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Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells

(afterhyperpolarizations/Ca-dependent conductances)

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ABSTRACT **Healthy bullfrog sympathetic ganglion cells often show a two-component afterhyperpolarization (AHP). Both components can be reduced or abolished by adding Ca-channel blockers or by removing external Ca. Application of a single electrode "hybrid clamp"—i.e., switching from current- to voltage-clamp at the peak of the AHP, reveals that the slow AHP component is generated by a small, slow, monotonically decaying outward current, which we call I_{AHP}. I_{AHP} is blocked by Ca-removal or by apamin and is a pure K current. It is slightly sensitive to muscarine and to tetraethylammonium ion but is much less so than muscarine-sensitive (I_M) and fast Ca-dependent (I_C) K currents. It also can be recorded in dual-electrode voltage-clamp experiments, where it is seen as a slow, small component of the outward tail current that follows brief depolarizations to 0 mV or beyond. I_C is seen as an early, fast, large component of the same tail current. Both components are blocked by Ca removal, but only the I_C component is blocked by low doses of tetraethylammonium ion. Thus, bullfrog ganglion cells exhibit two quite distinct Ca-dependent K currents, which differ in size, voltage-sensitivity, kinetics, and pharmacology. These two currents also play quite separate roles in shaping the action potential.**

Recent voltage-clamp work in bullfrog sympathetic ganglion cells (1–3) has established the existence of a large, rapid Ca-activated K current that is partly responsible for spike repolarization and early afterhyperpolarization (AHP). This current, which we term I_C, has several hallmarks: sensitivity to tetraethylammonium ion (Et_4N^+); insensitivity to muscarine, apamin, and barium; and a strong voltage dependence. It is carried by high-conductance channels like those in chromaffin cells and myotubes (4, 5). However, carefully impaled bullfrog neurons often show a second slow component of the afterhyperpolarization, which, while dependent on Ca-influx, seems to be inhibited by muscarinic-receptor activation (6) and rather insensitive to membrane potential (7). We have investigated this slow AHP component using voltage-clamp techniques, and we find that it is due to a Ca-dependent K current that, while it has some similarities to both I_C and the muscarine-sensitive K current, which we call I_M (8), is quite clearly distinct from them. We have named this previously poorly characterized current I_{AHP}.

MATERIALS AND METHODS

Fresh bullfrog ganglia were trypsinized (8) and bathed in control Ringer's solution containing 2.5 mM K, 115 mM Na, 4 mM Ca, 2.5 mM Tris (pH 7.2). Both single- and dual-electrode voltage-clamp techniques were used. In either case microelectrodes filled with 3 M KCl and with resistances of 20–35 MΩ were used. For single-electrode clamping, an Axoclamp 1 was used. Cycle rate was 3–5 kHz, with a duty cycle (ratio of time on to time off) of 1:1 or 1:2. Capacity compensation was continually adjusted to ensure complete headstage settling. For dual-microelectrode clamping, both current and voltage electrodes were provided with silver paint shields, as described (8). Responses were displayed on a Gould 2400 chart recorder and an oscilloscope. All drugs were obtained from Sigma. Temperature was about 23°C throughout.

RESULTS

Both single- and dual-microelectrode voltage-clamp techniques required relatively low-resistance electrodes, and these often inflict cell damage, manifested as low resting potential, high leakage, and short-duration passively decaying AHPs. However, long-duration AHPs could sometimes be recorded, especially with a single electrode and after some stabilization (Fig. 1*A*). To measure the current underlying the late AHP, we activated a switching voltage clamp shortly after the peak of the early AHP, bringing the potential back to rest (Fig. 1*B*). We term this procedure of switching from current clamp to voltage clamp "hybrid clamp." The outward current supplied by the clamp first decayed rapidly and then exhibited a prolonged exponential tail (Fig. 1*A*, trace f) with a time constant of 200–300 ms. After electrode stabilization, when the membrane leakage had substantially diminished, this small tail was capable of generating in the unclamped cell a large (10–30 mV) AHP lasting several hundred milliseconds (Fig. 1*A*, right-hand trace a). In particularly healthy cells, the slow AHP exhibited a rising phase, and the time to peak for the slow AHP component agreed with that expected for an exponentially decaying current injected into a passive isopotential cell (9).

The reversal potential of the small AHP current tail was studied by switching from current to voltage clamp, with or without interpolated single action potentials, and by varying the final command potential (Fig. 1*D*). The slowly decaying difference currents elicited in this way reversed at about −100 mV (−90 to −110 in different cells). In some cells outward rectification of I_{AHP} in 2.5 mM K was marked and hampered observation of reversal. This reversal potential shifted by 40 mV on increasing the external K concentration 5-fold, in exact agreement with the Nernst prediction for a pure K current.

The hybrid clamp suffers from the drawback that subtle changes in spike trajectory might influence the subsequent membrane currents. This was avoided by using a dual-microelectrode clamp to impose brief rectangular depolarizations resembling action potentials (Fig. 2). During the voltage step, a large inward–outward current sequence was observed. The large (50–100 nA) outward current generated during a 3-ms pulse to 0 mV from −40 mV was mostly abolished by removing Ca from the Ringer's solution, since the

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Abbreviations: AHP, afterhyperpolarization; I_C, fast Ca-dependent K current; I_{AHP}, slow Ca-dependent K current; I_M, muscarine-sensitive K current; Et_4N^+, tetraethylammonium ion.

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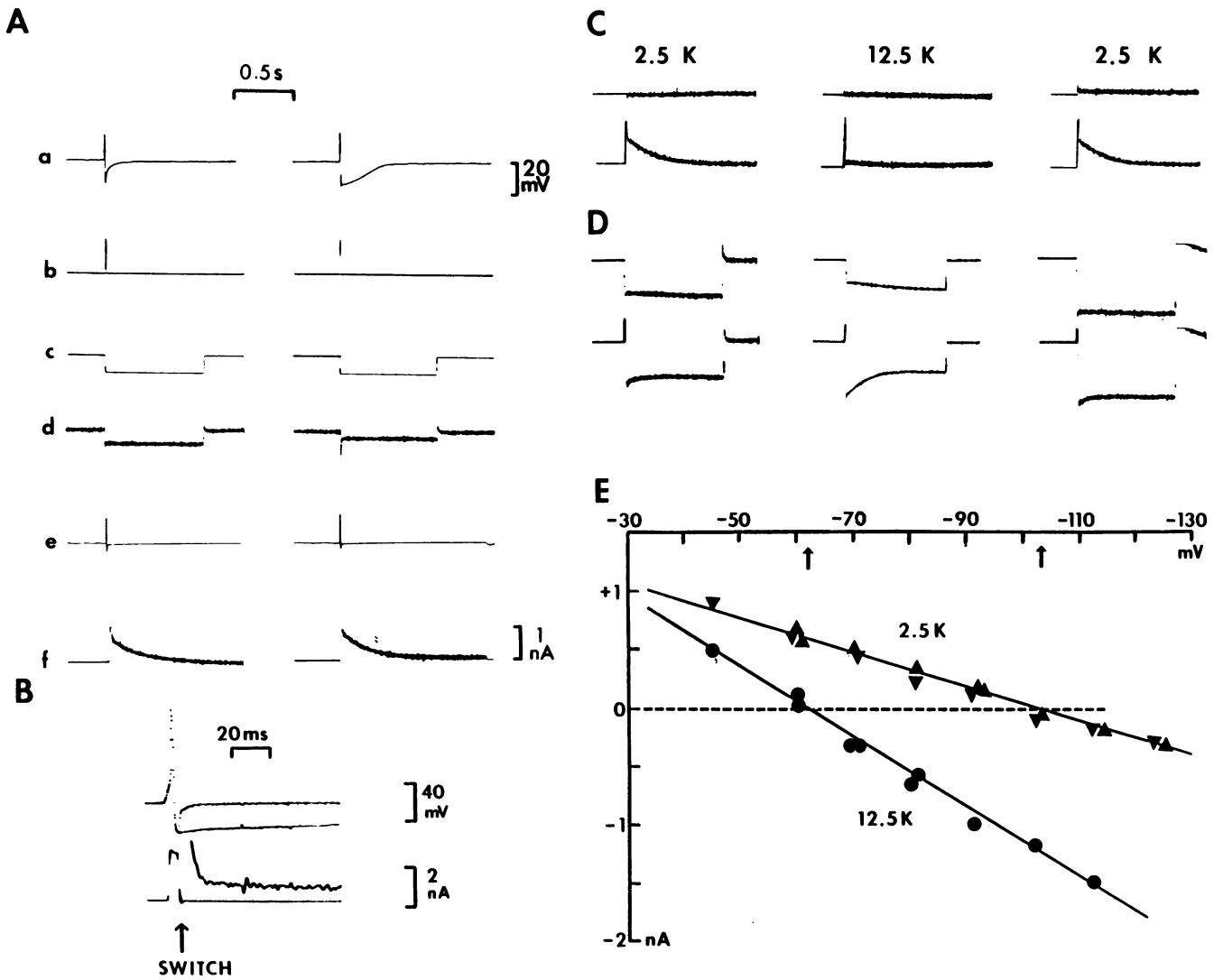

FIG. 1. Ionic basis of slow AHP in bullfrog sympathetic ganglion cells. (*A*) Records obtained shortly after impaling the cell (left-hand column; resting potential, −47 mV) and several minutes later (right-hand column; resting potential, −54 mV). Traces "a" show action potentials elicited by a 5-ms current pulse (shown at fast sweep in *B*). Right-hand trace a and the action potential in *B* (dotted because of 3-kHz sampling) show the well-developed AHP seen after electrode stabilization (compare with the left-hand trace a). Traces b show 5-ms current pulses distorted by the slow speed of the chart recorder. Traces d show membrane currents flowing during a 20-mV hyperpolarizing command (monitored in traces c) with a holding potential of −60 mV. The leakage resistance spontaneously increased from 47 MΩ (left-hand trace d) to 87 MΩ (right-hand trace d) as the electrode sealed in. Traces e and f show corresponding "hybrid clamp" experiments at these times. Traces e show voltage traces (same gain as for traces a) with a spike, the down-swing of the action potential, and voltage clamp to resting potential for the remainder of the record. Traces f show corresponding current records showing successively zero current level, 5-ms depolarizing current pulse (off scale), fast tail (mostly off scale), and slow tail; in both traces, the slow tails were exponential with extrapolated amplitudes of 0.80 nA (left-hand trace) and 0.85 nA (right-hand trace) and time constants of 309 ms (left) and 254 ms (right). (*B*) Timing of activation of switch clamp. Fast oscilloscope records of voltages (upper trace) and currents (lower trace) without and with activation of voltage clamp (arrow). The sweeps were the same as in the right-hand traces of *A*. Note that the voltage does not immediately settle to the command potential, so that a small part of the fast outward current tail could represent capacitative current. (*C*) Effect of increased K on I_{AHP}. Records (left to right) show I_{AHP} in 2.5 mM K, 12.5 mM K, and recovery in 2.5 mM K. The cell was voltage-clamped to a holding potential of −60 mV with (lower traces) or without (upper traces) a preceding action potential. (*D*) Same as for *C* except that the command potential was −110 mV rather than the holding potential. In both *C* and *D* the membrane potential was manually adjusted to −60 mV by passing the appropriate current prior to activating the voltage clamp. The difference between the lower and upper traces in both *C* and *D* represents I_{AHP} at the command potential. In *D*, I_{AHP} decayed exponentially with a time constant of 237 ms—i.e., similar to that at −54 mV in 2.5 mM K. The gain for *C* and *D* was as for traces f in *A* except for the center traces in *D*, which are at half gain. (*E*) Graphs of the I_{AHP} difference current amplitude at various command potentials determined before (▲), during (●), and after (▼) applying high-K Ringer's solution. The reversal potential shifts from −102 mV to −62 mV. Note that all records are from the same cell.

Ca-independent delayed rectifier develops slowly in these cells (8). The return to the holding potential revealed outward tail currents, composed of a large (10–20 nA) component decaying in a few milliseconds, and a small (≈1 nA) slow component corresponding to I_{AHP} (Fig. 2). The magnitude of the slow tail in the dual-electrode experiments also varied with external K in a Nernstian manner. Omission of Ca from the Ringer's solution reversibly abolished both the slow small tail and most of the fast large tail (Fig. 2*A*). Correspondingly, in hybrid clamp experiments, omission of Ca or addition of Cd (200–500 μM) slowed spike repolarization, reduced the early and abolished the late AHP, and abolished the postspike slow tail current. Thus, this K current is probably triggered by Ca influx. Dual-electrode voltage-clamp ex-

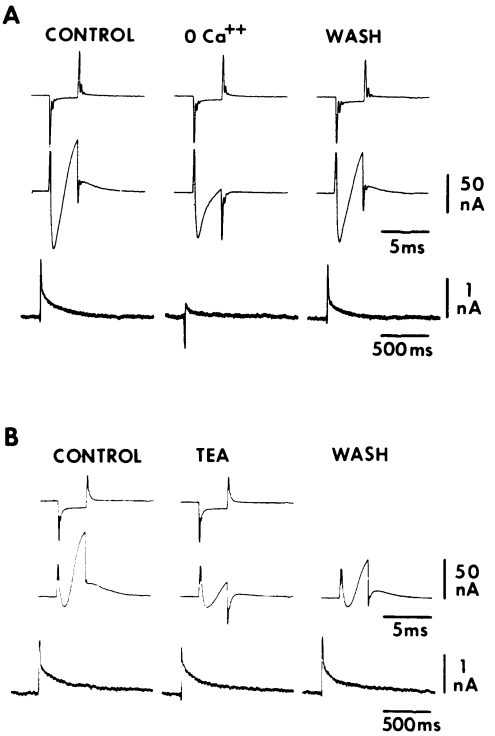

FIG. 2. Dual-electrode voltage clamp (for details, see ref. 8); differentiation of I_C and I_{AHP}. (*A*) Effect of zero-Ca Ringer's solution (Ca replaced by 4 mM Mg and 0.2 mM EGTA). Traces (left to right) show control, zero Ca, and recovery records. (*A Top*) Currents evoked by a hyperpolarizing command to −80 mV from a holding potential of −40 mV; command potential duration was 3 msec. (*A Middle*) Currents evoked by a depolarizing command to 0 mV from −40 mV. (*A Bottom*) I_{AHP} evoked by depolarizing command pulses as in *A Middle*. Note that *A Middle* and *Bottom* show the same responses but at vastly different sweep speeds and gains. (*B*) Effect of 3 mM Et_4NBr in another cell. Organization of traces and voltage-clamp protocol was as in *A*.

periments indicate that it is maximally activated by brief depolarizations (1–5 msec). In hybrid clamp, repetitive spikes generate an I_{AHP} of similar amplitude and longer duration (Fig. 3*A*).

The magnitude and time course of the slow AHP tail conductance did not obviously depend on the membrane potential (Fig. 1), in stark contrast to the behavior of I_C (1, 3–5). The effect of Et_4N^+ on I_C and I_{AHP} also contrasted: 3 mM Et_4N^+ reduced the fast tail component by about 70% (Fig. 2*B*); 1 mM Et_4N^+ was found to be about half-blocking for both I_C and the delayed rectifier (1, 10). However, either no or a small reduction in the slow tail component occurred (Fig. 2*B*). Similarly, in hybrid clamp experiments, Et_4N^+ slowed spike repolarization and reduced the early AHP but had only a small effect on the spike-triggered slow tail.

The fact that I_{AHP} is a slow, rather small, Et_4N^+-insensitive K current is reminiscent of I_M. However, though I_M and I_{AHP} have similar magnitudes and time courses near rest, at hyperpolarized potentials I_M decays much faster than I_{AHP}. Furthermore, removal of external Ca did not affect I_M (11).

It has been reported that the bullfrog AHP is shortened by muscarinic stimulation (6). We found in hybrid clamp experiments that 10 μM muscarine reduced I_{AHP} to 70% (±4% SEM, n = 11) of control with little effect on the time course (Fig. 3*B*). In the same set of cells, 10 μM muscarine decreased I_M to 18 ± 5% of control; 100 μM muscarine did not cause further reduction of either I_{AHP} or I_M. A small but consistent effect of muscarine on I_{AHP}, together with a dramatic effect on I_M, was confirmed in dual clamp experiments. The reported effects on the slow AHP itself probably reflect a combination of a small action on I_{AHP} and some muscarine-induced increase in leak conductance (11, 12). Indeed, at certain potentials, muscarine and Ba (see Fig. 3*A*, traces b) can enhance AHPs by abolishing I_M and, thus, increasing input resistance. Interestingly, Ba reduced both I_M and I_{AHP} in parallel (Fig. 3*A*).

There have been several conflicting reports on the action of apamin on Ca-activated K conductances in nerve cells. Previous work in bullfrog ganglia showed that I_C elicited by Ca-injection was insensitive to 100 nM apamin (3), but it seemed possible that I_{AHP} might be affected. Indeed, it was found in hybrid clamp experiments that apamin (2.5–25 nM) irreversibly knocked out at least 60% of I_{AHP} without affecting spike configuration, leakage, or I_M (Fig. 4). A similar differential action of apamin on Et_4N^+-insensitive and -sensitive Ca-activated K currents is seen in mammalian myotubes (ref. 13; see also refs. 14 and 15).

DISCUSSION

These experiments reveal the existence of a novel slow K current in bullfrog neurons, which, while sharing some of the characteristics of the previously described currents I_C and I_M, is sufficiently different that it probably reflects the operation of quite separate ionic channels. This current shows many of the properties that have been postulated for that underlying the slow AHPs of myenteric and hippocampal neurons, including sensitivity to muscarinic agonists (16–18). Coexisting but separate Ca-dependent outward currents with contrasting voltage- and Et_4N^+-sensitivity also have been seen in *Aplysia* neurons (19, 20), but unlike our data, the molluscan slow AHP current could not be reversed even in increased K and has been attributed to a decrease of resting inward current.

In bullfrog ganglion cells, which lack dendrites, I_{AHP} decays monotonically after a spike, even though the slow AHP may exhibit a rising phase. However, in mammalian neurons the current may itself show a rising phase (16, 21), possibly because Ca entering dendrites has to diffuse some distance to activate K channels. Mammalian neurons also may exhibit a fast, large I_C-like conductance (22). The very different properties of these two Ca-dependent K currents probably reflect the distinctive roles each plays in the electrical behavior of nerve cells. Thus, in bullfrog ganglion cells, I_C is well-suited to the role of spike repolarization. It activates very rapidly and is large enough to quickly recharge the membrane capacitance. As the membrane potential returns to rest and beyond, I_C rapidly turns off, even though internal Ca may still be raised. I_{AHP} is much better suited to producing prolonged hyperpolarizations, which may influence repetitive discharge characteristics.

Although I_{AHP} is clearly Ca-dependent, we have not shown unequivocally that it is activated by a rise in intracellular Ca. Four types of experiment could be performed to test this point. First, one might predict that the amplitude of the I_{AHP} tail after depolarizing clamp steps would decrease if the step were made sufficiently positive to reduce Ca entry. Second, intracellular injection of EGTA should reduce I_{AHP}. However, neither of these tests would be conclusive. The first type of test might fail if enough Ca could enter during the Ca tail currents to fully activate I_{AHP}. We have preliminary evidence that this test does in fact fail, possibly for this reason. The second test might fail if the normal resting intracellular Ca concentration was high enough to partly activate I_{AHP}, so that less was left to be activated following a spike. EGTA injection might then paradoxically increase I_{AHP} by decreasing the resting Ca concentration more than it decreased the peak concentration achieved in the wake of the action potential. Current clamp experiments indicate that in-

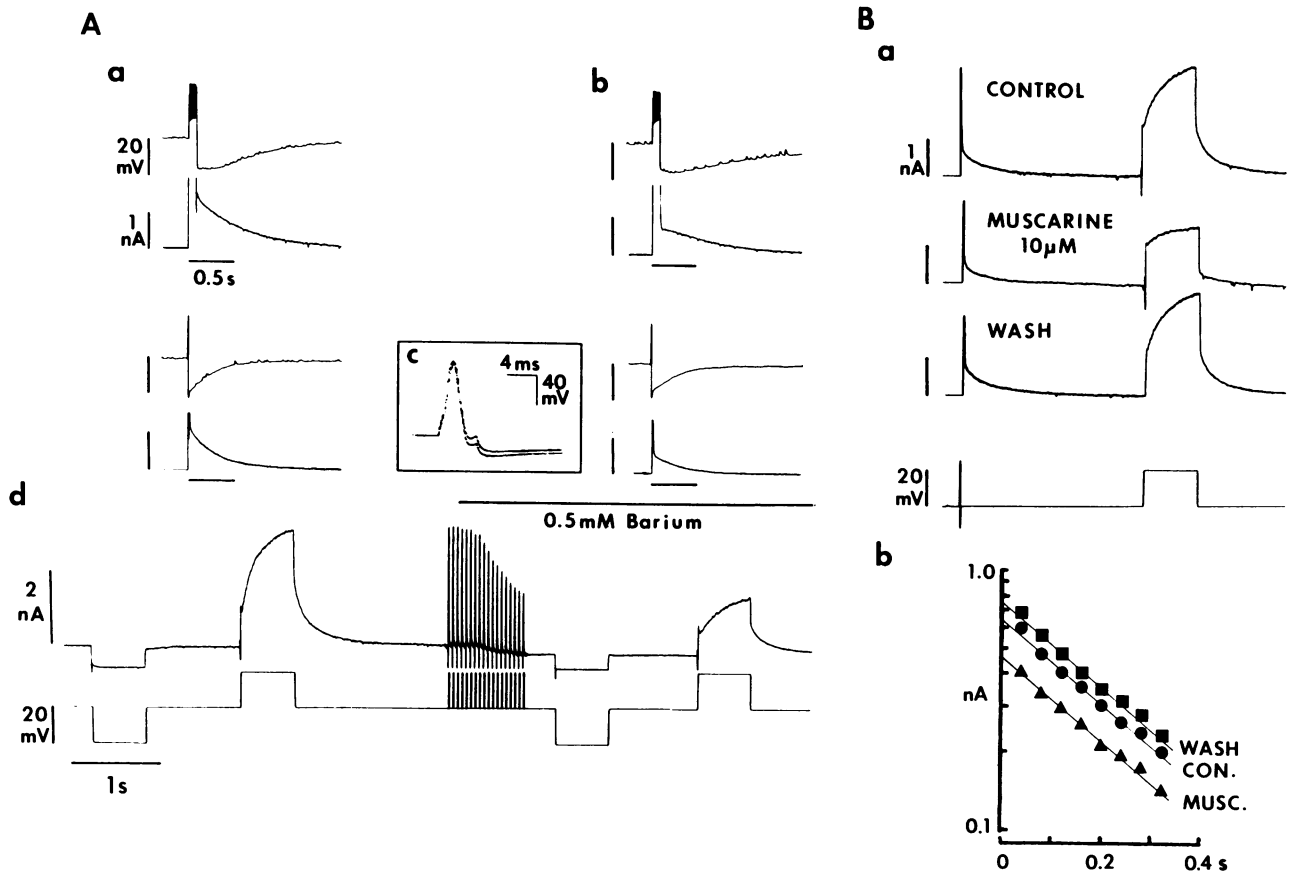

FIG. 3. Comparison of I_{AHP} and I_M with single-electrode clamping. (*A*) Effect of Ba on the AHP and I_{AHP} evoked by eight spikes (upper traces) or a single spike (lower traces). Traces: a, controls; b, in 0.5 mM Ba; c, superimposed action potentials before and during Ba addition; d, effect of 0.5 mM Ba on I_M evoked by a 20-mV command from the holding potential. Note that the trace was slowed 100-fold in the middle of the record. For all records in *A*, the holding potential was −50 mV. Hybrid clamp was used for traces a and b; current clamp was used for traces c; and voltage clamp was used for traces d. (*B*) Effect of muscarine. Current traces from the top show control, muscarine, and recovery records of I_{AHP} and I_M; recovery was speeded by perfusing 0.1 μM atropine. The bottom trace shows the voltage record. Membrane potential was manually held at −50 mV throughout, and I_{AHP} was elicited by activating a spike with a 2-ms current pulse and then voltage-clamping to −50 mV. The voltage clamp was left on while eliciting I_M (20-mV depolarizing command for 600 ms) and then was turned off in readiness for the next action potential. The corresponding semi-logarithmic plot of I_{AHP} is shown below the traces.

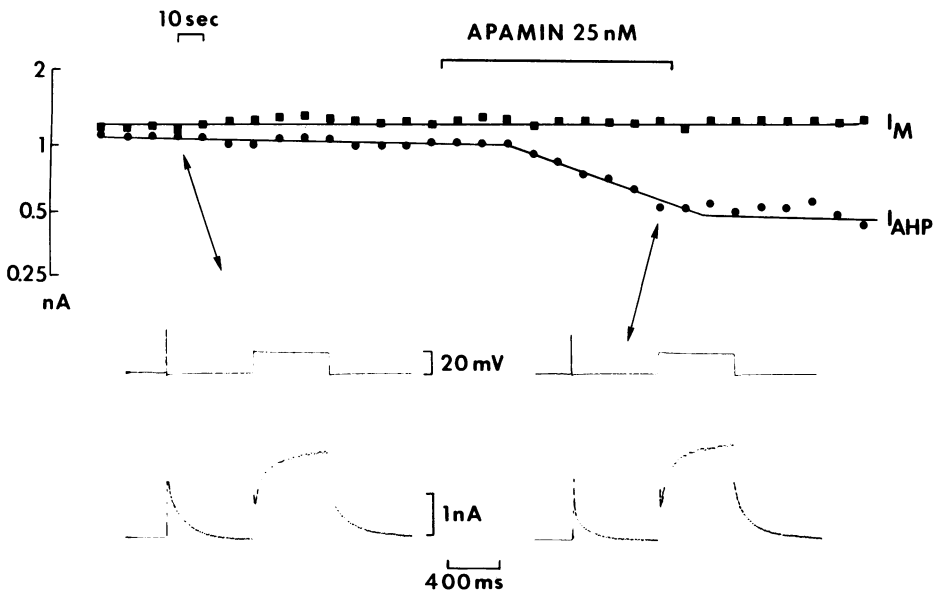

FIG. 4. Effect of apamin on I_M and I_{AHP} measured using single-electrode hybrid clamp. I_{AHP} and I_M were evoked every 10 s by using the protocol of Fig. 3*B*; 25 nM apamin applied for 100 s reduced I_{AHP} but not I_M. Sample records are shown from points indicated by arrows. I_{AHP} and I_M are plotted as extrapolated peak amplitudes of the tail currents. Apamin was obtained from Sigma (April, 1984) at 50% purity.

tracellular EGTA injection can curtail the slow AHP (7).

Third, iontophoretic injections of Ca should evoke mixtures of I_C and I_{AHP}, which should be separable on the basis of kinetics, voltage-dependence, and pharmacology. So far such experiments have only revealed I_C (1), possibly because traumatic impalement with low-resistance Ca-containing electrodes interferes with the labile AHP mechanism. Fourth, it should now be possible to record single I_{AHP} channels and measure their sensitivity to Ca in inside-out membrane patches.

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1. Adams, P. R., Constanti, A., Brown, D. A. & Clark, R. B. (1982) *Nature (London)* **296,** 746–749.
2. MacDermott, A. B. & Weight, F. F. (1982) *Nature (London)* **300,** 185–188.
3. Brown, D. A., Constanti, A. & Adams, P. R. (1983) *Cell Calcium* **4,** 407–420.
4. Marty, A. (1981) *Nature (London)* **291,** 497–500.
5. Pallotta, B. S., Magleby, K. L. & Barrett, J. N. (1981) *Nature (London)* **293,** 471–474.
6. Tokimasa, T. (1984) *J. Autonom. Nerv. Syst.* **10,** 107–116.
7. Kuba, K., Morita, K. & Nohmi, M. (1983) *Pflügers Arch.* **399,** 194–202.
8. Adams, P. R., Brown, D. A. & Constanti, A. (1982) *J. Physiol. (London)* **330,** 537–572.
9. Adams, P. R. (1980) in *Information Processing in the Nervous System,* eds. Pinsker, H. M. & Willis, W. D. (Raven, New York), pp. 109–121.
10. Hille, B. (1973) *J. Gen. Physiol.* **61,** 664–686.
11. Adams, P. R., Brown, D. A. & Constanti, A. (1982) *J. Physiol. (London)* **332,** 223–262.
12. Kuba, K. & Koketsu, K. (1976) *Jpn. J. Physiol.* **26,** 651–669.
13. Romey, G. & Lazdunski, M. B. (1984) *Biochem. Biophys. Res. Commun.* **118,** 669–674.
14. Blatz, A. L. & Magleby, K. L. (1984) *J. Gen. Physiol.* **84,** 1–23.
15. Barrett, J. N., Barrett, E. F. & Dribin, L. B. (1981) *Dev. Biol.* **82,** 258–266.
16. North, R. A. & Tokimasa, T. (1983) *J. Physiol. (London)* **342,** 253–266.
17. Hotson, J. R. & Prince, D. A. (1980) *J. Neurophysiol.* **43,** 409–419.
18. Cole, A. E. & Nicoll, R. A. (1984) *J. Physiol. (London)* **352,** 173–188.
19. Adams, W. B. & Levitan, I. B. (1985) *J. Physiol. (London),* in press.
20. Kramer, R. H. & Zucker, R. S. (1985) *J. Physiol. (London),* in press.
21. Lancaster, B. & Adams, P. R. (1984) *Soc. Neurosci. Abstr.* **10,** 872.
22. Gruol, D. L. (1983) *Soc. Neurosci. Abstr.* **9,** 680.