and 10 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid (HEPES). A pH of 7.4 was achieved with NaOH. Each ganglion was cleaned of connective tissue, cut in half, then incubated for 3 h at 32℃ in

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Ringer's solution containing the digestive enzymes trypsin (Sigma type-11, 2.5 mg ml^{-1}) and collagenase (Sigma type-1A, 0.5 mg ml^{-1}). After digestion, the fragments of ganglion were placed into Leibovitz's L-15 medium (Gibco 320-1415); 20% fetal bovine serum (Gibco 200-6140 AG) was added and the mixture diluted to 80% with water, followed by gentle pipetting until dissociated ganglion cells were recognized under the dissection microscope. The ganglion cells were suspended in the modified L-15 medium and stored for 2-10 days at 4-5℃ [15, 16].

Cells were pretreated with wortmannin (10 μM) for 3 h then subjected to the electrophysiological experiments with no added wortmannin in the superfusate (see below for its composition). The time between pretreatment and start of the electrophysiological experiments ranged from 0.5 to 2 h. Wortmannin was dissolved in dimethylsulfoxide (Sigma), and solutions (10 mM) were stored at −35℃. Cells pretreated only with the vehicle (0.01 vol%) for 3 h were used as a negative control.

Cells were pipetted into the recording chamber (0.5 ml total volume) then continuously superfused (2-3 ml min^{-1}) with Ringer's solution after they settled to the bottom of the chamber. Patch pipettes had a tip resistance of 1-2 MΩ when filled with a solution of the following composition (mM): 120 KCl, 2.5 $MgCl_2$, 0.32 $CaCl_2$ (free calcium brought to 100 nM with 1 mM 1, 2-bis(2-aminophenoxy) ethane-*N*, *N*, *N*', *N*'-tetraacetic acid (BAPTA)[6]), 10 HEPES, 1.15 Na_2ATP and 1.5 Na_2GTP (pH adjusted with KOH to 6.8). A reference electrode was an Ag-AgCl pellet (RC1, World Precision Instruments) placed in the superfusate downstream to the cells from which the whole-cell voltage-clamp recordings were made.

Voltage-clamp recordings were started approximately 5 min after the patch break and results were obtained during the next 30-45 min from cells with a resting potential between −52 and −70 mV and an action potential overshoot greater than 35 mV. After checking the action potential configuration, tetrodotoxin (TTX, 1 μM) and $CdCl_2$ (10 μM) were added to the Ringer's solution. This was the standard solution and will be referred to as the superfusate in the remaining sections of this paper. TTX and cadmium were used to block inward sodium and calcium currents, respectively [15, 16, 22]. $BaCl_2$ (1 mM) was added to the superfusate. When tetraethylammonium chloride (TEA)(10-30 mM) was used, the concentration of NaCl (normally 128 mM) was reduced by the same amount of TEA added, for isotonicity. TEA was used as a channel blocker and hence was a diagnostic tool for delayed rectifier [2, 22].

Outputs (current and voltage) from a sample-and-hold/voltage-clamp amplifier (Axoclamp-2B with an HS2A head stage, Axon Instruments) were digitized through an interface (ITC-16, Instrutech) at 2.5 kHz using commercially available software (Axodata version 1.22, Axon Instruments). This software was also used for subtracting, on-line, a time- and voltage-independent current (usually referred to as the leak current) using the P/4 protocol. Axograph (Axon Instruments, version 3.0) was used for off-line analysis including curve fitting by the simplex sum of squares errors.

The absolute zero current level was indicated beside each current trace in all figures but Fig. 3B. in which a positive (i.e., an upward) current represented an outward current (see [17] for review).

All reagents were obtained from Sigma with the exception of tetrodotoxin from Wako Pure Chemicals and tetraethylammonium chloride from Tokyo Kasei.

RESULTS

Absence of M-current in cells pretreated with wortmannin

Figure 1 shows voltage-clamp recordings obtained from a cell pretreated with wortmannin (10 μM) for 3 h (Fig. 1A) and a negative control cell pretreated only with the vehicle (0.01 vol%)(Fig. 1B). In both cases, the cell membrane potential (holding potential, −65 mV) was changed stepwisely for 0.5 s between −35 and −75 mV with −10 mV increments, and the observed current responses (and command pulses also) were superimposed.

Essentially the same results as seen in Fig. 1A were obtained with 5 other cells. The current-voltage (*I-V*) relationship in the 6 cells was 76 ± 18, 27 ± 5, -2 ± 9, -14 ± 7 and -22 ± 8 pA at −35, −45, −55, −65 and −75 mV, respectively, as indicated by filled triangles in Fig. 2A.

The open triangles in Fig. 2A represent

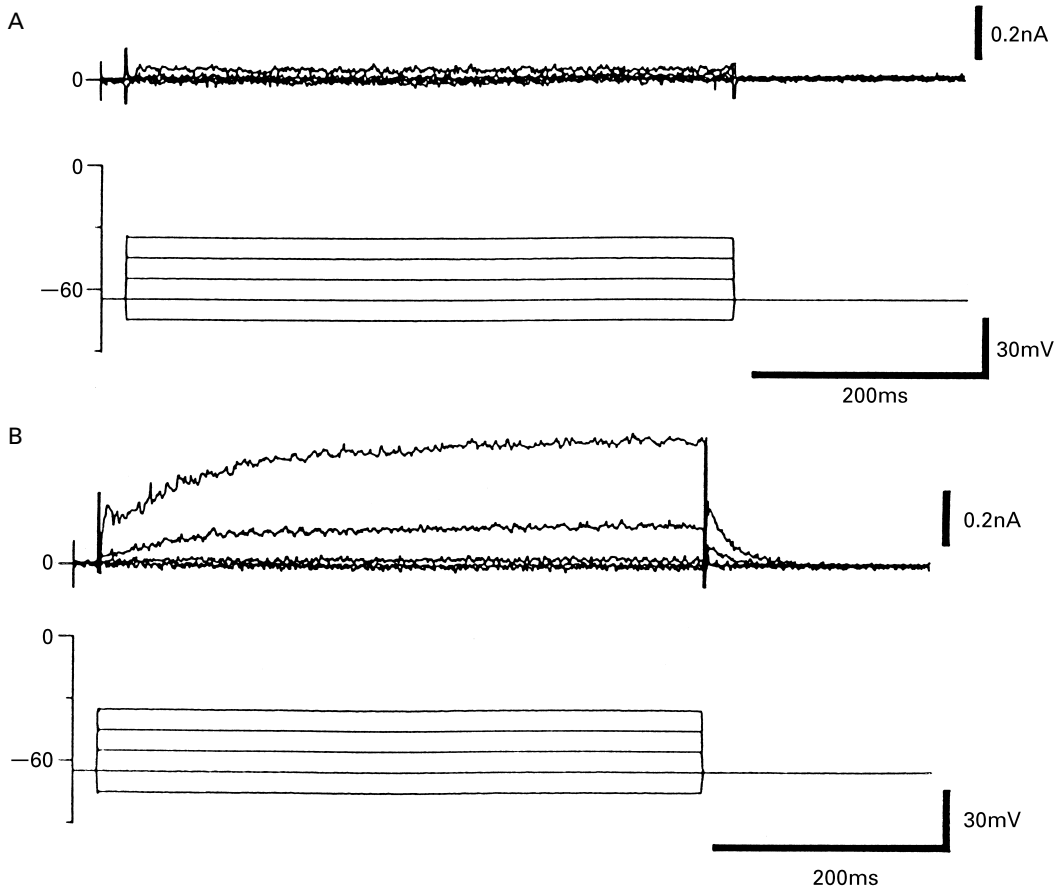

Fig. 1 Wortmannin actions on M-current. Results were obtained from a wortmannin-treated cell (A) and a control cell (B) in which superimposed traces denote currents (upper panel) and command pulses (lower panel). Relaxations on the current trace in (B) are due to the activating M-current during the command pulses followed by the deactivating tail after the termination of the command pulses. In this and in the following figures, the holding potential was −65 mV unless otherwise mentioned.

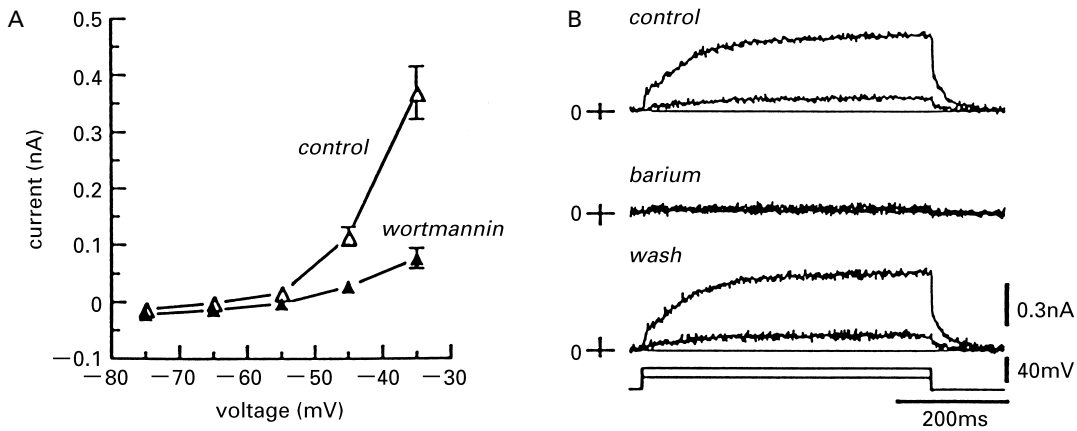

Fig. 2 Barium actions on M-current. (A) *I-V* curves based on the pooled data; filled triangles, wortmannin-treated cells ($n = 6$); open triangles, control cells ($n = 5$). (B) Sample recordings obtained from a control cell showing that barium (1 mM) eliminated M-current at −35 and −45 mV. Top, control (command pulse omitted); middle, barium (command pulse omitted); bottom, wash out. In this and in the following figures, command pulses were omitted in places for the simplicity of the illustration.

results obtained from the control cells ($n=5$). The *I-V* relationship was 370 ± 46, 115 ± 17, 19 ± 6, −1 ± 6 and −12 ± 6 pA at −35, −45, −55, −65 and −75 mV, respectively. Barium (1 mM) eliminated relaxations on the current trace (due to M-current [1, 2, 20, 21]) such as that in Fig. 1B in the 4 cells tested (Fig. 2B); the amplitude of the membrane current was decreased from 117 ± 14 to 26 ± 6 pA when measured at −45 mV. Corresponding values were 508 ± 34 and 59 ± 3 pA when measured at −30 mV. The difference between 27 ± 5 pA at −45 mV in wortmannin-treated cells (Fig. 2A) and 26 ± 6 pA in control cells, but in the presence of barium (1 mM), were not significant (unpaired *t*-test) at the 5% level.

These results were consistent with our previous observations that pretreatment of bullfrog sympathetic neurons with wortmannin (10 μM) resulted in irreversible loss of M-current [18].

Delayed rectifier in wortmannin cells

Figure 3 shows examples of our recordings for delayed rectifier in the present study. The cell membrane was depolarized from the holding potential of −65 mV to 3 different test potentials (−15, −30 and −45 mV) for 0.5 s (Fig. 3A) or to 0 mV for 20 s

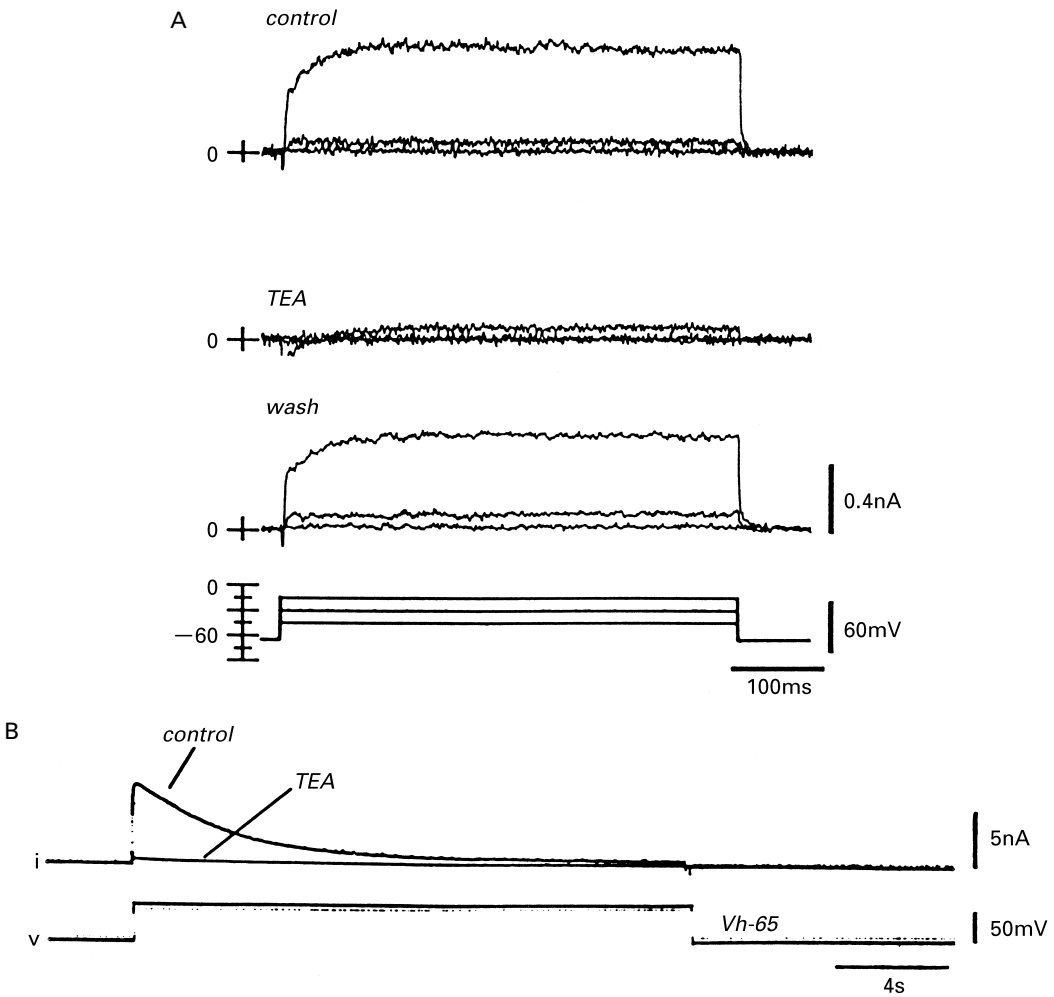

Fig. 3 TEA actions on delayed rectifier. (A) Current traces in response to stepwise depolarizations from −65 mV to 3 different test potentials (−15, −30 and −45 mV) for 0.5 s with P/4 protocol. Top, control; middle, TEA (20 mM); bottom, wash out. (B) Results from another cell. Recordings were obtained on a chart paper (zero current level not shown). Upper panel, control current superimposed by that in TEA (20 mM); lower panel, command pulses from −65 to 0 mV for 20 s.

(Fig. 3B), before and after adding tetraethylammonium (TEA)(20 mM) to the superfusate.

Our findings can be summarized as follows: first, the current underwent inactivation (unlike M-current [1, 2, 18]) of the slow time course (unlike a rapidly inactivating potassium current usually referred to as A-current [1, 2, 9, 19]); second, the inactivation was not complete (unlike A-current [9, 19]), thereby confirming our previous observations that the current has a non-zero steady-state conductance [22]; finally, the current was almost completely blocked by TEA, a channel blocker which has been used commonly for delayed rectifier-type potassium currents among excitable membranes [8], including bullfrog sympathetic neurons [1, 2, 22].

Actions of barium on delayed rectifier

The actions of barium (1 mM) on delayed rectifier were examined by activating the current at 0 mV for 0.5 s. The maximum time between wortmannin pretreatment and start of the whole-cell recordings was 2 h, during which the cells were continuously superfused with a wortmannin-free solution (see Methods). The amplitude of the current, corrected with the P/4 protocol for the leak current (see Methods), was measured at the end of the command pulses. The holding potential was fixed at −65 mV so that the A-current could not be activated from that potential [2, 9, 19]. The absence of the M-current was monitored with command pulses to −45 mV throughout the experiments.

Figure 4 shows an example of the recordings obtained in the present study. The

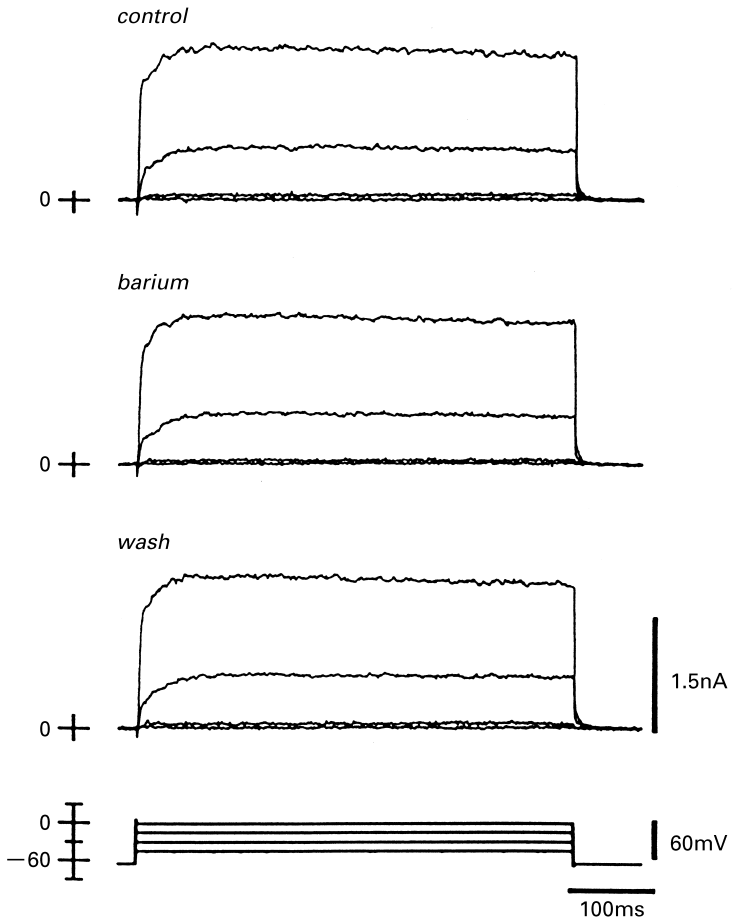

Fig. 4 Barium actions on delayed rectifier. Results were obtained from a single cell before (top), during (middle), and after (bottom) adding barium (1 mM) to the superfusate. P/4 protocol was used for 4 different test potentials (0, −15, −30 and −45 mV), from the holding potential of −65 mV.

amplitude of delayed rectifier was 2085, 1965, and 2040 pA before, during, and after adding barium (1 mM) to the superfusate for 3 min, demonstrating that barium inhibited the current only by about 5% in this particular example. In 10 cells, delayed rectifier averaged 2200 ± 107 pA (range, 1895-2770 pA) in the presence of barium (1 mM) as compared to 2308 ± 110 pA (range, 1945-2925 pA) in the controls, and 2085 ± 103 pA (range, 1790-2560 pA) after washout of barium. Differences were not significant at the 5% level (paired *t*-test) for any pair of samples.

DISCUSSION

These results have provided new and solid evidence that the delayed rectifier-type potassium current was largely insensitive to the block by barium in bullfrog sympathetic neurons.

Composition of the superfusate will be discussed with regard to the absence of aminopyridines (e.g., 4-aminopyridine) and the presence of cadmium. Four-aminopyridine (1-3 mM) has been widely used to block A-current but the drug is also known to block delayed rectifier in a variety of excitable membranes [8]. In order to avoid using 4-aminopyridine, the A-current was continuously inactivated by setting the holding potential at −65 mV [1, 2, 9, 19] throughout the experiments.

We used 10 μM cadmium to block an inward calcium current since otherwise the amplitude of delayed rectifier could not be measured. However, transition metals such as zinc and cadmium have recently been shown to displace the steady-state activation and inactivation curves of A-current to a depolarizing direction in many nerve and muscle cells [7], including bullfrog sympathetic neurons [9]. We examined the actions of barium (1 mM) on delayed rectifier but barium (and strontium also) can pass calcium channels more effectively than calcium [8]. This means that the concentration of cadmium should be low enough to avoid causing displacement in the A-current inactivation curve to potentials more positive than −65 mV, but high enough to prevent influx of barium ions though calcium channels. Our preliminary experiments gave approximately 10 μM as an appropriate concentration. An example is illustrated in Fig. 5 which shows that increasing the concentration of cadmium from 10 to 300 μM did indeed recruit A-current.

Barium (0.1-1 mM) produces membrane depolarizations associated with an increased input resistance of the cell membrane in unclamped bullfrog sympathetic neurons;

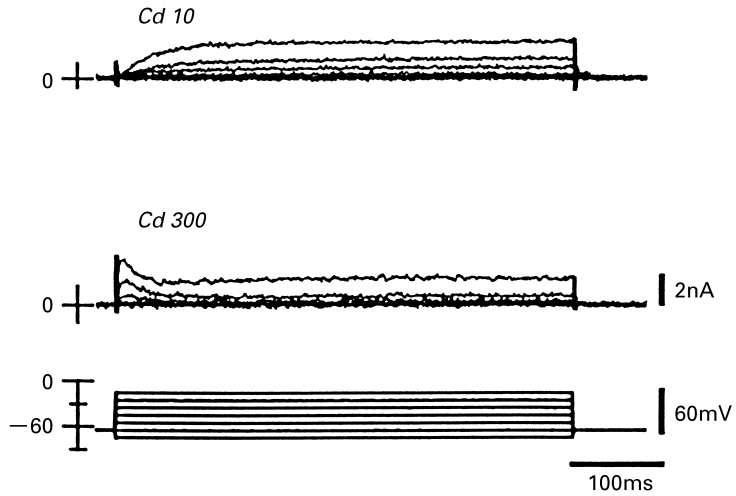

Fig. 5 Cadmium-induced recruitment of A-current. Results were obtained from a control cell superfused with a TEA-rich (30 mM) standard solution. The concentration of cadmium (10 μM) was then increased to 300 μM to detect the recruitment of A-current. Top, cadmium (10 μM); middle, cadmium (300 μM); bottom, command pulses from −65 mV to 7 different test potentials starting from −15 mV with −10 mV increments. TEA was used to eliminate delayed rectifier in such a manner that the recruited A-current could be emphasized.

the after-hyperpolarization of the action potential is greatly inhibited during depolarization [1, 5, 9, 13]. These characteristics have been suggested as resulting from inhibition of both M-current and delayed rectifier [9], or M-current alone [1, 5] for the depolarizations, and inhibition of a class of calcium-activated potassium current [13, 17] and/or delayed rectifier [9] for the after-hyperpolarization. It is clear from our findings that delayed rectifier cannot contribute to either the barium-induced depolarizations or to the barium-induced inhibition of the after-hyperpolarization.

In summary, we have demonstrated for the first time that the delayed rectifier-type potassium current was markedly insensitive to the block by barium in amphibian autonomic neurons.

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