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Potassium current suppression by quinidine reveals additional calcium currents in neuroblastoma cells

(voltage and Ca^{2+}-sensitive K^+ currents/repetitive activity/quinine)

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ABSTRACT **Quinine and quinidine have been evaluated with regard to their effects on the electrical activity of neuroblastoma cells. Under voltage-clamp conditions, we have found that quinine and quinidine block both the voltage-dependent and Ca^{2+}-dependent K^+ conductances. Blockage of the voltage-dependent K^+ channel is manifest as an increase in the amplitude and in the duration of the action potential. Blockage of the Ca^{2+}-dependent K^+ channel in Na^+-free (replaced by Tris) solutions containing 6.8 mM Ca^{2+} and tetraethylammonium ion or 4-aminopyridine (to block the voltage-dependent K^+ current) is seen as a further prolongation of the Ca^{2+} action potential and diminution of the after-hyperpolarization. A critical role of the Ca^{2+}-dependent K^+ conductance in modulation of the rate and duration of trains of Ca^{2+} action potentials is shown by the use of low concentrations (5–40 μM) of quinine or quinidine, which diminish the Ca^{2+}-dependent K^+ conductance in a graded manner. After complete blockade of K^+ currents, the peak Ca^{2+} currents are enhanced at all voltages, especially at values more positive than −30 mV, where a steady-state inward current appears as well. In this same voltage range, the decay of the Ca^{2+} currents exhibits two time constants—that of the transient inward current, which is about 20 msec, and a much slower (≈2000 msec) component. It is suggested that neuroblastoma cells have two types of calcium channels—one which generates the Ca^{2+} action potential and a second, distinguished by activation at more depolarized levels and by a slow rate of inactivation, which underlies the calcium entry necessary to activate the Ca^{2+}-dependent K^+ conductance.**

Nerve cells possess in their somata membrane several types of K^+-conductance mechanisms that subserve different functions and can be distinguished by their gating mechanisms, kinetics, and pharmacological properties (1, 2). In neuroblastoma cells, the repolarizing phase of the action potential is due to a voltage-dependent K^+ current and can be blocked by tetraethylammonium ion (Et_4N^+) (3). The prolonged after-hyperpolarization (AHP) that follows the action potential is due to a Ca^{2+}-dependent K^+ current. This current is refractory to Et_4N^+ or other known blockers of the voltage-dependent K^+ channels (4, 5). To date, there have been no effective methods to block the Ca^{2+}-dependent K^+ current other than those that simultaneously modify the intracellular levels of Ca^{2+}. Therefore, it has been difficult to isolate Ca^{2+} currents from the K^+ currents, to study the properties of each (6–10), and to evaluate their physiological consequences and interaction in such neuronal functions as synaptic plasticity, release of transmitters and hormones, and regulation of oscillatory behavior of membranes (11–15).

In contrast to the voltage-dependent K^+ conductance, a Ca^{2+}-dependent increase in K^+ permeability has been described in a variety of cells, both excitable and inexcitable (16–20). In erythrocytes and in β-pancreatic cells, quinine and its diastereomer quinidine have been found to inhibit the Ca^{2+}-dependent increase in K^+ permeability (13, 18). Reports of neural effects of quinine and quinidine have concentrated on the axonal membrane in which the voltage-dependent K^+ conductance is dominant and in which the Ca^{2+} and Ca^{2+}-dependent currents of the soma membrane are poorly developed. In the axonal membrane, they affect primarily the K^+ channels by a process of inactivation (21, 22).

We have studied the effects of quinine and quinidine on neuroblastoma cells under constant-current and voltage-clamp conditions. We report here that these agents suppress both the voltage-dependent and Ca^{2+}-dependent K^+ currents. Our observations further show that this suppression enhances the peak calcium currents and unmasks a persistent inward calcium current. Finally, we have studied the effects of quinine and quinidine on repetitively firing neuroblastoma cells and have obtained direct evidence for a physiological role of the Ca^{2+}-dependent K^+ conductance in the regulation of the frequency and duration of slow trains of action potentials.

MATERIALS AND METHODS

The present experiments were carried out on differentiated cells of mouse neuroblastoma clone N1E-115 and neuroblastoma–glioma hybrid line NG108-15 (T. Amano, B. Hamprecht, and M. W. Nirenberg, personal communication). Cells were grown to confluence in Dulbecco's modified Eagle's medium (GIBCO, no. 430-2100) containing 5% (vol/vol) fetal calf serum [and hypoxanthine (100 μM), aminopterin (1 μM), and thymidine (16 μM) for the NG108-15 cultures] at 37°C in an atmosphere of 10% CO_2/90% air. To obtain differentiated cells, confluent cultures were resuspended in growth medium containing 1 mM dibutyryl cyclic AMP for the NG108-15 cultures and 5 mM hexamethylenebisacetamide or 2% dimethyl sulfoxide for the N1E-115 cultures and plated into 35-mm plastic culture dishes.

Constant-current and voltage-clamp experiments with techniques as described (3) were performed 5–20 days after plating. For voltage-clamp experiments, cells were impaled with two microelectrodes filled with 3 M KCl that had resistances of 10–20 MΩ. Experiments were done at 20–24°C. For recording, the cells were bathed in balanced salt solutions buffered to pH 7.3 either with Hepes (Sigma) or with Tris. Normal solution (Hepes-buffered modification of Eagle's medium; Flow Laboratories, McLean, VA) contained 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mM NaH_2PO_4. For Na^+-free so-

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Abbreviations: Et_4N^+, tetraethylammonium; AHP, after-hyperpolarization.

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lutions, Tris was isosmotically substituted for Na^+. High Ca^{2+} solutions contained 6.8 mM $CaCl_2$. Et_4NCl (8–12 mM), 4-aminopyridine (2 mM; Sigma), quinine hydrochloride or quinidine sulphate (Sigma) were added to the dish or to a perfusing medium without osmotic compensation.

RESULTS

Effect on the Action Potential. Recent voltage-clamp studies on neuroblastoma cells have revealed voltage-dependent Na^+, K^+, and Ca^{2+} currents and a Ca^{2+}-dependent K^+ current (3–5). The voltage-dependent Na^+ and K^+ currents are responsible for the generation of the neuroblastoma fast-action potential and dominate the membrane behavior in normal solution. Et_4N^+, which blocks the voltage-dependent K^+ channels, causes a marked broadening of the fast-action potential (3). Quinine or quinidine (5–20 μM) exerted a similar effect on the fast-action potential (Fig. 1) At the higher concentrations (100–200 μM) used to block the Ca^{2+}-dependent K^+ permeability in erythrocytes (18) and in pancreatic beta cells (13) and to inactivate the K^+ currents in axonal membranes (21, 22), quinine and quinidine exerted local anesthetic effects on neuroblastoma cells and completely blocked the Na^+ component of the action potential. Low concentrations of these compounds enhanced the amplitude of the action potential evoked from the resting potential as does Et_4N^+. However, whereas Et_4N^+ enhances the amplitude and duration of the AHP that follows the action potential, quinine and quinidine reduced the AHP (Fig. 1A). The primary effect of Et_4N^+ is to block the voltage-dependent K^+ current,

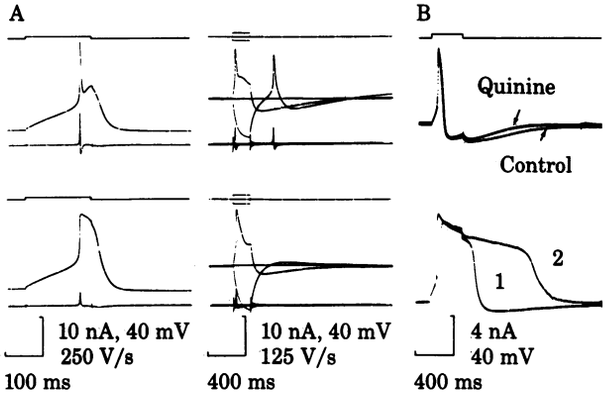

FIG. 1. Effects of quinine on the action potential of a neuroblastoma–glioma NG108-15 hybrid cell bathed in normal medium. (*A*) Control (upper) and after addition of 20 μM quinine (lower) to the bath. The uppermost trace in each record is the injected current, the middle trace is the membrane potential, and the lowest trace is the rate of change of the membrane potential (dV/dt). (Left) Broadening of the fast action potential after exposure to quinine. The action potential was elicited by a depolarizing current pulse applied at a hyperpolarized membrane potential (−85 mV). The decrease in action potential overshoot and rate of rise reflect the effects of quinine on the Na^+ component of the action potential. (Right) Slower sweep speed to show diminution of the AHP by quinine. The action potential was elicited from the resting potential (−55 mV). In addition to the prolongation of the action potential and the increase in overshoot, there was in this cell an increase in membrane resistance. In most cells, quinine induced no change in membrane resistance. (*B*). Dose-dependent effects of quinine on the electrical activity of a neuroblastoma–glioma NG108-15 hybrid cell perfused with normal Na^+ solution containing 6.8 mM Ca^{2+} and 8 mM Et_4N^+. Recordings are from the resting potential (−61 mV) to obtain a pronounced AHP. (Upper) Quinine at 10 μM affects only the AHP. (Lower) Quinine at 40 μM initially causes a prolongation of the action potential and a reduction in the magnitude of the AHP (trace 1). Thirty seconds later, the AHP is replaced by a prolonged after-depolarization (trace 2). The decrease in the fast spike reflects the effects of quinine on the Na^+ component. Upper trace is the injected current.

which explains the prolongation of the action potential. Because this K^+ current normally counteracts the inward Ca^{2+} current, its blockade enhances Ca^{2+} ingress, which explains the enhanced amplitude and duration of the action potential and that of the AHP (which is due to a Ca^{2+}-sensitive K^+ conductance) (3, 4). The suppression of the AHP by quinine and quinidine suggest that their mode of action is different from that of Et_4N^+ and, in particular, that they might directly block the Ca^{2+}-dependent K^+ conductance.

To examine this possibility, solutions containing elevated $[Ca^{2+}]_0$ and Et_4N^+ were used to suppress the voltage-dependent K^+ conductance and to enhance the Ca^{2+} conductance and the Ca^{2+}-dependent K^+ conductance. Fig. 1*B* shows the effects of different concentrations of quinine on a cell continuously perfused with a solution containing normal Na^+, 6.8 mM Ca^{2+}, and 8 mM Et_4N^+. Quinine at 10 μM diminished the amplitude and duration of the AHP but had little effect on the depolarizing and repolarizing phases of the action potential. At 40 μM, there was a dramatic change in the response. A prolonged plateau, close in its duration to that of the AHP, followed the depolarizing phase of the action potential. This plateau was abolished by the Ca^{2+} antagonists Ni^{2+}, La^{3+}, and Co^{2+}.

Effect on Repetitive Activity. It has been proposed that the AHP is involved in the generation of low-frequency repetitive discharge (14). As shown in Fig. 2*A*, slow trains of Ca^{2+} action potentials could be elicited by anodal-break stimulation in neuroblastoma cells perfused with a Na^+-free solution containing 6.8 mM Ca^{2+} and Et_4N^+. Under these zero external current conditions, the membrane behavior was dominated by inward Ca^{2+} currents and outward Ca^{2+}-dependent K^+ currents. Low concentrations of quinine (10–20 μM) diminished the AHP, shortened the duration of the train, and caused a more rapid redepolarization to threshold, thereby increasing the rate of discharge (Fig. 2*B*). At higher doses (50–100 μM; Fig. 2*C*), the AHP and the repetitive discharge were abolished and only one prolonged action potential remained, presumably terminated by the relatively rapid inactivation of the Ca^{2+} current (5).

Blockade of K^+ Currents. The results from the constant-current experiments suggest that quinine and quinidine exert their primary effects on the voltage-dependent and Ca^{2+}-dependent K^+ conductances. To elucidate their precise effect on membrane currents we performed voltage-clamp experiments. In normal solutions, the voltage-dependent K^+ conductance is the predominant outward current in neuroblastoma cells and can be seen well with a depolarizing step from a holding potential of −40 to 0 mV. Quinidine at 15 μM caused progressive decre-

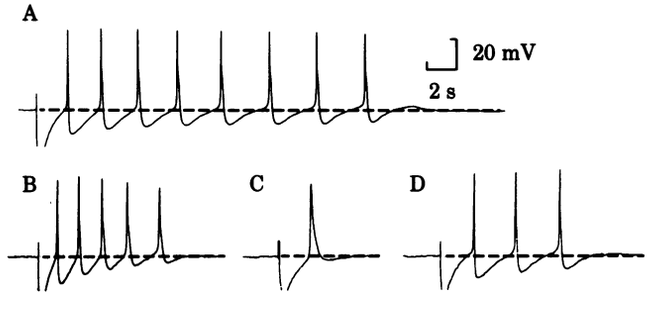

FIG. 2. Effects of different doses of quinine on the repetitive activity initiated by anodal-break stimulation in a neuroblastoma N1E-115 cell perfused with Na^+-free solution containing 10 mM Ca^{2+} and 10 mM Et_4N^+. The anodal-break pulse was applied at the resting potential (−55 mV). Records are of the control train (*A*) with 12 μM (*B*) and 100 μM (*C*) quinine in the perfusing solution, and during washout of quinine (*D*). Note in *B* the increase of the rate and in *C* the complete suppression of the repetitive discharge.

ment and eventual abolition of this current (Fig. 3A). Thus, quinidine suppressed the voltage-dependent K^+ current. This is in contrast to the effect of quinidine on the axonal membrane, where it has been shown to cause only an inactivation of the K^+ current, suggesting an action of quinidine on neuroblastoma cells that is not dependent upon open channels (21, 22).

In Na^+-free solution containing elevated $[Ca^{2+}]_o$ and Et_4N^+ or aminopyridines, the voltage-dependent K^+ conductance is blocked and the predominant outward current is a slowly rising Ca^{2+}-dependent K^+ current (5). This current can be seen well with depolarizing steps from holding potentials around −35 mV, levels at which steady-state inactivation of the transient Ca^{2+} current is essentially complete. Fig. 3B shows the effect of 20 μM quinidine on this outward current, elicited by a depolarization step from −40 to +10 mV. At this concentration, quinidine suppressed 90% of this current in a manner similar to its suppression of the voltage-dependent K^+ current. With higher concentrations of quinidine (40 μM), the outward currents were completely suppressed and were replaced by slowly inactivating inward currents. The partial blockage of the Ca^{2+}-dependent K^+ conductance that occurred with the lower concentration allowed the study of the effects of relatively minor decrements in the Ca^{2+}-dependent K^+ conductance on the repetitive activity. The slowly inactivating inward Ca^{2+} currents that were unmasked by higher concentrations of quinidine could provide the mechanism for the Ca^{2+} entry that activates the Ca^{2+}-dependent K^+ conductance.

Calcium Currents Studied in Isolation. Methods to avoid K^+ current contamination of Ca^{2+} currents either have not completely blocked K^+ currents, have interfered with Ca^{2+} currents as well, or have limited severely the voltage range in which currents could be studied (1). Quinidine and quinine offered us simple pharmacological tools to suppress all K^+ currents and to investigate the Ca^{2+} currents in isolation.

Fig. 4A shows a family of membrane currents elicited from a process-free round neuroblastoma cell bathed in a Na^+-free solution containing 6.8 mM Ca^{2+} before and after application of quinidine. Currents were measured during step depolarizations from −70 mV to various levels. A transient inward current appeared when the potential was stepped to −50 mV and reached its maximum peak amplitude at −20 mV. With larger depolarizations, outward membrane currents became prominent and abbreviated the time course and amplitude of the inward currents. With the largest depolarization, inward currents were completely swamped.

Seventeen minutes after the addition of quinidine, there was a decrease in the amplitude of the outward currents with a corresponding enhancement of the inward currents. By 35 min, equilibrium conditions were achieved, and depolarizing steps up to +20 mV elicited only inward currents. The quinidine-induced changes in the current–voltage (I–V) relationship for the peak inward and steady-state currents, after leakage correction, are shown in Fig. 4B. As in the controls, inward currents first appeared around −50 mV. However, the peak values of net inward currents were increased over the entire voltage range with a more pronounced increase at values more positive than −30 mV. The maximum peak inward current under control conditions was 2.25 nA and was achieved at −20 mV; after quinidine it was 4.3 nA and was reached with a voltage step to 0 mV. Consequently, although the voltage level for inward current activation remained the same, there was both an increase in amplitude and a voltage shift of maximum peak inward current in the depolarizing direction.

The transient peak inward Ca^{2+} current decreased in amplitude and flattened at values more positive than 0 mV and no transient inward or outward currents could be resolved with steps to values more positive than +70 mV. This null potential is higher than that previously measured in neuroblastoma cells in the presence of Et_4N^+, but is still lower than the theoretically predicted reversal potential for Ca^{2+}, probably due to the presence of large leakage currents with very large depolarizations (5, 7).

Steady-state outward K^+ currents under control conditions began around −40 mV and, with depolarizations to values more positive than 0 mV, these currents became so large as to dominate the total membrane currents. In the presence of quinidine, small outward currents were still detectable at very large positive potentials. However, in the voltage range from −30 to +20 mV, outward currents were replaced by steady-state inward currents.

The marked enhancement of the peak inward currents, especially at more positive potentials, suggested an additional component to the Ca^{2+} current that previously had been masked by K^+ currents. The existence of this current was substantiated by studies of the kinetics of inactivation of the Ca^{2+} current. Semilogarithmic plots of the time course of the calcium current at different test potentials are shown in Fig. 4 C and D. Under control conditions, the falling phase of the Ca^{2+} current is fitted by a single straight line with an inactivation time constant, τ_h, calculated from the slope of the line. τ_h is voltage dependent and increases with increasing depolarization (5). After exposure to quinidine, a second inactivation component becomes obvious, so that the inactivation process is best described by two straight lines. The first, τ_{h1}, is comparable to that

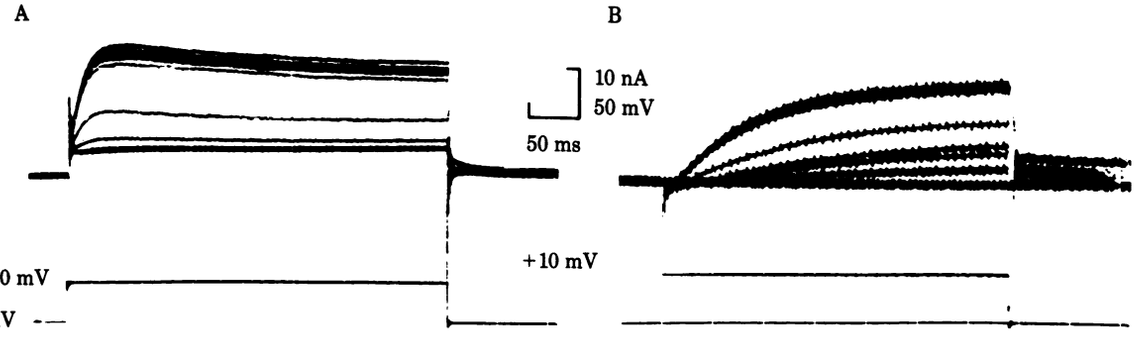

FIG. 3. Effect of quinidine on outward membrane currents recorded under voltage-clamp condition in N1E-115 cells. (A) Progressive blockade by 10 μM quinine of outward currents elicited in normal solution by a 350-msec step depolarization from a holding potential (V_h) of −40 to 0 mV, which was repeated every 30 sec. (B) Progressive blockade of 4-aminopyridine-resistant outward currents by 20 μM quinidine. The cell was bathed in a Na^+-free solution with 6.8 mM Ca^{2+} and 2 mM 4-aminopyridine. A step depolarization to +10 mV from V_h of −40 mV was applied every 30 sec. Also note the blockade of the pronounced tail currents. In both cases, outward currents were blocked within 30 min.

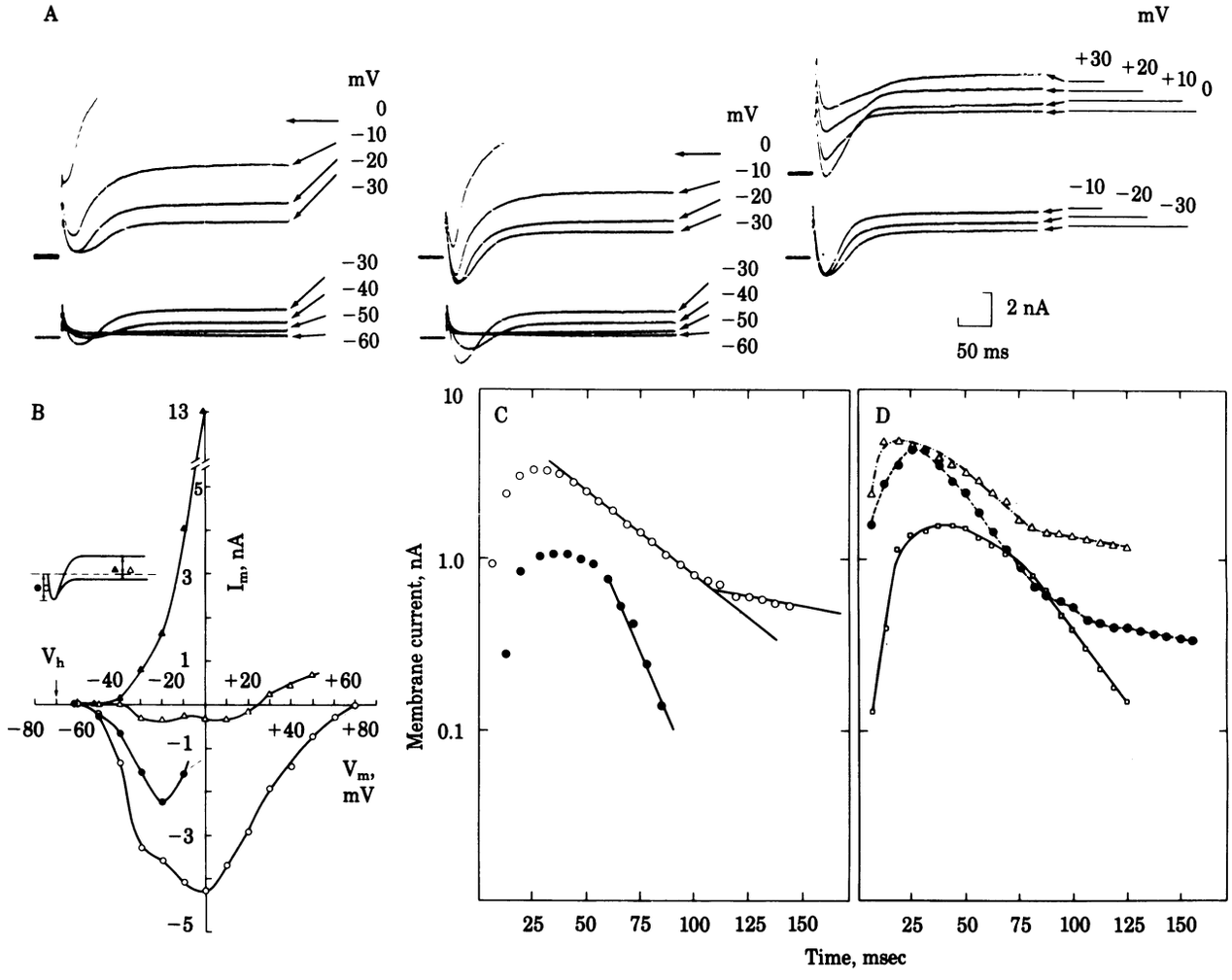

FIG. 4. (*A*) The effect of quinidine on membrane currents recorded from a round NG108-15 cell (66 μm in diameter) bathed in Na^+-free solution with 6.8 mM Ca^{2+}. All records were from a holding potential of −70 mV to various levels as indicated. (*Left*) Control traces. (*Center*) Seventeen minutes after application of 40 μM quinidine K^+ currents have been partially blocked and the Ca^{2+} currents enhanced. (*Right*) The final effects of quinidine (achieved after 35 min). There is complete blockage of outward currents and a further enhancement of inward currents. An estimate of leakage currents was calculated from equivalent pulses in the hyperpolarizing direction, which elicited linear responses over the voltage range shown. These levels are indicated to the right of each tracing. (*B*) Current–voltage (I–V) relationships for net peak Ca^{2+} current (○, ●) and net steady-state current (△, ▲), before (●, ▲) and after (○, △) the application of 40 μM quinidine. Values were obtained from the traces in *A* and corrected for leakage. V_h, holding potential; V_m, membrane potential; I_m, membrane current. (*C*) Semilogarithmic plot of the time course of the net current shown in *A* elicited by a voltage step depolarization to −30 mV, before and after addition of quinidine. Under control conditions (●), the decay is exponential with a single τ_h of 15 msec as calculated from the fitted line. After quinidine addition (○), the decay can be described by a fast (τ_{h1} = 43 msec) and a slow (τ_{h2} = 2069 msec) time constant, as indicated. (*D*) Semilogarithmic plots of the time course of the net inward current at various levels in the presence of 40 μM quinidine. ○, Monoexponential decay at −40 mV (τ_h = 25 msec); ●, two components at −10 mV (τ_{h1} = 27 msec, τ_{h2} = 1816 msec); △, two components at +10 mV (τ_{h1} = 39 msec, τ_{h2} = 1753 msec).

of controls (≈ 15–30 msec), but the second, τ_{h2}, is quite prolonged (≈2000 msec) and is seen only in the voltage range in which we find the steady-state inward current. Within that range, τ_{h2} exhibits little voltage dependence. Thus, the data is compatible with the existence of two types of Ca^{2+} channels in the neuroblastoma membranes. One type is activated at −50 mV, manifests relatively rapid inactivation kinetics and is responsible for the Ca^{2+} action potential. The other is activated at more positive potentials, exhibits slow inactivation, and may underlie the Ca^{2+}-dependent K^+ currents (cf. Fig. 3).

DISCUSSION

We showed here that quinine or quinidine block both the voltage-dependent and Ca^{2+}-dependent K^+ currents in neuroblastoma cells without blocking the Ca^{2+} currents. Therefore, these agents provide useful pharmacological tools to study the K^+ currents, to evaluate uncontaminated Ca^{2+} currents, and to assess directly the physiological roles of these currents in neuronal function. Quinidine's important clinical role in the treatment of cardiac arrhythmias has been attributed to its prolongation of the refractory period of the cardiac action potential, an effect ascribed by different investigators to depression of Na^+ or K^+ or Ca^{2+} or all fluxes in heart cells (23). In voltage-clamp studies of the axonal membrane, quinidine was found to suppress both the Na^+ and K^+ conductances, the latter preferentially (21, 22). Our observations in neuroblastoma cells, which in contrast to the axonal membrane manifest significant Ca^{2+} and Ca^{2+}-dependent K^+ conductances, show that quinidine, in addition to blocking the Na^+ and voltage-dependent K^+ channels, also blocks the Ca^{2+}-dependent K^+ currents without affecting Ca^{2+} currents. A complete block of the Ca^{2+}-dependent K^+ current was obtained with 40 μM, and partial suppression

could be achieved with concentrations as low as 5 μM. In contrast to the studies of the axonal membrane, we have no evidence that quinidine interacts with the open K^+ channel by inactivating it.

With the elegant elucidation of Ca^{2+} currents in the soma of the molluscan neuron (1), in the barnacle muscle (24, 25), and in the egg cell membrane (26, 27) came the realization that these currents were significantly contaminated by overlapping K^+ currents that proved difficult to eliminate (1, 16). In fact, it has remained unclear whether there exists more than one species of Ca^{2+} channel. The agents available to block the voltage-dependent K^+ current, such as Et_4N^+ or the aminopyridines, are ineffective in blocking the Ca^{2+}-sensitive K^+ current, or, like Ba^{2+}, must replace Ca^{2+} as the charge carrier. No simple pharmacological procedure has been described to date that can block K^+ currents completely while leaving Ca^{2+} currents intact.

Our results show that, in Na^+-free solutions, the peak inward currents in the presence of quinidine (*i*) are enhanced in magnitude, (*ii*) have the same gating range as in the absence of quinidine, (*iii*) can be activated over a much broader voltage range, and (*iv*) exhibit an inactivation phase with a single exponential time course at small depolarizations but with a second slower component at larger depolarizations.

These properties suggest that the Ca^{2+} channels showing an inactivation phase continue to function after quinidine treatment and that the fraction of inward current unmasked by quinidine at depolarized levels represents a separate population of Ca^{2+} channels that have different voltage sensitivities and kinetic properties. Alternatively, there could be one population of Ca^{2+} channels with intrinsically very complex voltage sensitivities and kinetics that become evident only after all outward currents are eliminated by quinidine. It is also possible that quinidine-insensitive K^+ currents or a direct effect of quinidine on calcium channels, or both, have combined fortuitously to induce the observed behavior of the calcium current.

A distinct variety of Ca^{2+} channels exhibiting slow inactivation kinetics might relate to several physiological phenomena. Such currents have been described in barnacle muscle and in molluscan neurons where, in association with slowly inactivating K^+ currents, they have been implicated in the generation of oscillatory behavior (15, 25, 28). In neuroblastoma cells, they exhibit a voltage dependency similar to that of the Ca^{2+}-dependent K^+ current and can explain the activation of the Ca^{2+}-dependent outward currents at membrane potentials at which the transient Ca^{2+} current is inactivated. Therefore, this type of channel can provide the mechanism for the Ca^{2+} entry that elicits the Ca^{2+}-dependent K^+ current.

The effects of quinine on the frequency and duration of trains of Ca^{2+} action potentials suggest an important role for the Ca^{2+}-dependent K^+conductance in control of information encoding in trains of action potentials. Subtle reductions in this current by low concentrations of quinine are reflected in abbreviation of the amplitude and duration of the AHP, with a consequent increase in the frequency of firing. Abolition of the current with higher concentrations blocks repetitive activity completely.

Even more intriguing are the potential roles of slowly inactivating currents in synaptic function. For example, Kandel's group (11, 12) has shown that transmitter release can be modulated by calcium entry into presynaptic terminals and that this entry is regulated primarily by the state of specific Ca^{2+} and K^+ conductance systems. They showed that one important physiological pathway to the enhancement of Ca^{2+} currents may be through a decrease of K^+ currents and suggested that a persistent Ca^{2+} current contributes to the regulation of transmitter release. This Ca^{2+} current could be directly evaluated only over a limited voltage range and with Ba^{2+} substitution for Ca^{2+} but appears to have characteristics similar to the persistent voltage-dependent Ca^{2+} current that we describe here in neuroblastoma cells. Furthermore, a variety of cellular processes that are triggered by Ca^{2+} accumulation, such as cyclic nucleotide synthesis (29), hormone secretion (30), or even synapse formation (31) may require prolonged Ca^{2+} influx. Hopefully, the use of quinine and quinidine to block K^+ currents while leaving Ca^{2+} currents intact will make them useful pharmacological adjuncts in the study of neuronal processes thought to be controlled by the interaction of these currents, such as synapse formation and plasticity.

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