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LARGE CONDUCTANCE Ca^{2+}-ACTIVATED K^+ CHANNELS ARE INVOLVED IN BOTH SPIKE SHAPING AND FIRING REGULATION IN *HELIX* NEURONES

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SUMMARY

1. The role of BK-type calcium-dependent K^+ channels (K^+_{Ca}) in cell firing regulation was evaluated by performing whole-cell voltage clamp and patch clamp experiments on the U cell neurones in the snail *Helix pomatia*. These cells were selected because most of the repolarizing K^+ current flowed through K^+_{Ca} channels.

2. U cells generated overshooting Ca^{2+}-dependent spikes in Na^+-free saline. In response to prolonged depolarizing current, they fired a limited number of spikes of decreasing amplitude, and behaved like fast-adapting or phasic neurones.

3. Under voltage clamp conditions, the K^+_{Ca} current had a slow onset at voltages that induced small Ca^{2+} entries. By manipulating the Ca^{2+} entry (either with appropriate voltage programmes or by changing the Ca^{2+} content of the bath), the K^+_{Ca} channel opening was found to be rate limited by the Ca^{2+} binding step and not by the voltage-dependent conformational change to the open state.

4. Despite the slow activation rate observed in voltage-clamped cells, 25–30% of the available K^+_{Ca} current was found to be active during isolated spikes. These data were based on patch clamp, spike-like voltage clamp and hybrid current clamp–voltage clamp experiments.

5. The fact that spikes led the slowly rising K^+_{Ca} current to shift into a fast activating mode was accounted for by the large surge of Ca^{2+} current concomitant with spike upstroke. The early calcium surge resulted in local increases in cytosolic calcium, which speeded up the binding of calcium ions to the closed K^+_{Ca} channels. From changes in the null Ca^{2+} current voltage, it was calculated that the submembrane $[Ca^{2+}]_i$ increased to 50–80 μM during the spike.

6. Due to their fast voltage dependence, K^+_{Ca} channels appeared to play no role in shaping the interspike trajectory.

7. Even in the fast activating mode, the K^+_{Ca} current had a finite rate of rise and was not involved in repolarizing short duration Na^+-dependent action potentials. The current became more and more active, however, when voltage-gated K^+ channels were progressively inactivated during firing.

8. The fast adaptation exhibited by U cells upon sustained depolarization was not paralleled by a recruitment of K^+_{Ca} channels because of the cumulative Ca^{2+} entries.

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During a spike burst, the K^+_{Ca} current progressively overlapped the depolarizing Ca^{2+} current, which ultimately stopped the firing. The early opening of K^+_{Ca} channels was ascribed to residual Ca^{2+} accumulation that kept part of the channels in the Ca^{2+}-bound state ready to be opened quickly by cell depolarization.

9. These data demonstrate that BK-type K^+_{Ca} channels may participate in a fast process (spike repolarization) as well as in long-lasting events (frequency regulation). This twofold role reflects the duality of the channel gating with its fast voltage-dependent control and its long-term calcium regulation.

INTRODUCTION

Two major types of calcium-activated K^+ currents (K^+_{Ca} currents) have been observed in a number of neurones including *Aplysia* neurones (Deitmer & Eckert, 1985), GH_3 pituitary cells (Ritchie, 1987), rat sympathetic neurones (Kawai & Watanabe, 1986; Smart, 1987) and rat hippocampal neurones (Lancaster, Nicoll & Perkel, 1991).

One K^+_{Ca} current (sometimes denoted the C-current) is a voltage-dependent, tetraethylammonium (TEA^+)- and charybdotoxin-sensitive current. It results from the opening of large-conductance (> 100 pS) BK channels. Although the molluscan K^+_{Ca} channels described in *Aplysia* (Belardetti, Schacher & Siegelbaum, 1986) and *Helix* neurones (Gola, Ducreux & Chagneux, 1990) have a relatively small unitary conductance (20–65 pS), they actually share the main properties of the BK-type channels. The second K^+_{Ca} current is poorly voltage dependent, insensitive to TEA^+ and charybdotoxin and can be either apamin sensitive (rat and bullfrog sympathetic ganglia) or apamin insensitive (hippocampal neurones). Since it is involved in prolonged after-hyperpolarization (AHP), this current is sometimes referred to as I_{AHP}; it results from the opening of small-sized channels or SK channels (Lang & Ritchie, 1987; Lancaster *et al.* 1991). The present classification of K^+_{Ca} channels in two groups has been challenged, however, by the description of several intermediate conductances (Reinhart, Chung & Levitan, 1989; Lang & Ritchie, 1990; Dryer, Dourado & Wisgirda, 1991).

On the basis of whole-cell voltage and current recordings and of unitary channel properties it is generally assumed that C- or BK channels are involved in active potential repolarization (Adams, Constanti, Brown & Clark, 1982; Gola, Hussy, Crest & Ducreux, 1986; Storm, 1987; Lang & Ritchie, 1987; Gola *et al.* 1990; Lancaster *et al.* 1991) and play a limited role, if any, in post-spike events and in the spike frequency regulation. Since SK and related Ca^{2+}-activated K^+ channels have little voltage dependence and are more sensitive to calcium than BK channels, they are ideally suited for acting in post-spike events and therefore for contributing to the spike frequency regulation (Madison & Nicoll, 1984; Lancaster & Adams, 1986; Lancaster *et al.* 1991).

Molluscan nerve cells, in which the C-current was originally described, display a palette of specific signalling properties and channel equipment, and thus provide an appropriate system for evaluating the role of ionic channels. The U cells in *Helix* ganglia (Lux & Hofmeier, 1982) are characterized by Ca^{2+}-dependent spikes and high sensitivity to repetitive firing, i.e. fast adaptation. Whole-cell voltage clamp and patch clamp experiments have demonstrated that the outward current in U cells

flows mainly through BK-type K^+_{Ca} channels (Gola *et al.* 1990). These cells can therefore be used to assess directly the role of neuronal BK-type channels.

A large number of data have been collected on the calcium sensitivity and voltage dependence of the BK-type K^+_{Ca} channels (reviewed by McManus, 1991). These data are mainly restricted, however, to steady-state conditions, and very little information is available about the calcium or voltage factor that rate-limits the channel opening. This information is critical to understanding the role of these channels in the cell encoding properties.

To answer these questions we have determined: (1) how voltage and calcium regulate the K^+_{Ca} current activation rate; (2) to what extent and in what situations the K^+_{Ca} channels may take part in shaping the spike, and (3) whether these channels may play a role in cell firing regulation.

Our results show that BK-type K^+_{Ca} channels may be involved in fast electrical events (i.e. spike repolarization) as well as in regulating the spike discharge (i.e. frequency adaptation).

METHODS

Experimental material

Experiments were performed on identified nerve cells from the central nervous system of the snail *Helix pomatia*. Most of the experiments were conducted on U cells; the letter 'U' refers to the fact that these cells fire spikes that depend only (uniquely) on calcium channels (Lux & Hofmeier, 1982). U cells were identified using the following morphological and electrophysiological criteria: (i) they form a cluster of ten to fifteen neurones located in the right parietal ganglion, in front of the right pallial nerve; (ii) they are silent with a −50 to −55 mV resting potential; (iii) they send off one or two axon branches into the right pallial nerve and are completely devoid of dendritic-like processes; (iv) consequently, they have no spontaneously occurring or stimulated synaptic inputs; (v) they have overshooting spikes with a large amplitude (80–110 mV, peak to peak) and long duration (10–20 ms at half-amplitude); (vi) they are unable to fire spikes at frequencies larger than 2·5–3 Hz.

A few additional experiments were conducted on another cell group, P cells in which spikes resulted from the activation of both sodium and calcium currents (Crest, Ehile, Pin, Watanabe & Gola, 1990*a*). P cells were identified according to the criteria described by Pin, Crest, Ehile, Jacquet & Gola (1990).

The suboesophageal ganglion complex was isolated and dissected out (see Pin *et al.* 1990 for details). It was continuously perfused with the following physiological saline (mM): NaCl, 75; KCl, 5; $CaCl_2$, 8; $MgCl_2$, 8; Tris (pH 7·5), 5. In Na^+-free saline, NaCl was replaced by either 90 mM Tris (Na^+-free saline) or 60 mM TEA^+ and 20 mM Tris (Na^+-free TEA^+ saline). Experiments were performed at room temperature (20–22 °C).

Calcium channels were blocked by adding either cadmium (0·5–1 mM) or lanthanum (0·2–0·5 mM) to the above salines or by substituting cobalt for calcium. The outward potassium currents were blocked by bathing the cell in the Na^+-free TEA^+ saline. The calcium-activated K^+ channels were specifically blocked with kaliotoxin, a toxin recently isolated from the venom of the scorpion *Androctonus mauretanicus mauretanicus* (Crest *et al.* 1992). The calcium currents were measured in the Na^+-free TEA^+ saline.

Whole-cell voltage and current recordings

Cells were impaled with two KCl (3 M)-filled microelectrodes (tip resistance 2–3 MΩ) and studied under conventional current clamp and voltage clamp conditions. Data were sampled at 0·5–1 kHz with a 16 bit A–D converter and stored on floppy disks. In voltage clamp experiments, capacitive and leak currents were evaluated by applying 20–50 mV hyperpolarizing voltage pulses. The holding potential was set at −50 mV. Intracellular injection of the calcium chelator EGTA (ethyleneglycol-bis-(β-aminoethylether)*N,N,N′,N′*-tetraacetic acid) was performed in voltage-clamped cells with a third intracellular electrode filled with 0·7 M EGTA (K^+ salt).

The ionic currents that flowed during cell firing were detected with a spike-like voltage clamp

pulse method based on an idea by Llinás, Sugimori & Simon (1982). Briefly, the neuronal signals evoked under current clamp conditions were stored and subsequently used to command voltage-clamped cells. The selective blockade of ionic channels was compensated for by a feedback current delivered by the voltage clamp amplifier. The compensating current mimicked the blocked current. Details on the way we used this method are given in Gola *et al.* (1986). This semi-analytical method gave an on-line evaluation of the involvement of ionic currents in the nerve cell signalling properties.

In order to quantify the contribution of the calcium and potassium currents to the U cell firing, we combined the above spike-like voltage clamp method with the hybrid current–voltage clamp method originally introduced by Bezanilla, Rojas & Taylor (1970). The hybrid clamp method involved switching from current to voltage clamp conditions during cell firing. The consecutive currents provided information about the state of the channels at the time when the voltage clamp was switched on.

Cell-attached patch clamp recordings

Ca^{2+}-dependent K^+ channels in U cells were detected at the unitary level with patch clamp electrodes. The method and the criteria used for channel identification are given in detail in Gola *et al.* (1990). To detect K^+ channels at negative cell potentials, patch pipettes were filled with a KCl-rich saline containing (mM): KCl, 115; $CaCl_2$, 8; $MgCl_2$, 8; Tris (pH 7·5), 5. In cell-attached recordings, this saline gave a potassium reversal potential of $\approx +10$ mV. Patch experiments were performed on U cells impaled with an intracellular electrode connected to a WPI model M701 amplifier.

RESULTS

Firing characteristics of U cells

The most prominent features of U cells firing properties are illustrated in Fig. 1*Aa*. The U cell spike was characterized by its large amplitude (overshoot, $+42 \pm 1{\cdot}6$ mV; post-spike hyperpolarization, $-54 \pm 1{\cdot}2$ mV; $n = 14$), its slow time course (depolarizing and repolarizing rates, $13{\cdot}5 \pm 1$ and $8{\cdot}8 \pm 0{\cdot}6$ V s^{-1}, respectively) and its long duration ($13{\cdot}9 \pm 0{\cdot}7$ ms at half-amplitude). Upon sustained depolarization induced by outwardly injected currents, the U cells fired a short burst of action potentials, the amplitude of which decreased progressively with time. The same changes in the shape of spike wave were observed when U cells were repeatedly fired even at a slow rate (1–2 Hz) with short current pulses. Beyond 3·5 Hz, the spike was rapidly attenuated and eventually aborted. The superimposed recordings in Fig. 1*Ab* exemplify the progressive decrease in the spike overshoot that preceded the firing blockade. These firing characteristics were not modified when the cells were bathed in the Na^+-free saline. U cells therefore fire Ca^{2+} spikes and can be classified as phasic or fast adapting neurones. Fast adaptation has been observed in a wide variety of nervous structures; it has been ascribed either to an all-or-none blockade of the spike generating mechanism or to the progressive activation of persistent currents (mainly carried by K^+ ions) that bring the potential under the spike threshold. None of these explanations accounted for the fact that the U cell spike was progressively reduced in amplitude during the burst, which suggested that spike repolarizing currents, mainly Ca^{2+}-dependent K^+ currents, were more and more actively recruited. This hypothesis was in line with the finding that repetitive firing caused a cumulative increase in the intracellular calcium in nerve cells possessing Ca^{2+} channels (Gorman, Hermann & Thomas, 1981; Delaney, Zucker & Tank, 1989). To elucidate this point, we have analysed the properties of the U cell Ca^{2+}-dependent K^+ current and its involvement in spike repolarizing and fast adapting mechanisms.

Calcium-dependent potassium currents

The set of ionic currents induced by pulse-depolarizing a U cell from −10 to +90 mV is shown in Fig. 1*B*. The outward current slowed down at large depolarizations. This slow current component was suppressed by preventing

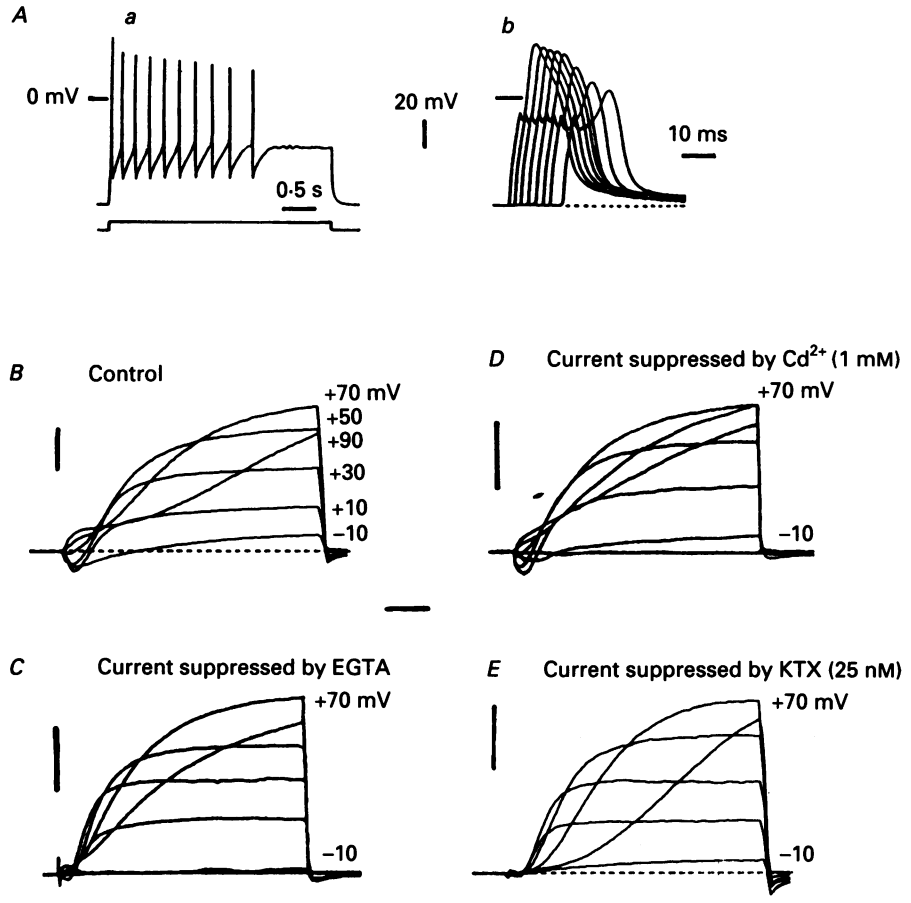

Fig. 1. Ca^{2+}-activated K^+ current in U cells. *A*, firing characteristics of U cells stimulated by either a prolonged current pulse (*a*) or repeated (3 Hz) short current pulses (*b*). *B*, membrane currents induced by voltage pulses at the levels indicated on the traces. Holding potential, −50 mV; cell bathed in the Na^+-free saline. *C–E*, identification of the Ca^{2+}-dependent K^+ component by either intracellularly injecting EGTA (*C*) or by adding Cd^{2+} (1 mM; *D*) or kaliotoxin (KTX, 25 nM; *E*) to the bath saline. The three sets of currents were obtained by subtracting the EGTA-, Cd^{2+}- and KTX-resistant currents from currents recorded under control conditions. Scale bars in *B–E*, 10 ms, 200 nA.

intracellular Ca^{2+} accumulation. Intracellular injection of the calcium chelator EGTA suppressed most of the outward current. The same result was obtained by adding calcium channel blockers to the bath saline (0·5–1 mM Cd^{2+}; 0·2–0·5 mM La^{3+}; 8 mM Co^{2+}). Sets of calcium-dependent currents obtained by both procedures are given in Fig. 1*C* (intracellular EGTA injection) and in Fig. 1*D* (effects of cadmium). The slow current component was also blocked by charybdotoxin (50 nM) and by kaliotoxin (25 nM, Fig. 1*E*). In all cases, this current had a characteristic slow activation rate

when voltages were pulsed over +30 mV. This is the main property of the Ca^{2+}-dependent voltage-gated K^+ current described by Woolum & Gorman (1981) and Lux & Hofmeier (1982). The fast outward current component resistant to EGTA and calcium channel blockers was therefore attributed to voltage-dependent K^+ channels

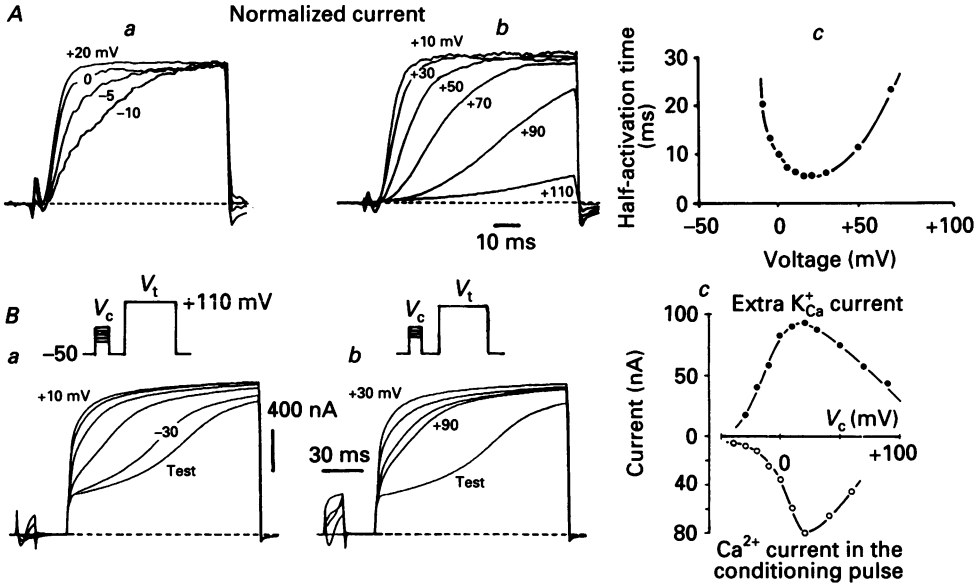

Fig. 2. Kinetic properties of the Ca^{2+}-activated K^+ current. *A*, KTX-sensitive currents induced by moderate (*a*) and large (*b*) depolarizing pulses. Currents normalized to the level reached at +20 mV. Holding potential, −50 mV; test pulse (V_t) incremented by 5 or 10 mV in *a* and 20 mV in *b*. KTX concentration, 25 nM. *Ac*, change in the half-activation time as a function of the voltage. The K^+_{Ca} current had a slow rate of activation at both moderate and large depolarizations. *B*, membrane currents induced by pulse depolarization at +110 mV following short (20 ms) conditioning pulses (V_c) at the level indicated on the current traces. The conditioning pulse increased by 10 mV step in *a* and 20 mV step in *b*. Its effect was quantified by measuring the extra K^+_{Ca} current obtained by subtracting the test current from the facilitated currents. *Bc*: ●, isochronal extra K^+_{Ca} current (measured 20 ms after the +110 mV test pulse was turned on) *versus* conditioning potential; ○, the calcium current flowing during the conditioning potential was further collected by bathing the cell in the Na^+-free TEA^+ saline. Both extra K^+_{Ca} currents and Ca^{2+} currents had peak values at +10 to +20 mV conditioning voltages.

(which will be referred to hereafter as K^+_V). In U cells, the K^+_V component (fast onset in the +90 mV current trace in Fig. 1*B*) was only detectable at large depolarizations. In the physiological voltage range ($V < +40$ mV) the K^+_V component amounted to less than 10% of the net outward current.

The K^+_{Ca} current activation is rate-limited by calcium

The K^+_{Ca} channel in molluscan nerve cells is of the BK-type. Its opening is dependent on both voltage and calcium (Gola *et al.* 1990). According to Kostyuk, Doroshenko & Tsyndrenko (1980) and Lux & Hofmeier (1982), calcium ions may have a permissive effect on the current activation, the rate of which may be under the control of a voltage-dependent sensor. Alternatively, since relatively large

increases in $[Ca^{2+}]_i$ are required for the K^+_{Ca} channel to open in response to membrane depolarization, the Ca^{2+} binding step might be the rate-limiting factor (Müller, Swandulla & Lux, 1989; Gola *et al.* 1990). In the following experiments we re-evaluated these hypotheses by correlating the activation rate of the macroscopic K^+_{Ca} current with the size of the underlying Ca^{2+} current.

In order to accurately determine its time course, the K^+_{Ca} current was specifically blocked by adding 25 nM kaliotoxin. The kaliotoxin-sensitive currents, normalized to the steady-state level reached at +20 mV, are displayed in panel *A* of Fig. 2. They were obtained from a U cell depolarized at either moderate (from −30 to +20 mV, Fig. 2*Aa*) or large (+10 to +110 mV, Fig. 2*Ab*) potentials. The K^+_{Ca} current induced by large depolarizations was more and more delayed and slowed down as the pulse potential increased. The slowing down that occurred at large positive potentials has been observed in similar K^+_{Ca} currents in molluscan and mammalian nerve cells (Gorman & Thomas, 1980; Dryer *et al.* 1991). Nevertheless, no information on the current activation rate at moderate depolarizations was available. Assuming the existence of a voltage- or calcium-dependent regulation, this information was critical since maximum Ca^{2+} currents occurred in the +10 to +30 mV range. The traces in in Fig. 2*Aa* show that the K^+_{Ca} current induced by moderate depolarizations (between −10 and +10 mV) had a slow onset like those observed at large depolarizations. The change in the time to half-activation of the K^+_{Ca} current with voltage (Fig. 2*Ac*) demonstrated that the K^+_{Ca} current had a fast onset at voltages that produced large calcium currents. This finding suggested that this current was rate-limited by the Ca^{2+} entry. This conclusion was further tested in the experiments displayed in Figs 2*B* and 3.

In their original description of the K^+_{Ca} current properties in U cells, Lux & Hofmeier (1982) reported that a short conditioning depolarization speeded up the K^+_{Ca} current induced by a subsequent large depolarization. They interpreted the current onset facilitation as an alternative means of current activation through a voltage-dependent process. Actually, this facilitating process turned out to be directly related to the amount of calcium ions that entered the cell during the conditioning pulse. The two sets of current recordings in Fig. 2*B* were obtained from a U cell pulse-depolarized at +110 mV; this test pulse was applied 25 ms after a short (20 ms) conditioning pulse which was intended to produce a Ca^{2+} entry. The traces in *Ba* in Fig. 2 were obtained with conditioning pulses (from −50 to +10 mV) that speeded up the test K^+_{Ca} current, whereas in *Bb*, further increasing the conditioning pulse level (from +30 to +90 mV) resulted in a progressive slowing down of the facilitated test current. To quantify the current onset facilitation we measured the extra current as the facilitated test current minus the control test current. The calcium current that flowed during the conditioning pulse was further determined by bathing the cell in a TEA^+-containing saline. The current–voltage curves in Fig. 2*Bc* show that the extra K^+_{Ca} current had a peak at conditioning voltages that produced large calcium currents.

These results strengthen the hypothesis that calcium limited the K^+_{Ca} current onset. Both series of experiments were based, however, on experimental procedures that did not exclude the possibility that a peculiar voltage-dependent process may have regulated the K^+_{Ca} current onset. Additional data in favour of our hypothesis are

given in Fig. 3*A*. Conditioning pulses at +20 mV that induced large Ca^{2+} entries resulted in fast activating K^+_{Ca} currents, whatever the test pulse level (Fig. 3*Ab*). Under these conditions no more obvious change in the current activation rate occurred when the test pulse was changed from −10 to +90 mV. We also noted that

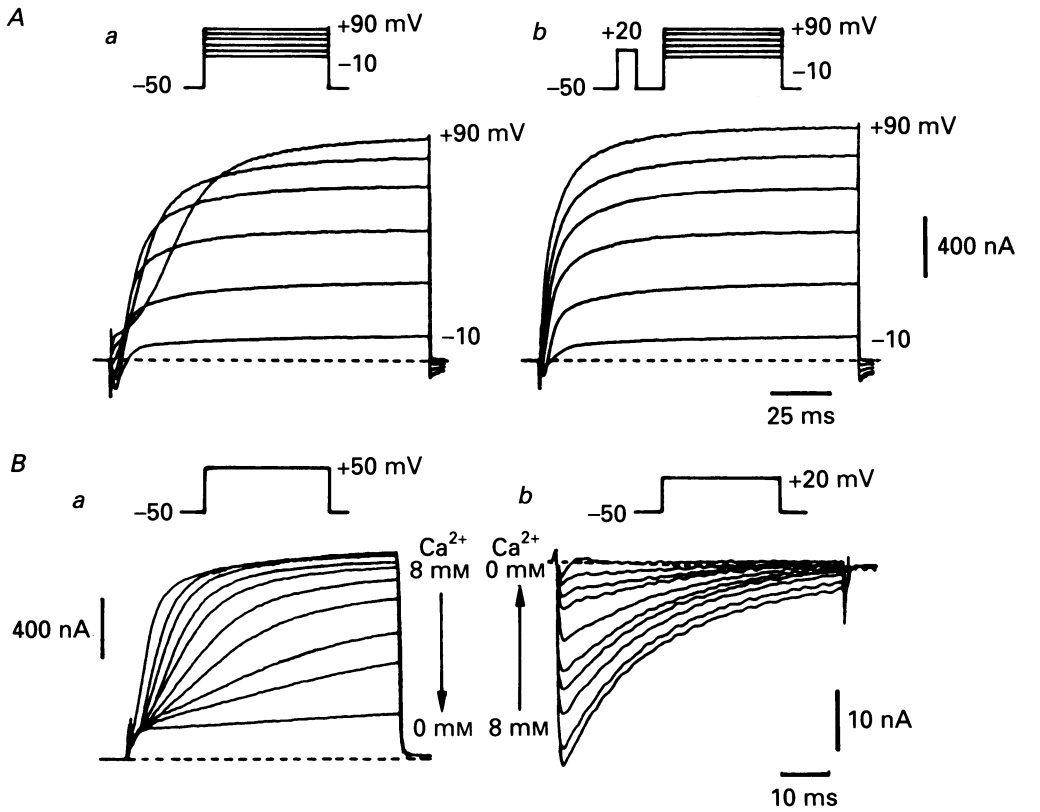

Fig. 3. Changes in the K^+_{Ca} activation rate with calcium. *A*, the Ca^{2+}-induced K^+_{Ca} current facilitation was not voltage dependent. Currents induced by pulse depolarizations (increased by 20 mV steps) after (*b*) a conditioning pulse (+20 mV, 20 ms) and with no conditioning pulse (*a*). *B*, effect of gradually reducing the bath Ca^{2+} saline on the K^+_{Ca} current (*a*) and on the Ca^{2+} current (*b*). Experiments performed in the same cell bathed in the Na^+-free saline (*a*) and then in the Na^+-free TEA^+ saline (*b*). Test pulses: +50 mV in *a*; +20 mV in *b*. Holding potential, −50 mV. The K^+_{Ca} current decreased and slowed down at low Ca^{2+} concentrations.

the steady-state test K^+_{Ca} current had almost the same level whether it was preceded (Fig. 3*Ab*) or not (Fig. 3*Aa*) by a conditioning calcium entry, i.e. the onset facilitation was not paralleled by a recruitment of open K^+_{Ca} channels.

In the above procedures, the amount of calcium that entered the cell was manipulated by changing the cell potential. To definitely rule out the possibility that a voltage-dependent process may have been involved in the regulation of the K^+_{Ca} activation rate, the calcium current at a given voltage was changed by altering the calcium content of the bath saline. The shift from the normal saline (8 mM Ca^{2+}) to a Ca^{2+}-free saline resulted first in a progressive slowing down of the K^+_{Ca} current onset

and then in its disappearance (Fig. 3*Ba*). Lowering the bath calcium content from 8 to 2 mM had no significant effect on the steady-state K^+_{Ca} current, whereas the time required to half-activation increased 2- to 3-fold. This kinetic change was not paralleled by any similar change in the kinetics of the underlying calcium current (Fig. 3*Bb*).

From this set of experiments, we concluded that the K^+_{Ca} current activation was rate-limited by the calcium channels' density but not by a voltage-dependent process. The voltage-dependent opening might occur when the channels were in a Ca^{2+}-bound state, resulting in current facilitation with the two-pulse voltage programme used in Figs 2*B* and 3*A*. This fast activation did not, however, reflect the actual kinetics of the voltage sensor since Ca^{2+} entries induced by several conditioning pulses resulted in an additional increase in the activation rate (not illustrated). Therefore, even in the facilitated mode, the activation of the K^+_{Ca} current was still rate-limited by the intracellular calcium concentration.

Single K^+_{Ca} channel openings during U cell spikes

The main conclusion drawn from the above results was that the Ca^{2+}-activated K^+ current had a slow activation rate under physiological conditions. It has nevertheless been demonstrated that this current may take part in spike repolarization (Gola *et al.* 1986, 1990; Lang & Ritchie, 1990). The aim of the experiments described in this and the subsequent sections was to evaluate to what extent and by means of what mechanisms Ca^{2+}-dependent K^+ channels can be brought into action by isolated spikes.

Single K^+_{Ca} channels were detected in the cell-attached mode and identified by comparing macroscopic whole-cell K^+_{Ca} currents with reconstructed currents obtained by averaging unitary channel currents (Gola *et al.* 1990). For that purpose, cell-attached patch recordings were performed on U cells impaled with an intracellular KCl-filled microelectrode. The recordings in Fig. 4 were obtained from a membrane patch containing two identical channels with a 40 pS unitary conductance. The patch depolarization induced channel openings, the probability of which had a peak value at potentials ranging from +10 to +30 mV (Fig. 4*A* and *D*). At larger depolarizations (> +50 mV), the opening probability (P_o) decreased (Fig. 4*B*) and the ensemble current became slower. These were the main characteristics of the whole-cell K^+_{Ca} current triggered by cell depolarizations at levels that approached the reversal potential for Ca^{2+} ions. Large patch depolarizations were able, however, to open the channels when they were preceded by a whole-cell spike (Fig. 4*C*). This protocol mimicked the two-pulse program we used to facilitate the K^+_{Ca} current activation (see Fig. 2), i.e. the spike induced a Ca^{2+} entry large enough to facilitate the opening of the K^+_{Ca} channels elicited by the subsequent large patch depolarization. The fact that the P_o–V relationship of the channels had the same shape as the whole-cell K^+_{Ca} current indicated that Ca^{2+} channels were present in the patch together with the K^+_{Ca} channels. The Ca^{2+} channels were not detected at the unitary level due to their small conductance and to the subtraction procedure we used to eliminate capacitive and leak currents.

In order to be able to detect K^+_{Ca} channel openings at both negative and positive potentials during spiking periods, the patch pipette was filled with a KCl-rich saline

(see Methods), which shifted the reversal potential (E_{rev}) of K^+ ions to $\approx +10$ mV and increased the unitary channel conductance to 60–65 pS. When the cell was fired (Fig. 5*Aa*), the patch electrode (clamped at 0 mV) recorded the spike-induced capacitive and leak currents (smooth trace in Fig. 5*Ab* and occasional single K^+_{Ca}

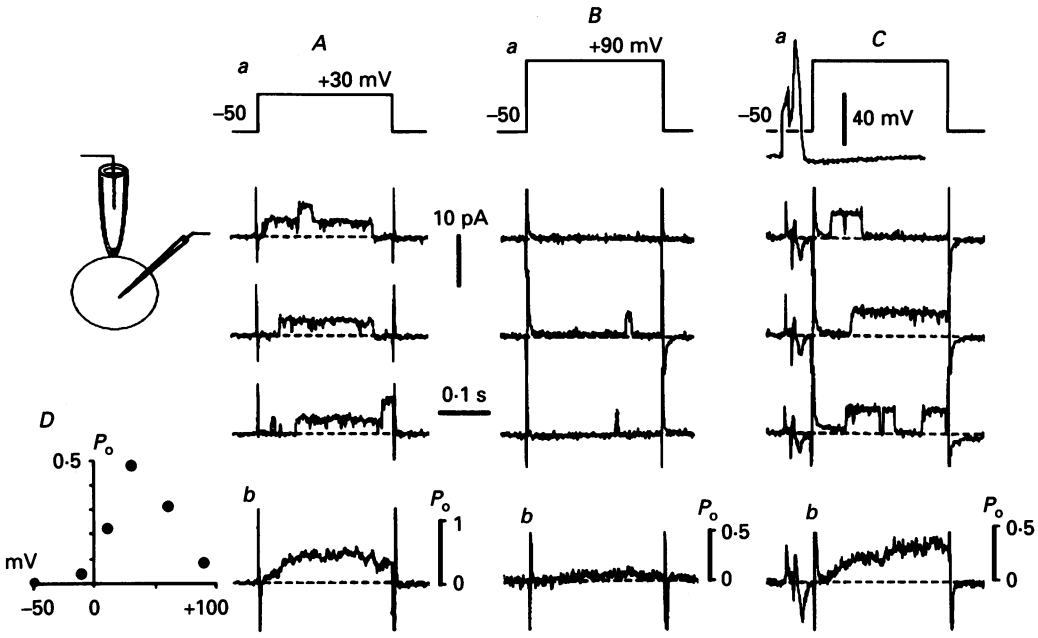

Fig. 4. Criteria used to identify K^+_{Ca} channels in cell-attached patches. U cell impaled with an intracellular electrode. Patch pipette filled with physiological saline. The patch membrane contained two channels. *Aa*, channel openings in response to three successive patch depolarizations from −50 mV (cell holding potential) to +30 mV. *Ab*, change in channel opening probability, P_o, obtained by dividing the averaged patch current ($n = 14$) by twice the unitary current. Peak $P_o = 0{\cdot}48$. *B*, same series with the patch depolarized at +90 mV. Peak $P_o < 0{\cdot}1$. *C*, the +90 mV pulse was applied 50 ms after a spike (upper traces). Peak $P_o = 0{\cdot}45$. *D*, peak P_o as a function of patch potential (without preceding spike).

channel events that superimposed on these currents (Fig. 5*Ab*). Series of recordings with or without channel currents were averaged giving traces 1 and 2, respectively, in Fig. 5*Ac*. The difference between these traces yielded the averaged K^+_{Ca} current that flowed during the spike (Fig. 5*Bb*). The corresponding conductance change was obtained by dividing the averaged K^+_{Ca} current by the K^+ driving force $V-E_{rev}$. The result showed that the K^+_{Ca} current started to activate after the spike overshoot, that it peaked when the spike repolarized to about 0 mV and that it was rapidly deactivated when the cell repolarized (Fig. 5*C*). From the averaged and unitary K^+_{Ca} currents we calculated that the opening probability of the channel reached a peak value of 0·25–0·3 during the spike repolarizing phase. This experiment also demonstrated that the K^+_{Ca} current was rapidly turned off during the early post-spike hyperpolarization and therefore did not contribute significantly to shaping the post-spike voltage trajectory.

The K^+_{Ca} current operates in the fast activating mode during spiking

The hybrid voltage clamp method (see Methods) was used to evaluate the whole-cell K^+_{Ca} current that was activated by the spike. The principle of the method is illustrated in Fig. 6*Aa*. The spike (actually a digitized stored spike used to command

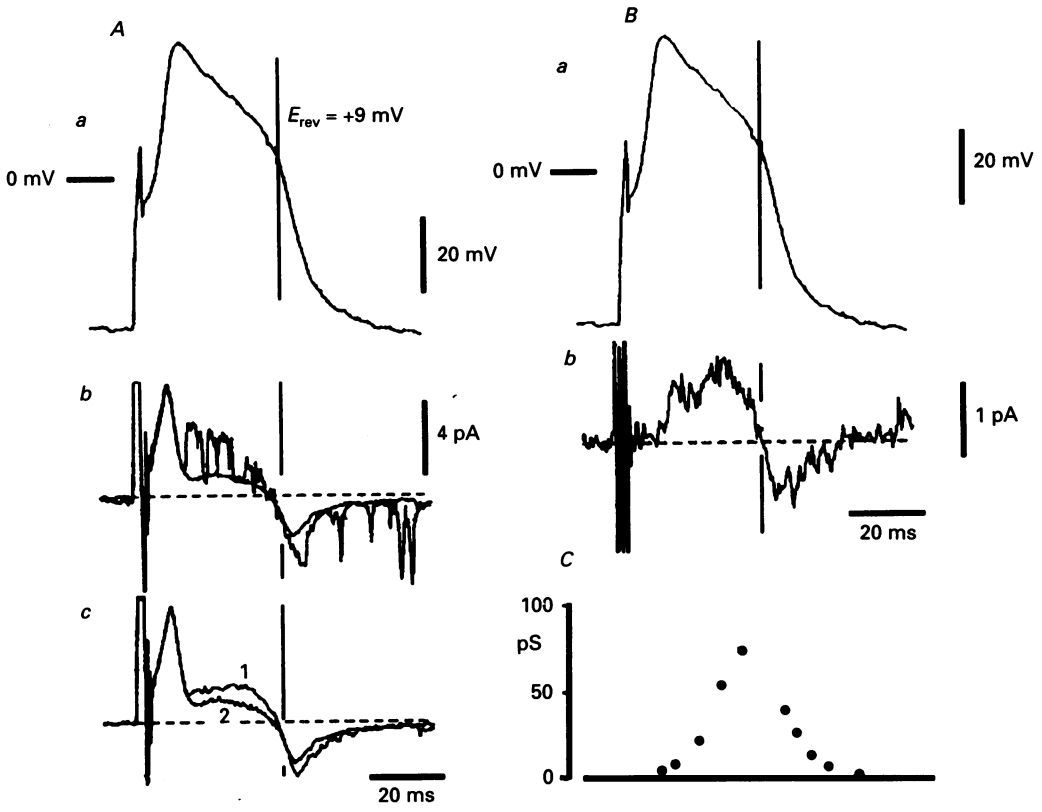

Fig. 5. Opening of K^+_{Ca} channels during U cell spikes. Cell-attached recording of K^+_{Ca} channels. Patch pipette filled with a KCl-rich (115 mM) saline. Cell impaled with an intracellular microelectrode and fired regularly (0·1 Hz) with short current pulses. *Aa*, intracellular cell recording. *Ab*, patch pipette currents. Channel currents superimposed on the spike-induced capacitive and leak currents (smooth trace). *Ac*, ensemble currents obtained by averaging recordings containing channel currents (trace 1, $n = 42$), and those with no channel currents (trace 2, $n = 6$). *B*, averaged channel current (*b*) during U cell spike (*a*). Trace *b* obtained by subtracting trace 2 from trace 1 in *Ac*. *C*, K^+_{Ca} channel conductance changes. The conductance was obtained by dividing the averaged channel currents (trace *Ac*) by the K^+ driving force (voltage in *Ba* minus $E_{rev} = +9$ mV). The K^+ reversal potential (vertical lines in *A* and *B*) was given by the zero current level of the averaged ionic current (in *Ac*). The K^+_{Ca} channel started to open when the spike overshot: its opening probability peaked when the spike repolarized and rapidly decreased to zero as the cell repolarized. This patch contained three K^+_{Ca} channels.

the voltage clamp amplifier) was interrupted and the voltage was set at a large positive level (+70 mV in Fig. 6*Aa*). The consecutive current gave two significant items of information.

Firstly, as compared to the control current (step depolarization from resting to +70 mV, left-hand recording in Fig. 6*Aa*), this consecutive current had the fast onset characteristic of the facilitated state described in Figs 2*B* and 3*A*. This facilitation developed progressively during the spike and reached a steady level when

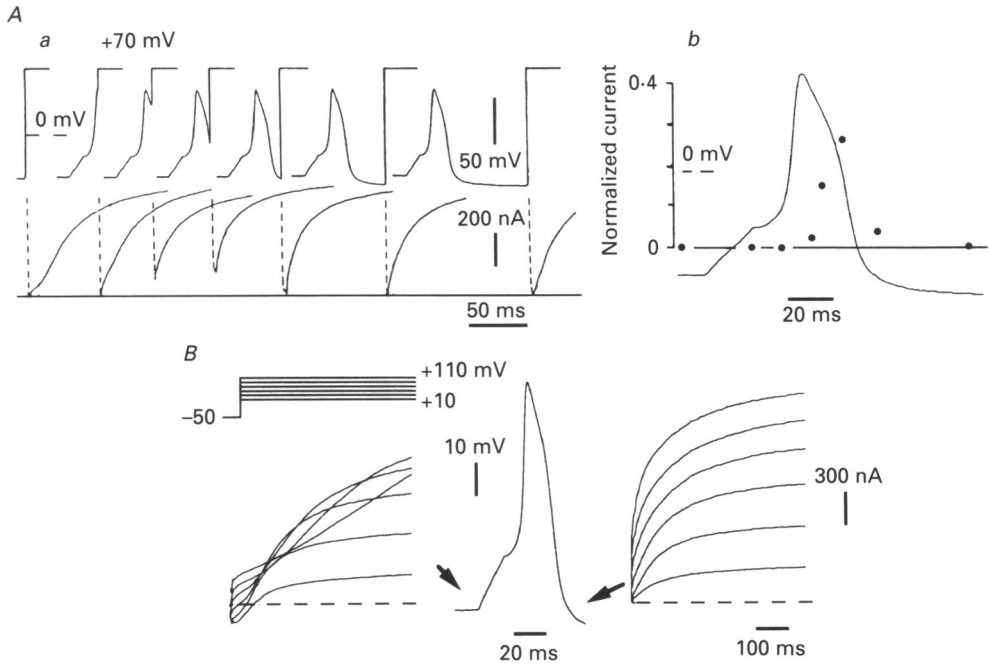

Fig. 6. Quantitative evaluation of K^+_{Ca} current increase in U cell spikes. *Aa*, hybrid current clamp–voltage clamp method. U cell voltage-clamped and subjected to a spike-like voltage clamp pulse. Upper traces, the spike was interrupted at various times and the cell voltage was set at +70 mV. Lower traces, currents recorded in response to the +70 mV pulse. Left-hand recording, control current in the absence of spikes. *Ab*, the ratio of the initial current when the spike was interrupted to its steady-state level gave the relative increase in the K^+_{Ca} current (dots) induced by the spike. In this experiment, 28% of the available K^+_{Ca} current participated in the spike repolarization. *B*, set of membrane currents induced by voltage pulses (increased in 20 mV steps) applied before and after a spike-like voltage clamp pulse.

the cell repolarized. The two sets of current recordings in Fig. 6*B* show that the spike-induced current facilitation was independent of the test voltage. It should also be noted that the facilitation was still present during the post-spike hyperpolarization (right-hand recording in Fig. 6*Aa*).

Secondly, the amount of K^+_{Ca} current, already activated at the time the spike was interrupted, was deduced from the initial level of the consecutive current. This initial current increased progressively during the spike and peaked when the spike was half-repolarized. The ratio of the initial to the steady-state levels of the consecutive current gave a quantitative evaluation of the K^+_{Ca} current active during the spike. This ratio (black dots superimposed on the spike in Fig. 6*Ab*) confirmed that 25–30% of the available whole-cell K^+_{Ca} current participated in the spike repolarization. It

also confirmed the preceding conclusion that the K^+_{Ca} current was rapidly deactivated during the early post-spike repolarization. This deactivation occurred in spite of the fact that the Ca^{2+}-induced facilitating process persisted for half a second (Fig. 6*Aa*) and it must therefore have been due to the fast voltage-dependent closing of K^+_{Ca} channels.

Ca^{2+} and K^+_{Ca} currents' contribution to the spike shape

The time-course of the Ca^{2+} and K^+_{Ca} currents during the spike was further evaluated using the spike-like voltage clamp method as previously described (Gola *et al.* 1986). The K^+_{Ca} current contribution to the spike wave shape was first extracted either by adding TEA^+ (20 mM) to the Na^+-free saline or by injecting EGTA. Further addition of Cd^{2+} (1 mM) or La^{3+} (0·2–0·5 mM) to the bath saline gave the Ca^{2+} current. This method revealed that the calcium current that flowed during U cell spikes had two successive peaks corresponding to the rising and late repolarizing phases (Fig. 7*Aa*). Concomitantly, the K^+_{Ca} current had a peak of 147 ± 20 nA ($n = 15$) which was almost synchronous with the second Ca^{2+} current surge (Fig. 7*Aa*). The K^+_{Ca} current peak corresponded to a conductance of $g_{K,Ca} = 2{\cdot}25\ \mu S$ (with a potassium reversal potential at -65 mV). The maximum K^+_{Ca} conductance in U cells was evaluated from the K^+_{Ca} current induced by voltage pulses of increasing amplitude that followed a conditioning pulse (10 ms at $+20$ mV) applied in order to facilitate the K^+_{Ca} current onset. This conductance was $7{\cdot}5 \pm 0{\cdot}9\ \mu S$. Almost 30% of the available K^+_{Ca} current was therefore activated during the spike. It is likely that the first entry of calcium ions, induced by the spike, played the role of the conditioning pulse in the two-pulse program used to facilitate the K^+_{Ca} current activation, i.e. it was large enough to produce a significant increase in the intracellular calcium concentration.

The quantity of Ca^{2+} ions that entered the cell during the spike was determined by numerical integration of the Ca^{2+} flux (Fig. 7*Aa*). It ranged from 3·4 to $21{\cdot}5 \times 10^{-15}$ mol of Ca^{2+} $cell^{-1}$ $impulse^{-1}$ (mean, $7{\cdot}2 \pm 1{\cdot}1$ in 15 cells). This quantity was scaled to the cell surface assuming that the specific membrane capacitance was $1\ \mu F\ cm^{-2}$. The input cell capacitance was obtained from the capacitive current produced in response to voltage ramps (slope, 10–20 $V\ s^{-1}$). In the above fifteen U cells, the cell capacitance varied from 2·4 to 8·5 nF ($4{\cdot}4 \pm 0{\cdot}2$ nF). The quantity of calcium ions that entered the cell was highly correlated (correlation coefficient, 0·91) with the cell capacitance (Fig. 7*Ab*). The variability of this quantity therefore reflected neuronal size variations rather than heterogeneous membrane properties. The slope of the regression line in Fig. 7*Ab* gave the number of Ca^{2+} ions that entered the cell per unit area: $1{\cdot}63 \pm 0{\cdot}3 \times 10^{-12}$ mol μF^{-1} $impulse^{-1}$. This calcium influx appeared large enough to raise the Ca^{2+} concentration over 10–20 μM within the 1 μm depth of the superficial cytoplasm.

The spike-like voltage clamp method was applied to U cells in which the K^+_{Ca} current was progressively blocked by 20 mM TEA^+. The spikes were collected in the course of TEA^+ effect. Full K^+_{Ca} current blockade was then obtained by increasing the TEA^+ concentration to 60 mM. The stored signals were then applied to the voltage-clamped cell before and after adding Ca^{2+} channel blockers (Cd^{2+} or La^{3+}). The results are displayed in Fig. 7*B*.

As expected from the blockade of K^+_{Ca} channels, TEA^+ induced spike lengthening,

positively shifted the overshoot and prolonged the second Ca^{2+} entry (Fig. 7*Ba*). The most significant finding was that the Ca^{2+} current concomitant with the spike overshoot was progressively reduced (arrow in Fig. 7*Ba*) as the spike increased in amplitude. We also noted that the first calcium surge and the spike depolarizing rate

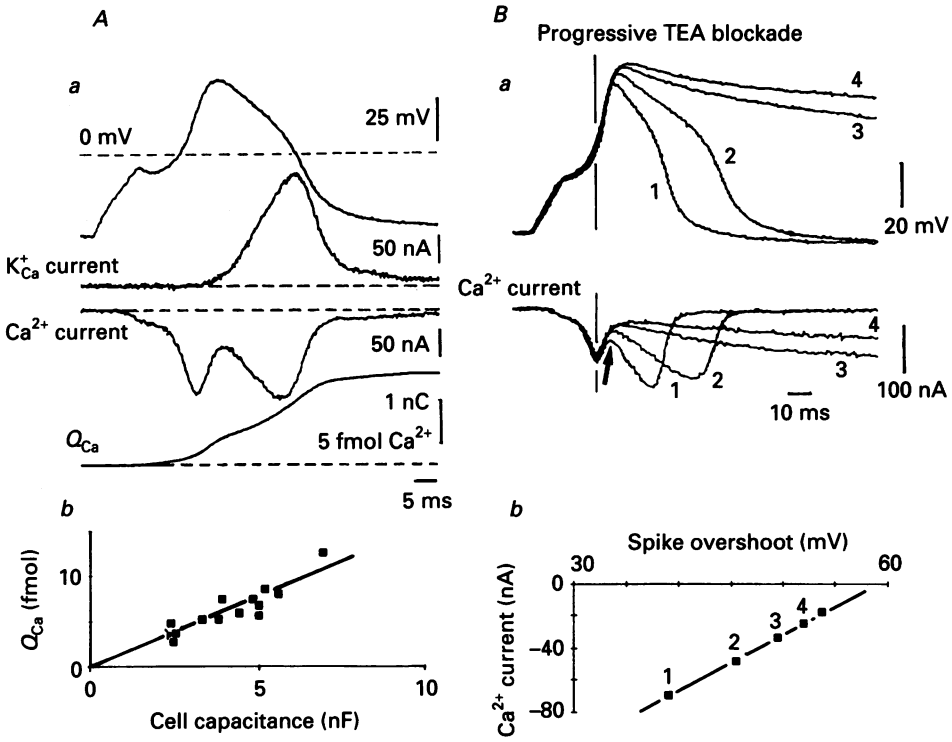

Fig. 7. Timing of Ca^{2+} and K^+_{Ca} currents changes in U cell spikes. Ionic currents flowing during U cell spikes were determined using the spike-like voltage clamp method. *Aa*, U cell spike recorded, stored and further applied to the same voltage-clamped cell. K^+_{Ca} current and Ca^{2+} current: compensation current delivered by the voltage clamp amplifier when K^+_{Ca} channels and Ca^{2+} channels were successively blocked (see Methods for details). The Ca^{2+} current had two successive peaks. Q_{Ca}, quantity of calcium ions that entered the cell during the spike, obtained by integrating the Ca^{2+} current. *Ab*, data from fifteen U cells; Q_{Ca} (in fmol) as a function of the cell membrane capacity (in nF). The regression line corresponds to a specific calcium flux of $1{\cdot}63 \times 10^{-12}$ mol μF^{-1} impulse^{-1}. *B*, same protocol as in *A* applied to a TEA^+-treated cell. *Ba*, spikes collected in the course of TEA^+ action and corresponding calcium currents were aligned with the first Ca^{2+} surge. Note the sag in the Ca^{2+} current (arrow) corresponding to the spike overshoot. *Bb*, plot of the Ca^{2+} current during the sag and spike overshoot changes induced by TEA^+. The extrapolated linear relationship gave a null calcium current at +58 mV.

were not affected by TEA^+. We therefore took the Ca^{2+} channels at the spike overshoot to be in the same state although the overshoot had increased by 10–12 mV. The sag in the Ca^{2+} current at the spike overshoot therefore resulted from the decrease in the calcium driving force as the cell potential approached the Ca^{2+} reversal potential. This reversal potential was determined by plotting the spike overshoot and the corresponding Ca^{2+} current (Fig. 7*Bb*). The extrapolated null Ca^{2+}

current voltage was about +60 mV (from +58 to +64 mV in four experiments). In conventional voltage clamp experiments, this null-current voltage ranged from +100 to +130 mV (Eckert & Tillotson, 1978). In a cell bathed in the normal saline the reversal potential at +60 mV corresponded to an intracellular calcium

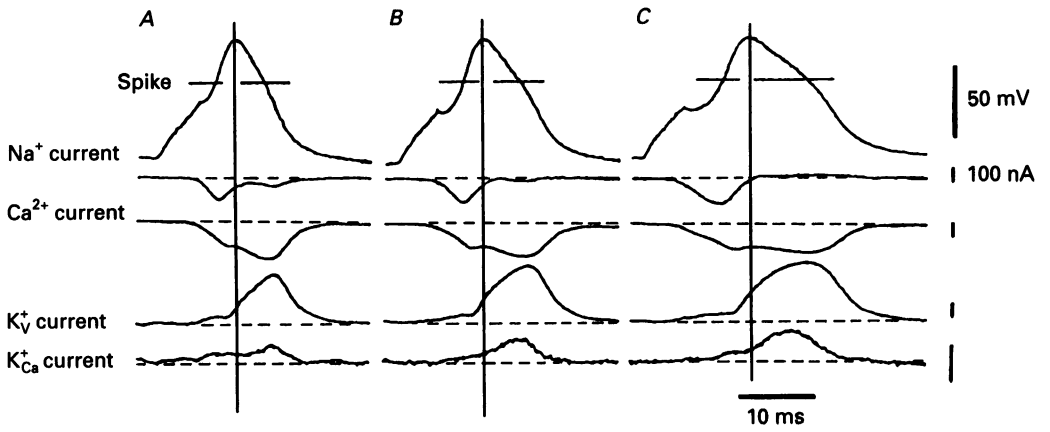

Fig. 8. K^+_{Ca} current contribution to Na^+- and Ca^{2+}-dependent spikes. Experiment performed on P cells. Ionic current contribution determined with the spike-like voltage clamp method. The ionic components active during the spike were sequentially separated by (1) bathing the cell in a Na^+-free saline (Na^+ current); (2) injecting EGTA (K^+_{Ca} current); (3) adding 60 mM TEA^+ to the Na^+-free saline (K^+_V current) and (4) substituting Co^{2+} for Ca^{2+} in the TEA^+ saline (Ca^{2+} current). *A*, short duration spike from a resting P cell. *B* and *C*, elongated spikes collected in the same P cell fired at 5 Hz. The K^+_{Ca} current contribution to the spike repolarization became more and more consistent as the spike enlarged. Note the presence of a sustained Ca^{2+} current in enlarged spikes.

concentration of 80 μM. The first surge of Ca^{2+} current during the spike upstroke therefore induced a large increase in the free calcium, at least in the close vicinity of the inner mouth of the Ca^{2+} channels. This high level of $[Ca^{2+}]_i$ may have facilitated the opening of the K^+_{Ca} channels.

The data obtained with the various experimental protocols we used converge to indicate that K^+_{Ca} channels constitute a powerful repolarizing mechanism in *Helix* U cells. These cells are characterized, however, by their long spike duration due to the low density of voltage-gated Na^+ and K^+ channels. Are K^+_{Ca} currents still active in *Helix* cells generating short duration action potentials? To solve this question, we applied the spike-like voltage clamp method to P cells in which the inward current flowed through Na^+ and Ca^{2+} channels and the outward current, through K^+_V and K^+_{Ca} channels (Crest *et al.* 1990*a*). In the experiment shown in Fig. 8, The K^+_V and K^+_{Ca} currents induced by voltage pulses (not illustrated) contributed in almost equal amounts to the total outward current.

In P cells, the spike depolarization was mainly sustained by a transient sodium current (Fig. 8*A*). The Ca^{2+} current still had the two phases observed in U cells but the main Ca^{2+} entry occurred during the spike repolarization. The K^+_{Ca} current did not contribute significantly to the repolarization, which was mainly due to the activation of the K^+_V current.

The same procedure was applied to spikes, the duration of which was increased by firing the cell at 5 Hz. Two relevant events were observed: long-lasting spikes were sustained by a persistent calcium current (Fig. 8*B* and *C*) and the K^+_{Ca} current became increasingly active as the spike lengthened. K^+_{Ca} currents, even when working

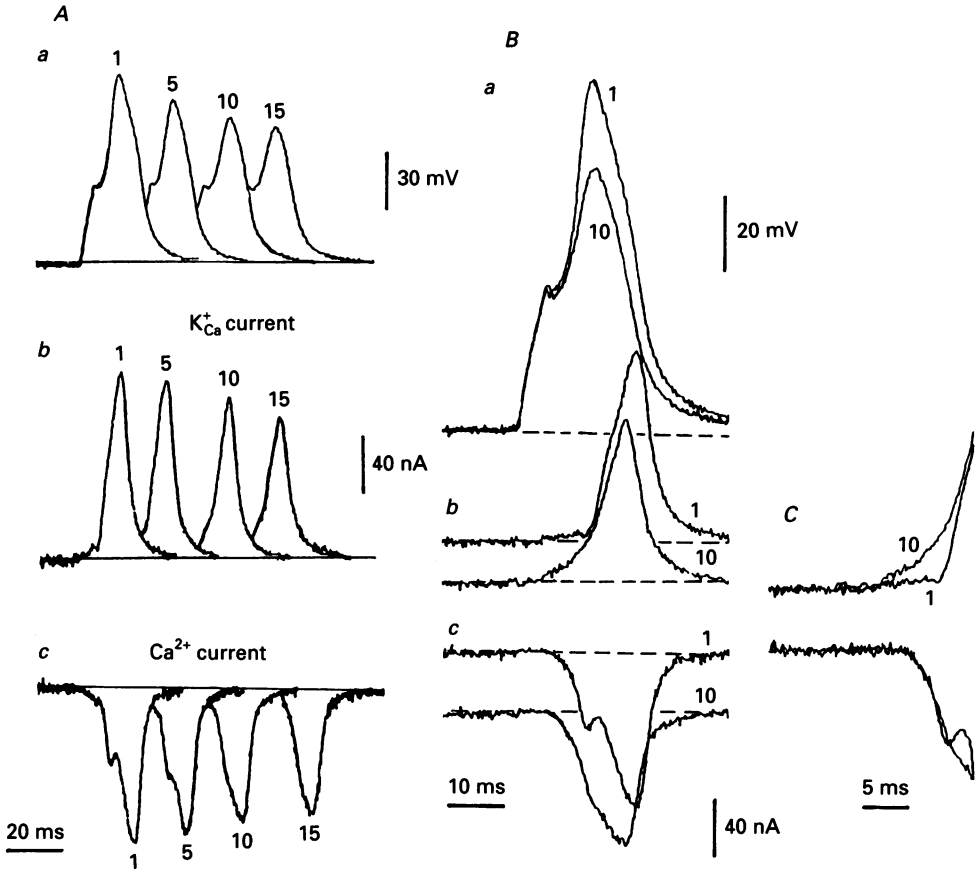

Fig. 9. U cell firing blockade induced by early activation of the K^+_{Ca} current. *Aa*, U cell spikes triggered by 3 Hz current pulses. Numbers attached to the recordings refer to the number of stimulations. The entire spike burst was recorded and then applied to the voltage-clamped cell. Ca^{2+} (*b*) and K^+_{Ca} (*c*) currents were obtained as described in Fig. 7*A*. Both currents decreased in amplitude during the burst. *B* and *C*, detail of the first and tenth spikes. The K^+_{Ca} current (*Bb* and *C*) in the tenth spike started to activate as the cell depolarized and thus partly counteracted the Ca^{2+} current (*Bc* and *C*).

in the facilitated mode, therefore have a finite rate of rise which limited their involvement in the spike repolarization.

Firing regulation by K^+_{Ca} channels

When U cells were fired repeatedly, even at low frequencies (3–5 Hz), the spike overshoot progressively decreased, which ultimately resulted in firing blockade. This behaviour was not specific to U cells since it was observed in nerve cells in which the

Ca^{2+}-dependent K^+ current was the main spike repolarizing current (Galvan & Sedlmeir, 1984; Alger & Williamson, 1988). This finding suggested that successive Ca^{2+} entries during firing might produce cumulative recruitment of Ca^{2+}-dependent K^+ channels.

This hypothesis was tested by determining the Ca^{2+} and K^+_{Ca} currents that flowed during successive spikes. For this purpose, the spike-like voltage clamp method was applied to individual spikes within a burst. Spike bursts elicited at 3–5 Hz in a U cell were recorded in the Na^+-free saline and then reinjected into the same voltage-clamped cell. The K^+_{Ca} current contribution was determined from the compensation current evoked when the cell was bathed in the Na^+-free saline supplemented with 60 mM TEA^+. The Ca^{2+} current was further obtained by adding 1 mM Cd^{2+} or by substituting Co^{2+} for Ca^{2+} (8 mM) in the TEA^+ saline. The data from one such experiment are given in Fig. 9. From the first to the fifteenth spikes, the overshoot shifted from +42 to +12 mV (Fig. 9*Aa*). This change was not paralleled by any increase in the repolarizing K^+_{Ca} current (Fig. 9*Ab*) nor by any significant decrease in the Ca^{2+} current (Fig. 9*Ac*); both currents were moderately reduced (by 20%) during the burst in such a way that their ratio remained almost constant. The fast adaptation exhibited by U cells could therefore not be attributed to a change in the net balance between depolarizing and repolarizing currents.

The firing adaptation mechanism was determined by closely examining the current time course. The superimposed recordings in Fig. 9*Ba* show the first and tenth spikes from the burst illustrated in Fig. 9*A*. The corresponding K^+_{Ca} and Ca^{2+} currents are displayed in *Bb* and *Bc*, respectively, of Fig. 9. The Ca^{2+} current in the first spike had the two peaks described in the previous section. The two peaks fused progressively during the burst as expected from the fact that the spike overshoot became less positive. Nevertheless, the Ca^{2+} current time course was not significantly altered during the successive spikes (see details in Fig. 9*C*). As already mentioned, no additional facilitation or recruitment of K^+_{Ca} channels occurred during the burst. The most significant modification was observed in the time course of the K^+_{Ca} current. As shown in Fig. 9*Bb* and *C*, the K^+_{Ca} current in the tenth spike had an early activation which started at the same time as the Ca^{2+} current. The early K^+_{Ca} current activation counteracted the depolarizing Ca^{2+} current; it was observed when U cells were fired at frequencies of more than 1·5–2 Hz. This is consistent with the fact that the Ca^{2+}-induced K^+_{Ca} current facilitation vanished within 0·5–0·8 s after a brief Ca^{2+} entry (exponential decay with a 0·2–0·3 s time constant). The early activation of the K^+_{Ca} current evoked by repeated firing was therefore due to the voltage-dependent opening of the K^+_{Ca} channels that were still in the voltage-sensitive Ca^{2+}-bound state due to the residual calcium accumulated by the preceding spikes.

The frequency-induced early activation of K^+_{Ca} channels was also observed at the unitary level. The experiment illustrated in Fig. 10*A* was performed on a U cell fired regularly at either 0·5 or 2·5 Hz. A patch pipette sealed to the neurone recorded the current from three K^+_{Ca} channels. The averaged patch currents induced by firing the cell at each frequency are displayed in Fig. 10*Ab*. The peak opening probability of the K^+_{Ca} channels showed no significant change in response to firing the cell at either 0·5 or 2 Hz. At 2 Hz, the K^+_{Ca} current occurred earlier, however, and was faster than at 0·5 Hz, which reduced the overshoot and shortened the spike.

When performing these experiments, the patch electrode was occasionally sealed to a region containing voltage-gated K^+ channels and was devoid of K^+_{Ca} channels. In whole-cell voltage clamp experiments, we have observed that K^+_V channels played a limited role in U cells. Accordingly, K^+_V channels were found to be clustered in a few

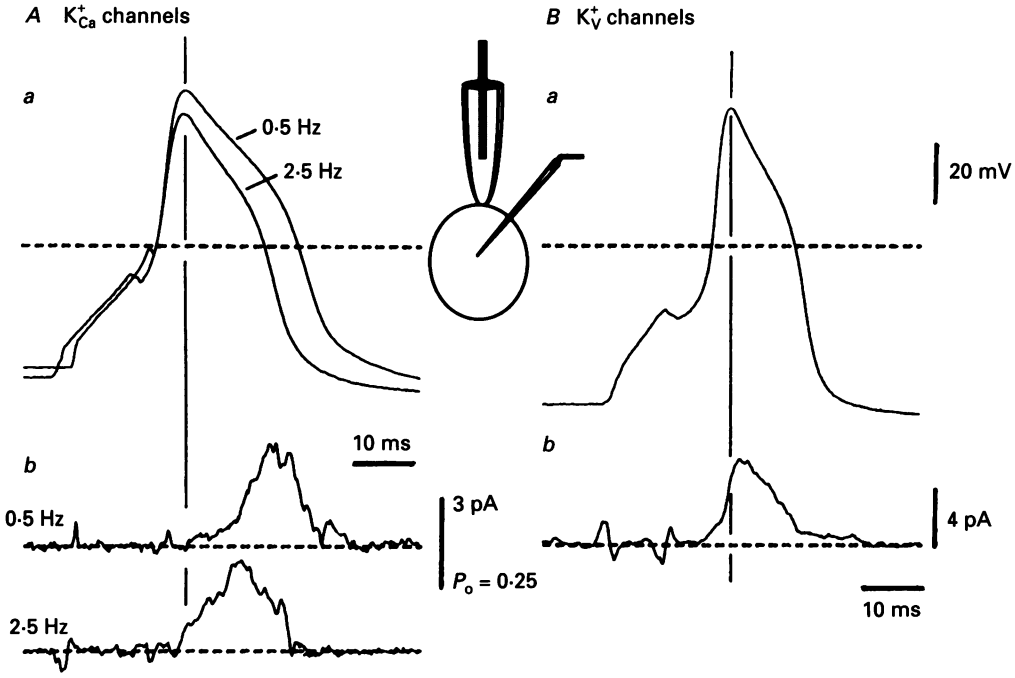

Fig. 10. Frequency-induced early activation of K^+_{Ca} channels. Experiment performed in a U cell fired with a current pulse applied to the intracellular recording electrode. Patch electrode filled with the physiological saline. *A*, patch sealed to a region containing three K^+_{Ca} channels. *Aa*, spikes in response to repetitive stimulations applied at 0·5 and 2·5 Hz. *Ab*, corresponding averaged ($n = 20$) patch currents. Leak and capacitive currents subtracted using current recordings devoid of channel openings. *B*, patch sealed to a region containing voltage-gated K^+ channels. *Ba*, cell firing at 0·5 Hz. *Bb*, corresponding averaged ($n = 20$) K^+_V current. Leak and capacitive currents subtracted using current recordings obtained with the patch hyperpolarized by 60 mV.

discrete areas containing an unresolved number of these channels. The recording in Fig. 10*B* shows the averaged K^+_V current from one such cluster. The K^+_V current had a faster rate of activation than the K^+_{Ca} current even when repetitive firing speeded up the K^+_{Ca} current. These data are in line with those obtained with the spike-like voltage clamp method applied to cells having both channel types.

DISCUSSION

The main aim of this study was to evaluate to what extent BK-type large conductance K^+_{Ca} channels are involved in cell firing regulation. Due to their dual regulation by both calcium and voltage, we demonstrate that these channels may take part in both spike repolarization and firing frequency regulation.

Based on the effects of channel blockers and calcium chelators on the spike shape, several authors have concluded that BK channels and voltage-gated delayed rectifiers may be co-activated during the spike repolarization and in the initial phase of the after-hyperpolarization (MacDermott & Weight, 1982; Hounsgaard & Mintz, 1988; Mosfeldt Laursen & Rekling, 1989; Lang & Ritchie, 1990). These conclusions imply that BK channels have a short opening latency in response to both cell depolarization and increase in intracellular calcium concentration.

In experiments performed on either excised BK channels or channels incorporated into lipid bilayers, the Ca^{2+} requirements and voltage sensitivity of channel opening were exactly evaluated (see for review McManus, 1991). These experiments yielded little information, however, about the activation time course of the corresponding currents in response to step changes in voltage and calcium. Ikemoto, Ono, Yoshida & Akaike (1989) observed that step changes in $[Ca^{2+}]$ (up to 1 mM) at the inner face of excised BK channels induced openings with latencies of several tens of milliseconds (see also Brett, Dilger, Adams & Lancaster, 1986). Whole-cell voltage clamp experiments have shown that BK currents have a relatively slow activation time course in comparison with voltage-activated K^+ currents. This applies to *Helix* neurones (Lux & Hofmeier, 1982), rat sympathetic neurones (Galvan & Sedlmeir, 1984), anterior pituitary cells (Ritchie, 1987), leech neurones (Johansen, Yang & Kleinhaus, 1987) and chick ciliary ganglion neurones (Dryer *et al.* 1991). The reverse was found to occur, however, in bullfrog sympathetic neurones (Adams *et al.* 1982; MacDermott & Weight, 1982).

In spite of the slow time course of the macroscopic current, recordings of unitary currents in cell-attached patches have shown that the BK channel opening probability peaks within a few milliseconds of the spike upstroke at values as large as 0·25–0·4 (Lang & Ritchie, 1987; Gola *et al.* 1990). These findings suggested that whole-cell BK currents observed under voltage clamp conditions did not give reliable information on their involvement in freely firing cells, presumably because of the way Ca^{2+} ions enter the cell in these different situations (see below).

BK currents are rate-limited by calcium

It was soon recognized that BK channels are controlled by both calcium and membrane potentials (Gorman & Thomas, 1980; Woolum & Gorman, 1981; Lux & Hofmeier, 1982). The fact that the current became slower at large positive potentials led to the hypotheses that it had a particular voltage dependence (Woolum & Gorman, 1981) and that Ca^{2+} ions played a permissive role in the activation of the current that was then kept activated by depolarization (Lux & Hofmeier, 1982). These hypotheses were challenged, however, by the suggestion that the channel may have an intrinsic voltage sensor with fast kinetics while the channel opening may be rate-limited by the intracellular $[Ca^{2+}]$ (Smart, 1987; Müller *et al.* 1989; Gola *et al.* 1990). This is in line with the fact that the sensitivity of neuronal maxi K^+_{Ca} channel gating to calcium is low (see review by McManus, 1991). The various protocols we used to manipulate the inflow of Ca^{2+} ions gave results which were all consistent with the idea that the Ca^{2+} binding step was the rate-limiting factor. They also indicated that even when large Ca^{2+} inflows were produced, the channels were far from working under Ca^{2+} saturating conditions. Similar conclusions were reached upon analysing

a related K^+_{Ca} current present in a restricted number of *Helix* neurones (Crest, Watanabe & Gola, 1990*b*).

The sensitivity of neuronal BK channels to calcium requires to be evaluated by examining the channel behaviour separately under steady-state conditions and in response to step changes in $[Ca^{2+}]_i$ or voltage. The steady-state current from hippocampal K^+_{Ca} channels subjected to step changes in $[Ca^{2+}]$ was found to be sensitive to $[Ca^{2+}]$ in the 1–10 μM range. At these concentrations, the current onset was consistently delayed and had a slow activation rate (half-activation of several hundreds of milliseconds; Ikemoto *et al.* 1989). Between 10 μM and 1 mM $[Ca^{2+}]$, although the steady-state P_o saturated, the activation time course was speeded up. Increase of intracellular $[Ca^{2+}]$ to more than 50 μM would therefore be necessary for K^+_{Ca} current to have an onset fast enough to be significantly activated by isolated spikes.

K^+_{Ca} current involvement in spike repolarization

The main conclusion reached in the preceding section was that the K^+_{Ca} current activation rate was critically dependent on the instantaneous local intracellular calcium concentration rather than on the cell potential. This large sensitivity to calcium helps to explain the repolarizing role played by K^+_{Ca} currents. We observed that a single spike shifted the current to its fast activating mode. This resulted from the large Ca^{2+} inflow concomitant with the spike upstroke. Increases in $[Ca^{2+}]_i$ to several micromolar after a spike train have been detected using Ca^{2+}-sensitive dyes and electrodes (Gorman & Thomas, 1980). Much larger increases (up to 0·3 mM) probably occur in the space immediately adjacent to Ca^{2+} channels (Müller & Connor, 1991; Adler, Augustine, Duffy & Charlton, 1991; Llinás, Sugimori & Silver, 1992). The change in the null-current potential of the Ca^{2+} inflow in TEA^+-treated spikes that we observed with the spike-like voltage clamp method was indicative of local $[Ca^{2+}]_i$ increases of more than 50 μM. Under these conditions, K^+_{Ca} channels rapidly bound calcium and opened as long as the cell voltage was more positive than the threshold for the voltage-gated transition to the open state to occur. In addition, the second Ca^{2+} entry that occurred during the spike repolarization contributed to the fast activation rate of the K^+_{Ca} current. This second Ca^{2+} surge, reminiscent of the late Ca^{2+} entry observed in presynaptic spikes (Llinás, Steinberg & Walton, 1981), resulted from the increased driving force as the cell repolarized and thus had the properties of tail currents in voltage clamp experiments.

The hypothesis that spikes induce a large Ca^{2+} concentration build up at the cytosolic face of the membrane would account for the powerful repolarization that K^+_{Ca} currents exert in the Ca^{2+}-dependent U cell spikes. Using various methods we calculated that 25–30 % of the available K^+_{Ca} current was activated during isolated U cell spikes. This figure is surprisingly close to that of the voltage-gated delayed rectifier active in squid axon spike repolarization (Bezanilla *et al.* 1970). Likewise, the Ca^{2+} inflow during firing corresponded to a net transfer of $3{\cdot}2 \times 10^{-12}$ positive charges cm^{-2} $spike^{-1}$, which is very comparable to the Na^+ influx in *Sepia* and *Loligo* axons: $3{\cdot}5$–$3{\cdot}8 \times 10^{-12}$ mol cm^{-2} $spike^{-1}$ (Keynes & Lewis, 1951). This comparison shows that the Ca^{2+}–K^+_{Ca} system is analogous to the Na^+–K^+_V system as far as the mode of action potentials generation is concerned. There is, however, about one order in magnitude in the time scale between the spikes generated by the two systems. The

question therefore arises as to whether K^+_{Ca} currents may still be active in spikes generated by both systems. The experiment we have performed in *Helix* cells fulfilling these requirements showed that, although facilitated by calcium, K^+_{Ca} currents had a finite activation rate and played a limited role in shaping the spike. This role became crucial, however, when repetitive firing tended to depress the delayed rectifier due to cumulative voltage-induced inactivation.

Firing frequency adaptation induced by BK channels

Due to their fast voltage dependence, BK-type K^+_{Ca} currents persist a few tens of milliseconds after the spike and do not contribute to shaping the interspike trajectory. Most authors have therefore assumed that these channels are not directly involved in cell firing regulation (Lancaster *et al.* 1991). This function has been ascribed to SK channels characterized by their high affinity to calcium and low sensitivity to voltage. Due to their gating characteristics, these channels are mainly active in post-firing periods. The corresponding currents last seconds after a spike. They clamp the cell potential close to E_K (potassium equilibrium potential) and therefore limit the firing frequency. In U cells, we obtained no evidence supporting the existence of any such currents. U cell spikes are followed by brief after-hyperpolarizations which are insensitive to apamin and can be blocked by TEA^+ and charybdotoxin. Patch clamp experiments failed to reveal the existence of any Ca^{2+}-dependent channels resembling SK channels. Alternatively, the cumulative increase in $[Ca^{2+}]_i$ occurring during firing may progressively recruit BK-type K^+_{Ca} channels, which would result in phasic responses. This process does not appear to give U cells their phasic characteristic. We observed that (1) no recruitment of active K^+_{Ca} channels occurred in response to repeated firing and (2) during successive spikes, the K^+_{Ca} current tended to overlap the Ca^{2+} current, thus reducing and ultimately preventing its depolarizing tendency.

These findings are directly relevant to the gating properties of the maxi K^+_{Ca} channels. In the 0·5–0·8 s period that follows a U cell spike, $[Ca^{2+}]_i$ returns to its resting level (Tsien, 1988). During this period, K^+_{Ca} channels remain in the closed state due to their voltage dependence. Nevertheless, some of the channels are still in the Ca^{2+}-bound state, i.e. ready to be quickly opened by cell depolarization. When spikes occur during this period, Ca^{2+}-bound channels open in the fast mode as soon as the cell voltage reaches the threshold for the transition from the Ca^{2+}-bound closed state to the open state to occur. Since this threshold roughly coincides with that of the Ca^{2+} current (Crest *et al.* 1990*a*), both currents overlap. The process develops progressively during firing, up to a point where the facilitated K^+_{Ca} current overwhelms the Ca^{2+} current and thus stops the firing.

Since BK-type maxi- K^+_{Ca} channels from various nerve cells have common gating properties, we feel that the frequency adaptation mechanism proposed here may operate, although in varying degrees, in all the cells generating Ca^{2+}-dependent spikes.

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