Potassium Current Activated by Depolarization of Dissociated Neurons from Adult Guinea Pig Hippocampus

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ABSTRACT Currents were generated by depolarizing pulses in voltage-clamped, dissociated neurons from the CA1 region of adult guinea pig hippocampus in solutions containing 1 μM tetrodotoxin. When the extracellular potassium concentration was 100 mM, the currents reversed at -8.1 ± 1.6 mV (n = 5), close to the calculated potassium equilibrium potential of -7 mV. The currents were depressed by 30 mM tetraethylammonium in the extracellular solution but were unaffected by 4-aminopyridine at concentrations of 0.5 or 1 mM. It was concluded that the currents were depolarization-activated potassium currents. Instantaneous current-voltage curves were nonlinear but could be fitted by a Goldman-Hodgkin-Katz equation with $P_{\text{Na}}/P_{\text{K}} = 0.04$. Conductance-voltage curves could be described by a Boltzmann-type equation: the average maximum conductance was 65.2 ± 15.7 nS (n = 9) and the potential at which $g_{\text{K}}$ was half-maximal was -4.8 ± 3.9 mV (mean ± 1 SEM, n = 10). The relationship between the null potential and the extracellular potassium concentration was nonlinear and could be fitted by a Goldman-Hodgkin-Katz equation with $P_{\text{Na}}/P_{\text{K}} = 0.04$. The rising phase of potassium currents and the decay of tail currents could be fitted with exponentials with single time constants that varied with membrane potential. Potassium currents inactivated to a steady level with a time constant of ~450 ms that did not vary with potential. The currents were depressed by substituting cobalt or cadmium for extracellular calcium but similar effects were not obtained by substituting magnesium for calcium.

INTRODUCTION

Depolarization of excitable membranes rapidly activates a sodium conductance and a delayed potassium conductance, often referred to as the delayed rectifier, that promotes return of the membrane potential to its resting level (Hodgkin and Huxley, 1952a). In central neurons, the presence of other outward currents in addition to the voltage-activated potassium current (see, e.g., Barrett et al., 1980; Halliwell and Adams, 1982; Segal and Barker, 1984; Adams and Galvan, 1986) has made it difficult to record the characteristics of the delayed rectifier. We have used the...
whole-cell voltage-clamp technique (Hamill et al., 1981) to study the characteristics
of voltage-activated potassium currents in neurons dissociated (Numann and Wong,
1984) from the CA1 region of slices of guinea pig hippocampus. A preliminary
report of some of these results has appeared elsewhere (Sah et al., 1987).

M E T H O D S

The methods of preparation of cells, voltage clamp, and data capture and analysis have been
described previously (Sah et al., 1988). Dissociated cells, identified as neurons on the basis of
morphology, were voltage-clamped using a single-electrode, switching voltage-clamp device
(Axonclamp 2A, Axon Instruments, Inc., Burlingame, CA) in conjunction with a whole-cell
patch pipette. This method combines freedom from series resistance errors afforded by the
switching voltage clamp with the better frequency response provided by patch electrodes with
a lower resistance than intracellular electrodes. An added advantage of the patch electrodes
was the opportunity to modify the intracellular solution. The pipette solution contained (mil-
limolar): 130 KF, 10 KCl, 10 HEPES, 10 EGTA, titrated to a pH of 7.2 with KOH. With the
relatively high concentrations of EGTA and KF, the free intracellular calcium concentration
would have been <2 nM (DeCoursey et al., 1984), and this should have minimized the contri-
bution of calcium-activated currents to the outward current (see Discussion). The bath solu-
tion contained (millimolar): 125 NaCl, 5.0 KCl, 25 NaHCO₃, 2.5 CaCl₂, 1.2
NaH₂PO₄, 11 glucose, pH 7.3–7.4 when bubbled with 95% O₂/5% CO₂. When the potassium
concentration was altered, KCl was substituted for NaCl. In experiments in which the effects
of inorganic calcium channel blockers were investigated, calcium was replaced by either
cobalt or cadmium on an equimolar basis, NaHCO₃ was replaced by Na-Tris, and NaH₂PO₄
was left out. Tetrodotoxin (TTX; Sigma Chemical Co., St. Louis, MO) was always added to
solutions at a concentration of 1 μM to block sodium channels (Sah et al., 1988). The osmo-
aility of the pipette solution and the bath solution was 290–300 mosmol/liter.

Curves were fitted to data using a modified Levenberg-Morrison-Marquardt algorithm
(Osborne, 1976), which was implemented in FORTRAN by A. R. Miller (1981). Tetraethy-
alammonium bromide (TEA-Br) and 4-aminopyridine (4-AP) were obtained from Sigma
Chemical Co. All recordings were made at room temperature (22–24°C).

R E S U L T S

Depolarization-activated Outward Currents

In the solutions used (containing 1 μM TTX), only outward currents were generated
by depolarizing voltage pulses, as illustrated in Fig. 1 A: these currents were in
response to voltage steps to −20 mV, and then in 20-mV steps to +80 mV from a
holding potential of −80 mV. The steady state current-voltage relationship mea-
sured from such currents recorded in the same cell is shown in Fig. 1 B. It can be
seen that the outward current became apparent at −40 mV and its amplitude
increased steadily with increasingly positive voltage steps.

When the external potassium concentration was raised to 100 mM, the current
was inward at potentials more negative than −10 mV. Currents generated by volt-
age steps to potentials from −20 to +60 mV under these conditions are shown in
Fig. 1 C (different cell) and a current-voltage plot can be seen in Fig. 1 D. The null
(zero current) potential in the solution containing 100 mM K⁺ was −4 mV, close to
the calculated potassium equilibrium potential (E钾) of −7 mV. In five cells, E钾 was
-8.1 ± 1.6 mV in solutions containing 100 mM K⁺. These observations suggest that the outward current was carried predominantly by potassium ions.

Effects of TEA and 4-AP

Many potassium currents are blocked by internal or external application of TEA (Armstrong, 1966; Hille, 1967; Segal and Barker, 1984). The effect of externally applied TEA on outward current in these cells is illustrated in Fig. 2. The outward currents in A were generated by voltage steps to +40 mV from a holding potential of -80 mV. Addition of 30 mM TEA to the external solution caused a marked reduction in the amplitude of the currents within 5–10 min. A similar effect was seen in two other cells. Although cells were never held long enough to demonstrate reversal of the depression, spontaneous rundown of the outward current was not seen in other experiments in which cells were held for similar periods of time, or in the presence of lower concentrations of TEA (10 or 20 mM; n = 4). It was therefore concluded that the outward current was depressed by 30 mM TEA. The current-voltage curves for the potassium current before and after exposure of the cell in Fig. 2 A to TEA are shown in Fig. 2 B. The amplitude was reduced at all voltages. Outward currents were also markedly reduced when the pipette (intracellular) solu-
Current-Voltage Relationship

Instantaneous currents were measured using a two-pulse procedure (Hodgkin and Huxley, 1952b). A cell was voltage-clamped to +40 mV for 10 ms (holding potential, -80 or -100 mV) to activate the potassium conductance, and the membrane potential was then stepped to a potential between +30 and -130 mV (Fig. 3 B). The currents generated in this way are shown in Fig. 3 A. The amplitudes of the instantaneous currents measured 400 µs after the voltage step are plotted against clamp potential in Fig. 3 C. The relationship appeared reasonably linear in the voltage range in which the potassium currents are generated (i.e., more positive than -50 mV), but a better fit to all the data points was obtained by applying the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

\[ I = F^2V(w - xy)/(RT(1 - x)), \]  

(1)
where \( w = [K]_o + [Na]_o(P_{Na}/P_K), x = \exp(-VF/RT), y = [K]_i + [Na]_i(P_{Na}/P_K), F, R, \) and \( T \) have their normal meaning, and \( V \) is membrane potential. The line through the points in Fig. 3 B, drawn with \( P_{Na}/P_K = 0.04 \), gave a null potential of \(-68 \text{ mV}\). In some cells, instantaneous currents were fitted with a single exponential and their peak amplitude was calculated by extrapolation back to the time of the onset of the voltage step. No significant change in the current-voltage relationship was introduced by this procedure.

**Conductance-Voltage Relationship**

Conductance \((g)\) was calculated from \( I/(V - E_o) \) (Hodgkin and Huxley 1952b), where \( I \) is the current amplitude and \( E_o \) is the null potential. The conductance-voltage relationship for the cell in Fig. 3 A is shown in Fig. 3 D. The solid line through the points shows the best (least-squares) fit of the Boltzmann equation,

\[
g_K = g_{K,(\text{max})}/[1 + \exp [(V' - V)/k]],
\]

(2)
to the data, where \( g_K \) is the potassium conductance at voltage \( V, g_{K,(\text{max})} \) is the maximum potassium conductance, \( V' \) is the voltage at which \( g_K \) is half-maximal, and \( k \) is a constant. In the cell shown in Fig. 3 D, the peak potassium conductance was 61 nS and the parameters \( V' \) and \( k \) were \(-4.1 \) and \( 14.2 \text{ mV} \), respectively. The average values of \( g_{K,(\text{max})}, V', \) and \( k \) in nine cells were \( 65.2 \pm 15.7 \text{ nS}, -4.8 \pm 3.9 \text{ mV}, \) and \( 13.6 \pm 1.3 \text{ mV} (\text{mean} \pm 1 \text{ SEM}) \).
Ion Selectivity

As expected for a potassium-selective channel, the null potential shifted toward more positive potentials as the external potassium concentration was raised (e.g., Fig. 1 D). Average null potentials measured in 22 neurons at different extracellular potassium concentrations are shown plotted against the extracellular potassium concentration, [K]o, in Fig. 4. The straight line is the Nernst relationship that would be expected to apply if the channels were permeable only to potassium. The curved line was drawn according to the Goldman-Hodgkin-Katz equation, assuming that the channel was permeable only to sodium and potassium with a permeability ratio, $P_{Na}/P_{K}$, of 0.04 (cf. Hodgkin and Horowicz, 1959).

![Figure 4](https://example.com/figure4.png)

FIGURE 4. Relationship between extracellular potassium concentration and null potential. The average null potential (± 1 SEM) is plotted against potassium concentrations of 100 (n = 4), 50 (n = 2), 5 (n = 16), and 2 (n = 2) mM. The straight line shows the Nernst relationship for an intracellular potassium concentration of 150 mM. The curved line is drawn according to the Goldman-Hodgkin-Katz equation with a $P_{Na}/P_{K}$ of 0.04.

Activation Kinetics

Although some of the potassium currents turned on sigmoidally, in order to compare the currents recorded in dissociated neurons with depolarization-activated potassium currents in other cells, the rising phase of the potassium current was fitted with the following equation (Hodgkin and Huxley, 1952c):

$$I_K = I_K^{(\text{max})}[1 - \exp(t/\tau)]^n.$$  \hspace{1cm} (3)

The value of the exponent $n$ was determined empirically by plotting $\ln[I_K/I_K^{(\text{max})}]^{1/n}$ against time for various values of $n$. The value of $n$ that gave the best straight line was 1 (10 neurons) and values of $\tau$ were obtained from the slope of such lines. The broken lines in Fig. 5 A show fits of Eq. 3 (exponent $n = 1$) to a family of potassium currents recorded in one of the neurons.
Tail currents obtained on repolarization to different potentials could also be fitted with a single exponential (not shown). Values of $\tau$ obtained from the activation and turn-off of the instantaneous currents of potassium currents recorded in two cells (circles and squares) over a range of potentials are shown in Fig. 5B. We noticed that tail currents decayed more slowly in solutions containing raised potassium concentrations, but this phenomenon was not examined further.

**Inactivation**

In response to prolonged depolarizing steps, the potassium currents slowly decayed, as illustrated in Fig. 6A. Currents decayed to a plateau level and the decay to this level could be well described by a single-exponential process, as illustrated in Fig. 6B, which shows the decay of a current generated by a voltage step to +40 mV plotted semilogarithmically against time. The time constant of inactivation obtained by fitting a straight line to the points (Fig. 6B) was 450 ms. The decay time constants measured in this way for currents generated by voltage steps to −20, 0, +20, and +40 mV were 425, 450, 370, and 450 ms, respectively.

Steady state inactivation was studied by holding the cell for 2 s at different membrane potentials and then activating the potassium current with a 10-ms step to +40 mV. Potassium currents recorded in this way in a cell held at −110 and −50 mV are shown in Fig. 7A. The current amplitude was clearly smaller at the latter potential. The steady state inactivation parameter $h_a$ was calculated by dividing the peak current at each holding potential by the peak current generated from a holding potential of −110 mV; a plot of $h_a$ against potential is shown in Fig. 6B. The solid line through the points was drawn according to (Hodgkin and Huxley, 1952b):

$$h_a = 1/[1 + \exp((V - V')/k)],$$

where $V'$ is the potential at which $h_a$ is 0.5 and $k$ is a constant. For the cell shown in
FIGURE 6. Time course of inactivation. (A) Currents generated in response to 1-s depolarizing voltage pulses to potentials from -20 to +60 mV (20-mV increments). (B) Semilogarithmic plot of the decay of the current to a steady level ($I - I_s$) during a pulse to +60 mV. The broken line shows the best (least-squares) fit of a single exponential to the points (time constant, 450 ms).

FIGURE 7. Voltage dependence of steady state inactivation. (A) Currents generated in response to a voltage step to +40 mV from holding potentials of -110 (above) and -50 (below) mV. (B) Plot of $h_n$ (calculated from the ratio of the steady state current amplitude with a holding potential of $V$ to the amplitude with a holding potential of -110 mV). The solid line is the best (least-squares) fit to Eq. 4 and gave a $V'$ of -75 mV and a $k$ of 27 mV.
Fig. 7, the parameters $V'$ and $k$ were $-75.9$ and $26.9$ mV, respectively. Average values from four cells were $-67.7 \pm 11.6$ and $26.6 \pm 5.6$ mV (mean $\pm$ 1 SEM).

**Effects of Cobalt and Cadmium**

Divalent ions that block calcium channels have been shown to block depolarization-activated potassium currents in human T cells (DeCoursey et al., 1985). We therefore examined the effects of cobalt and cadmium on the depolarization-activated potassium current in hippocampal cells. The effect of substituting cobalt for calcium in the extracellular solution is shown in Fig. 8. The currents were generated by voltage steps to $+20$, $+40$, and $+60$ mV in cobalt solution (A) and after the return to control solution (B). (This sequence was chosen to demonstrate that the effect was not due to deterioration of the cell.) The current-voltage relationships during exposure to cobalt (circles), during washout with control solution (squares), and during re-exposure to cobalt (triangles) are shown in C. It can be seen that cobalt reduced the peak amplitude of the potassium current at all voltages. This effect was reversible and was seen in all three neurons exposed to cobalt. The instantaneous current-voltage relationships and the conductance-voltage relationships before (circles) and during (squares) exposure to cobalt are shown in Fig. 9. The lines through the points in A were fitted as in Fig. 3 A with a $P_{Na}/P_{K}$ value of 0.03 in control.
solution and in cobalt solution. The lines in Fig. 9 B were fitted with $g_K(\text{max})$, $V'$, and $k$ values of 55.7 nS, 3 mV, and 20.4 mV in control solution (circles) and 23.4 nS, 4.6 mV, and 17.1 mV in cobalt solution (squares). It is clear that the effect of cobalt cannot be explained by a shift in the voltage dependence of activation.

It seemed possible that the effects of the divalent calcium channel blockers could be due to blockage of a potassium conductance activated by a calcium current (Meech and Standen, 1975) too small for us to see. To test this hypothesis, experiments were done in which the calcium in the extracellular solution was replaced with magnesium. This caused an increase in the peak potassium current of 50 and 20% in two cells, had no effect in two other cells, and decreased the peak current by 40% in the other. We concluded that cobalt has a direct blocking effect on the depolarization-activated potassium current that is not dependent on depression of calcium current.

The effect of replacing calcium with cadmium (2.5 mM) in the extracellular solution was examined in two cells. In both, there was a reduction in the peak amplitude...
of the outward current and an increase in the rate of inactivation. Lower concentrations of cadmium (0.1–0.5 mM) had no effect on the potassium current.

**DISCUSSION**

The depolarization-activated potassium current ($I_K$) in dissociated guinea pig hippocampal CA1 neurons that we have described in this article has characteristics very similar to those of $I_K$ in other cells: it is activated at potentials more positive than $-50$ mV and can be described by Hodgkin-Huxley-type equations, as in squid axon (Hodgkin and Huxley, 1952a), skeletal muscle (Adrian et al., 1970; Stanfield, 1975; Pappone, 1980), human T lymphocytes (Cahalan et al., 1985), and rat sympathetic neurons (Belluzzi et al., 1985b). The relative permeability of the channel to sodium and potassium ($P_{Na}/P_K$) of $\sim 0.04$ is similar to the value in squid axon (Binstock and Goldman, 1971; Bezanilla and Armstrong, 1972), snail neurons (Reuter and Stevens, 1980), and skeletal muscle (Gay and Stanfield, 1978). The currents activated with an exponential time course, as in rat sympathetic neurons (Belluzzi et al., 1985b). In contrast, a power of 4 or higher of a first-order process is needed to describe $I_K$ in squid axon (Hodgkin and Huxley, 1952c), skeletal muscle (Adrian et al., 1970; Stanfield, 1975; Pappone, 1980; Beam and Donaldson, 1983), and human T lymphocytes (Cahalan et al., 1985), and a power of 2 provides a better fit in mouse neuroblastoma cells (Moolenar and Spector, 1978). The activation time constants of $I_K$ over a range of potentials in dissociated hippocampal neurons were about two orders of magnitude faster than in cultured hippocampal neurons at the same temperature (Segal and Barker, 1984; Rogawski, 1986). We do not know the reason for the difference: internal perfusion may have altered the kinetics of the channel, or channel opening may be slower than normal in cultured neurons. We favor the latter explanation because the time course of $I_K$ in nonperfused CA1 pyramidal neurons in hippocampal slices (French, C. R., and P. W. Gage, unpublished observations) is similar to that described here.

Potassium conductance was found to increase e-fold per 13 mV depolarization at potentials around $V'$. This is similar to the voltage sensitivity of $g_K$ in cultured hippocampal neurons (Rogawski, 1986) and in rat sympathetic neurons (Belluzzi et al., 1985b). However, $g_K$ in squid axon (Hodgkin and Huxley, 1952b) and in skeletal muscle (Pappone, 1980; Beam and Donaldson, 1983) is much more steeply voltage dependent.

From estimates of the surface area of the dissociated neurons, based on the assumption of a specific membrane capacity of 1 µF·cm⁻², the maximum potassium conductance was $7.7 \pm 2.0$ mS·cm⁻² ($n = 9$). This value is of the same order as the maximum potassium conductance in rat sympathetic neurons (17 mS·cm⁻²; Belluzzi et al., 1985b) and cultured neuroblastoma cells (12 mS·cm⁻²; Moolenar and Spector, 1978) but is somewhat smaller than the peak potassium conductance in squid axon (36 mS·cm⁻²; Hodgkin and Huxley, 1952b). Assuming a single-channel conductance of 18 pS (Conti and Neher, 1980; Rogawski, 1986) gives a channel density of about four channels per square micrometer in dissociated neurons, which is of the same order as the one to two channels per square micrometer measured from single-channel records in cultured hippocampal neurons (Rogawski, 1986).
The depolarization-activated potassium conductance could play an important role in restoring the resting membrane potential during an action potential. The maximum $g_K$ is similar in magnitude to the maximum transient sodium conductance (~10 mS·cm$^{-2}$; Sah et al., 1988). Furthermore, it can be seen from Fig. 5 that, at potentials near the peak of an action potential (approximately +40 mV), $I_K$ activates with a time constant of ~1 ms. Assuming a $Q_{10}$ of 2.5 for the activation time constant (Pappone, 1980; Beam and Donaldson, 1983), the time constant of activation at 37°C would be ~0.2 ms. $I_K$ would therefore contribute significantly to the repolarization of action potentials in these cells (cf. Störm, 1987).

Possible Contributions from Other Currents

Hippocampal pyramidal cells have been shown to have many other currents apart from $I_K$ that can be recorded in the soma (for review, see Adams and Galvan, 1986). Most of these currents, both inward and outward, are activated over the same voltage range as $I_K$. In this study, the transient (Sah et al., 1988) and persistent (French and Gage, 1985) sodium currents were blocked by adding 1 μM TTX to extracellular solutions. We have shown previously (Sah et al., 1988) that calcium currents (Johnston et al., 1980; Brown and Griffith, 1983) in internally perfused dissociated CA1 neurons are either absent or very small (see also Kay et al., 1986). No sign of inward currents was seen in any of the cells described in the present study.

A fast transient outward current (A-current; Connor and Stevens, 1971; Neher, 1971) has been described in hippocampal CA3 neurons (Gustafsson et al., 1982) and is also present in CA1 neurons in hippocampal slices (Sah, P., unpublished observations). In dissociated CA1 neurons, a transient outward current was never seen. The reason for this is not known. It may be that the A-current in these cells is calcium dependent (e.g., Segal and Barker, 1984; Galvan and Sedlmeir, 1984; see, however, Beluzzi et al., 1985a) and was depressed under our recording conditions (see below). It is also possible that, like the calcium current (Kostyuk, 1984), the A-current is labile and is "washed out" during cell dissociation and/or internal perfusion. Another possibility is that the A-current is present only in dendrites and is thus removed during the dissociation procedure (see Rogawski, 1986). We did not specifically look for an M-current (Halliwell and Adams, 1982), but it activates relatively slowly and would not have contributed to any marked extent to the currents described here.

Hippocampal pyramidal cells also have several calcium-activated potassium currents that activate over the same voltage range as $I_K$ (Brown and Griffith, 1983; Sah et al., 1985; Lancaster and Adams, 1986). It can be seen from Fig. 1 that the current continued to increase in amplitude during depolarizing pulses up to +80 mV. It is clear that there is no region of negative slope resistance in the steady state current-voltage relation that would be expected if there were a calcium-activated component of the current (Meech and Standen, 1975; Galvan and Sedlmeir, 1984; Marty and Neher, 1985). There are several possible explanations for the absence of a calcium-activated potassium current in these cells. First, the calcium current in dissociated CA1 neurons is very small or absent (Sah et al., 1988). Second, it has been shown that calcium-activated potassium current can be suppressed by having strong calcium buffers in the pipette solution (Marty and Neher, 1985; Ritchie,
The presence of a high concentration of fluoride as well as 10 mM EGTA in the perfusing solution would have reduced the intracellular calcium concentration to <2 nM (DeCoursey et al., 1984). The high-conductance, calcium-activated potassium channels in skeletal muscle and sympathetic neurons require a free calcium concentration of at least 100 nM to activate (Barrett et al., 1982; Gardner, 1986). It seems unlikely, therefore, that there would have been any significant contribution from a calcium-activated potassium current to the outward current.

Intracellular perfusion of cells has been found in some studies to alter the voltage dependence of ionic currents (Fernandez et al., 1984), and intracellular fluoride has been shown to alter the kinetics of $I_K$ in squid axon (Chandler and Meves, 1970; Adams and Oxford, 1983). We did not look systematically for shifts in the voltage dependence of the delayed rectifier in this study. Recordings were obtained in many cells, however, for periods as long as 30 min, but no significant shifts in the voltage dependence of $I_K$ were observed over this time. All the results presented in this study were obtained with fluoride as the major anion in the pipette solutions and it is possible that the time course of $I_K$ is normally faster than reported here.

**Effects of Cobalt and Cadmium**

It was surprising that the calcium channel blockers cobalt and cadmium (Hagiwara and Byerly, 1981) depressed $I_K$. However, cobalt, manganese, and nickel have been reported to depress a noninactivating voltage-activated potassium conductance in cat motoneurons (Schwindt and Crill, 1981) and to depress $I_K$ in human T lymphocytes (Matteson and Deutsch, 1984; Decoursey et al., 1985). Cadmium and some of the heavier divalent cations also increase the rate of decay of $I_K$ in human T lymphocytes (Decoursey et al., 1985). Experiments on squid axons have also indicated that there may be an external divalent cation-binding site that can alter the time course of $I_K$ (Stanfield, 1975; Eaton and Brodwick, 1980; Gilly and Armstrong, 1982). Extracellular barium, for example, has been shown to block $I_K$ (Armstrong and Taylor, 1980; Armstrong et al., 1982), and it has recently been suggested (Armstrong and Lopez-Barneo, 1987) that binding of calcium ions may be essential for maintaining the integrity of depolarization-activated potassium channels. It is possible that a similar site in hippocampal neurons may also control the probability of opening or conductance of delayed rectifier channels. Another possibility is that the depolarization-activated potassium channels do not form a homogeneous population (Dubois, 1981; Conti et al., 1984; Gardner, 1986) and that the calcium channel blockers block one of the subpopulations.

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