Br. J. Pharmacol. (1992), **106**, 751–755

The selective action of quinacrine on high-threshold calcium channels in rat hippocampal cells

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1 The whole-cell patch-clamp technique has been used to examine Ca channel currents carried by Ba (I_{Ba}) in rat hippocampal neurones.

2 Quinacrine selectivity decreased the high-threshold current activated by membrane depolarization from a holding potential of −70 mV. Neither the low-threshold Ca channel current nor the fast tetrodotoxin (TTX)-sensitive sodium current were affected by quinacrine.

3 Bath application of quinacrine caused a dose-dependent reduction of the peak amplitude of I_{Ba}. This effect was fast, voltage-independent, reversible and had a K_d of 30 ± 5 μM.

4 The quinacrine-induced block did not change the time-course and the voltage dependence of I_{Ba} activation and deactivation. The inhibition revealed no use-dependence, ruling out an open channel block by quinacrine.

5 *p*-Bromophenacyl bromide had no effect on I_{Ba} suggesting the lack of involvement of phospholipase A_2 in the action of quinacrine. In addition, the quinacrine-induced block was not related to the calmodulin pathway and internal quinacrine did not affect the peak amplitude of I_{Ba}.

6 The effect of quinacrine on the amplitude of I_{Ba} was dependent of the external pH, and suggested that only the single-protonated form of the drug can bind to the channel receptor with a K_d of 3 μM. Quinacrine and other substituted acridines can thus be useful for pharmacological and structure-activity studies of Ca channels.

Keywords: High-threshold calcium current; hippocampal neurones; quinacrine

Introduction

Quinacrine is used as an antihelmintic and antiprotozoan agent (Chou *et al.*, 1984). It has reported to bind to DNA (Darzynkiewicz *et al.*, 1984), to inhibit phospholipase A_2 (Schweitzer *et al.*, 1990) and calmodulin (Prozialeck & Weiss, 1982) and to block potassium channels in rat melanotrophs (Kehl, 1991). As part of the pharmacological characterization of neuronal Ca channels the effects of quinacrine were investigated. The results described in this paper show that the drug exerts a specific action on a high-threshold Ca channel current in neurones.

Methods

Acutely isolated pyramidal neurones from rat hippocampus were prepared by conventional techniques (Yaari *et al.*, 1987) with slight modifications. Briefly, following the dissection of one hippocampus from the brain of rat embryos (18–19 days gestation), tissue chunks (1 mm^3) were prepared and placed into Earle's saline containing 1% of antibiotic-antimycotic liquid, 0.4% glucose and $Mg_2SO_4 \cdot 7H_2O$ 1.5 mg ml^{-1}; they were then washed twice with serum-free BME medium with 1% L-glutamine, 0.44% glucose, 0.375% insulin and 0.25% penicillin-streptomycin. After dissociation by trituration, the cells were plated on poly-L-ornithine coated tissue-culture dishes, each containing 2 ml of incubation medium (BME medium enriched with 10% horse serum). The plates were kept in a humified incubator at 37°C. To suppress the proliferation of glial cells, on the 4th day of cultivation the cell culture medium was supplied with 1 μM cytosine-β-D-arabinofuranoside hydrochloride. The cells were used for the experiments 8–14 days after plating.

The whole-cell currents were recorded by conventional patch-clamp technique using the amplifier EPC-7 (List Electronics, Darmstadt-Eberhardt, Germany) with about 70% compensation of the series resistance. Patch pipettes were pulled in two steps from a soft Kimax glass (Witz Scientific, Maumee, Ohio, U.S.A.) and heat polished with a final inner tip diameter between 1 and 1.5 μm. Their resistance ranged from 1 to 3 MOhm when filled with the standard pipette solution and the seal resistance was from 1 to 10 GOhm. The holding potential was −70 to −60 mV, a potential at which the inactivation of low-threshold Ca channels is complete.

For recording of this current component, the holding potential was set to −100 or −110 mV; 3–5 min was allowed for equilibration of patch pipette contents with the cytoplasm before recording was begun, although interfering potassium currents usually washed out earlier. Data were stored and digitized at 10 kHz, for analysis with PC programme. Records were correlated for linear leak and capacitance currents. All mean data are given ± s.e.mean of the number of experiments given in parentheses. All the experiments were carried out at room temperature (18–22°C).

The standard recording solution contained (mM): $BaCl_2$ 5, NaCl 120, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 20 (adjusted to pH 7.5 with NaOH), glucose 10, and tetrodotoxin (TTX) 0.001 to block the fast sodium current. In some experiments TTX was not added. The patch electrodes were filled with a solution containing (mM): CsCl 100, tetraethylammonium (TEA) chloride 40, glucose 10, ethyleneglycol-*bis*-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 100, HEPES 10 (adjusted to pH 7.5 with CsOH). To record the sodium current through the Ca channel $BaCl_2$ was replaced by 5 μM EGTA.

All chemicals and drugs were obtained from Sigma and culture reagents were from Gibco. Solutions containing quinacrine dihydrochloride were prepared fresh from 10 mM stock solutions. Experiments were performed under dimmed

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lights to prevent photoinactivation of the test drugs. Solution changes were carried out with the fast perfusion system (Konnerth *et al.*, 1987), consisting of a large bore six-barrelled pipette placed 30–60 μm from a patched cell.

Results

Reduction by quinacrine of the peak amplitude of high-threshold Ca channels

In each cell examined the Ba current decreased after the application of quinacrine (Figure 1). The effects of the drug were similar for both rat hippocampal and chick sensory neurones, but only the data obtained for the former cell type are shown. The block developed within several seconds and wash-out of quinacrine was accomplished in 10–20 s with complete removal of the block in most cases. The block of Ca channel current by quinacrine was concentration-dependent but it was independent of the test voltage.

With voltage steps from the holding potential of −100 mV, both high- and low-threshold Ba currents could be observed. Quinacrine blocked only the high-threshold current (Figure 1). Nowycky *et al.* (1985) suggested a division of high-threshold Ca channels into L- and N-type. N-type channels are largely inactivated at holding potentials more positive than −70 mV. Quinacrine always blocked the same amount of current elicited by test pulses from the holding potential of −100 and −70 mV. This result rules out the effect of the drug on N-type channels, if present in the cells examined.

The drug was equally effective in blocking the Na current through the Ca channel in Ca-free solutions (Figure 2). In cells exposed to 36 μM quinacrine the peak amplitude of the current carried by Ba, Ca and Na decreased by 49 ± 4% ($n = 12$), 45 ± 6% ($n = 10$), 47 ± 8% ($n = 8$), respectively, indicating that the drug blocks the Ca channel independently of its conductive mode. Quinacrine had no effect on TTX-sensitive Na current. As shown in Figure 3, the drug application selectivity suppressed the steady component of the total current while the amplitude of fast transients remained unchanged.

Lack of effect of quinacrine on Ca channel kinetics

Quinacrine did not change the time-course and the voltage-dependence of whole-cell Ca channel current. Inactivation kinetics was not changed by quinacrine (Figure 4a) and no shift in the voltage-dependence of the steady state inactivation was observed in 5 cells exposed to 36 μM quinacrine (data not shown).

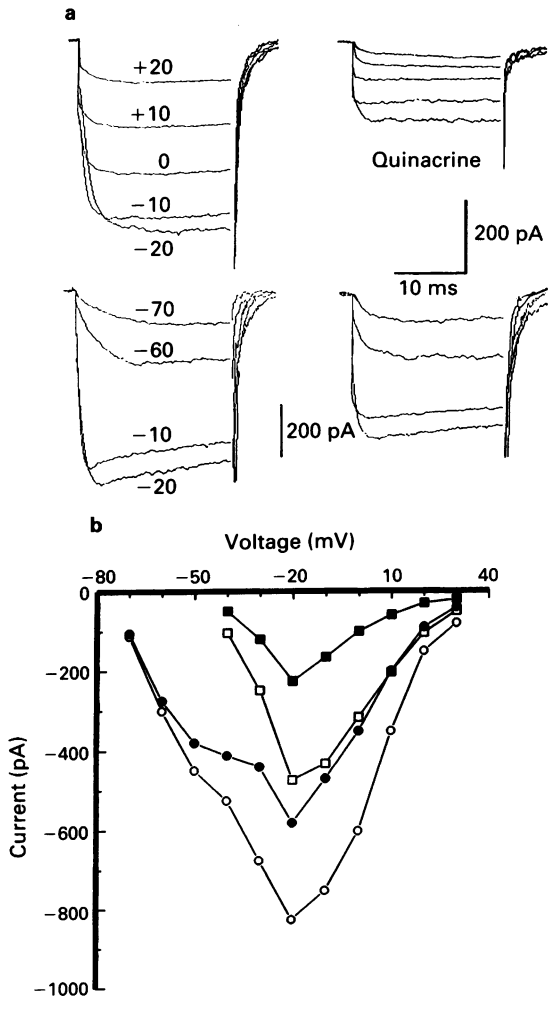

Figure 1 Quinacrine block of Ca channel current. (a) The traces show Ba currents recorded before and at equilibrium after 2 min application of 36 μM quinacrine in standard external solution. Recordings were made from holding potentials of −70 mV (top) and of −100 mV (bottom) for different depolarizing steps as indicated (in mV). (b) The dependence of peak currents on applied voltage for the same cell recorded before (open symbols) and after the application of quinacrine (solid symbols) for a holding potential of −100 mV (circles) and of −70 mV (squares). Note the unchanged peak current amplitudes between −70 and −50 mV which are attributable to low-threshold Ba currents.

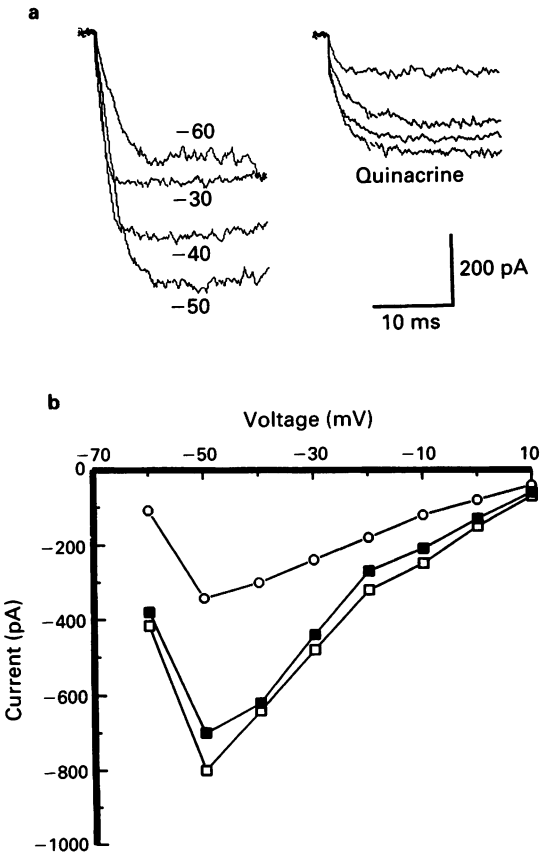

Figure 2 Quinacrine block of the sodium current through the Ca channel. (a) The traces show currents recorded before and 2 min after application of 36 μM quinacrine in Ca-free external solution. Recordings were made from the holding potential of −90 mV for different depolarizing steps in (mV) as indicated. The holding potential was made more negative regarding the shift of the activation curve of high-threshold Ca channels in solutions free of divalent cations. (b) The dependence of peak currents on the applied voltage for the same cell recorded before (□) 2 min after quinacrine application (○) and 2 min after wash-out of the drug (■).

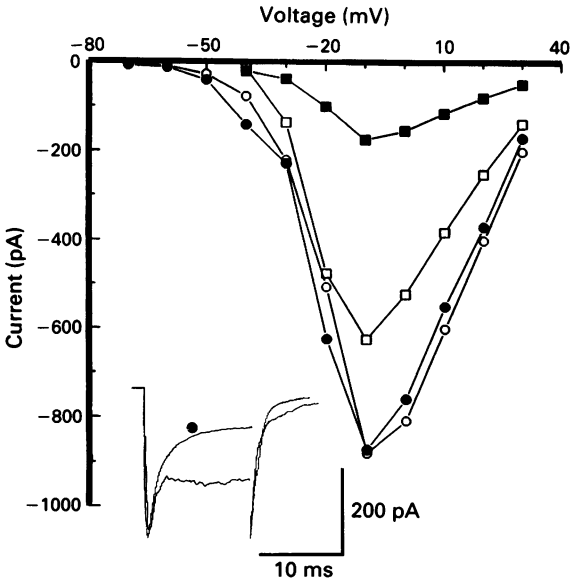

Figure 3 Quinacrine block of fast Na and slow Ba currents. The dependence of peak currents on applied voltage for the same cell recorded in tetrodotoxin TTX-free standard external solution before (open symbols) and at equilibrium after 2 min application of 50 μM quinacrine (solid symbols). Holding potential, −100 mV. Circles – peak current; squares – steady current measured at the end of 15 ms depolarizing steps. The inset shows two superimposed inward currents during test pulses from −100 to −30 mV, with and without the drug.

Like local anaesthetics acting on the fast sodium channels and some organic Ca antagonists, the quinacrine-induced block might possess use-dependence, originating from the block of open channels. Three lines of evidence, however, are against this possibility. First, the deactivation time constant was not affected by quinacrine (data not shown), although many drugs which block open channels alter tail current kinetics. Secondly, the time-course of the Ba current observed during long depolarizing pulses was unaffected by quinacrine (Figure 4a). Finally, no additional block was observed when the Ba current was evoked by a high-frequency train of depolarizing pulses. Quinacrine produced a fast decline of I_{Ba} and the block was still developing even when the stimulation was interrupted and the membrane was held at −70 mV (Figure 4b).

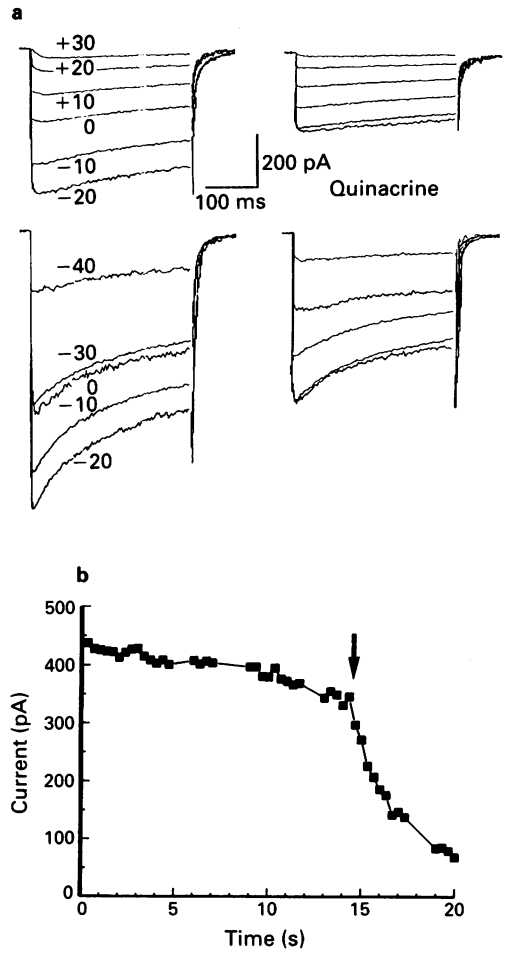

Figure 4 Absence of use-dependence in the quinacrine-induced block. (a) Ba currents recorded from the holding potential of −70 mV (upper row of records) and −100 mV (lower row of records), with voltage steps (in mV, as indicated near corresponding traces) before (left panel) and after 2 min application of 30 μM quinacrine (right panel). (b) Ba currents elicited by a train of 40 ms-long test pulses to 0 mV from holding potential of −70 mV delivered with a frequency of 3 s^{-1}. The pulse protocol was interrupted several times, indicated by the intervals between the experimental points. During these periods the cell membrane was held at −70 mV. Application of μM quinacrine is indicated by an arrow. Six other cells demonstrated similar behaviour.

pH-dependence of quinacrine block

The dose-response curve of the quinacrine block of the high-threshold Ba current is shown in Figure 5. The Hill coefficient was 0.97 ± 0.03 suggesting 1:1 quinacrine binding to the corresponding receptor in the channel. The dissociation constant was 30 ± 5 μM (confidence limits 19–41 μM). The drug can exist in several forms with different charge. One positive charge is located at the aminoacridine ring nitrogen, having a pK_a of 8.2, whereas the pK_a of the tertiary amine of the side chain is 10.2. Thus, at normal pH, quinacrine is mostly present as a single charged cation. Since the hydrophobicity of the ring might be an important determinant of binding, the pH-dependence of the quinacrine-induced block of the Ca channel current was studied.

Figure 6a shows that I_{Ba} was progressively reduced by successive applications of standard recording solution containing 36 μM quinacrine with increased external pH. Apppplication of acidic solutions produced brief transients in the current at holding potential due to the pH-activated non-selective conductance, $I_{Na(H)}$ (Krishtal & Pidoplichko, 1980; Konnerth *et al.*, 1987). In six out of ten cells examined, this current was absent but quinacrine revealed the same amount of I_{Ba} block. The quinacrine-induced block at different pH

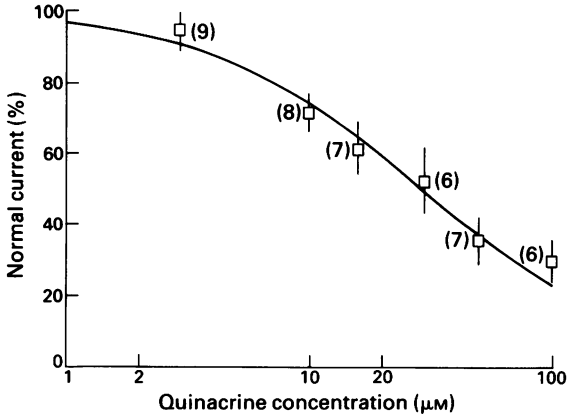

Figure 5 The dose-response curve of quinacrine block of high-threshold Ca channel current carried by Ba. For each cell examined the normalized amplitude ($I_{quinacrine}/I_{control}$) was measured for 2 to 3 different concentrations of quinacrine. All data are mean values with s.e.mean (vertical bars) and with number of cells given in parentheses. The solid line represents the least squares fit to a Michaelis-Menten equation with a K_d of 30 μM.

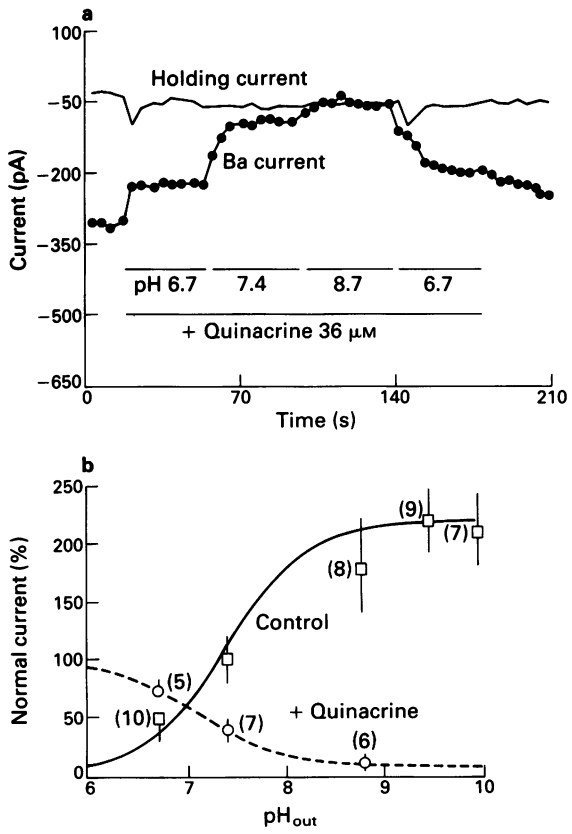

Figure 6 Dependence on pH of the block of Ca channel current by quinacrine. (a) Shown are the peak Ba currents (●) and the current at holding potential (solid line). The data were received after a transient current response which appears with fast application of acidic solutions. The test pulses to 0 mV from −70 mV holding potential lasted 40 ms and were delivered every 3 s. Horizontal bars indicate the periods of application of standard external solution containing 36 μM quinacrine at different pH. (b) The pH-dependence of peak Ba currents shown by (□). A new steady value of I_{Ba} was established within 1 min of cell perfusion with standard external solution of a different pH. The pH-dependence of the quinacrine block obtained as the ratio of peak currents at a given pH in the presence and in the absence of the drug shown by (○). All data are mean values with s.e.mean (bars) and number of cells denoted in parentheses. Curves represent the least squares fits to Michaelis-Menten equations. The solid curve was drawn according to $I_{Ba}(\%) = 220/(1 + 10^{7.4-pH})$ and the dotted line was drawn evaluated according to the equation $I_{Ba}(\%) = 100 \times K_Q/(K_Q + C_Q^+)$ with the concentration of single-protonated quinacrine given by $C_Q^+ = C_Q/(1 + 10^{pK-pH})$, where the total quinacrine concentration C_Q is 36 μM and $pK = 8.2$ for protonation of the nitrogen in the aminoacridine ring.

should be corrected for the pH-dependence of Ca channel current shown in Figure 6b. This effect is due to I_{Ba} increase with intracellular alkalinization as has recently been shown in sensory neurones (Mironov & Lux, 1991a). The block produced by quinacrine at different pH can be described as the binding of its single-protonated form to the channel with a dissociation constant of 3 μM, see the legend to Figure 6b.

External action of quinacrine

The voltage-independence of the quinacrine-induced block of the Ca current may indicate an intracellular target. Quinacrine has been reported to affect potassium current by inhibiting phospholipase A_2 (Schweitzer *et al.*, 1990). However, 10 μM *p*-bromophenacyl bromide, another inhibitor of this enzyme, was without effect either on Ca channel current or its block by quinacrine. An inhibition of calmodulin activity by quinacrine has also been demonstrated (Prozialeck & Weiss, 1982) but the Ca channel current was only increased after bath application of the calmodulin antagonists, trifluoperasine and W-13 (Mironov & Lux, 1991b). Thus, the mechanisms underlying this effect seem to be different from the quinacrine action on Ca channels observed in this study.

The results of experiments when quinacrine was included in the pipette solution are in line with data mentioned above. The persistence of the inward current at 0 mV with 36 μM internal quinacrine (−210 ± 60 pA, $n = 6$ vs. −285 ± 104 pA in 12 control cells and vs. −99 ± 31 pA in 36 μM external quinacrine, $n = 6$) indicated that there was little or no effect of quinacrine when it was applied internally.

Discussion

It has been shown that quinacrine blocks specifically high-threshold Ca channel currents blocked by quinacrine while no effect was observed on the low-threshold Ca channel and the fast Na channel. The fast transient potassium current I_K^f has been shown to be inhibited by quinacrine, 70% inhibition by 100 μM quinacrine at pH 8.5 (Figure 7 in Kehl, 1991). Our data show that Ca channels are more sensitive since a 90% block of I_{Ba} was produced by 36 μM quinacrine at pH 8.8 (cf. Figure 6).

Internal application of the drug had little if any effect on I_{Ba}. Inhibitors of phospholipase A_2 and calmodulin did not mimic the action of quinacrine which was reported to affect these two enzymes. These results speak against an intracellular action of quinacrine.

Amongst the possibilities that might explain the action of quinacrine observed in this study are a reduction of the probability of channel opening and/or a decrease of the single channel conductance. The absence of use-dependence rules out the modulated receptor-hypothesis suggested to describe the block of the fast Na current by local anaesthetics (Hille, 1977) and of the Ca channels by other organic antagonists (Lee & Tsien, 1983).

The effect of quinacrine was essentially similar for the two cell types examined, hippocampal neurones of the rat and peripheral sensory neurones of the chicken. For neuronal Ca channels the drug efficiency appears similar to that of other Ca antagonists (this only concerns the comparison of dissociation constants determined in electrophysiological studies). The data suggest that a quinacrine-binding site may be a distinct structural element common to neuronal high-threshold Ca channels.

The quinacrine molecule consists of a hydrophobic portion, the heterocyclic aminoacridine ring with the aliphatic side chain attached. The pH-dependence of the quinacrine-induced block indicates that only a single-protonated form can bind to the channel receptor. At pH 7.2 only about 10% of total quinacrine is active, resulting in a difference between the apparent and the putative dissociation constants of one order of magnitude. This information may provide a useful clue to increase the drug efficiency. Chemical modification in the aminoacridine ring can be made by introduction of nucleophylic substituents which will pump the electronic density on an aromatic nitrogen. The resulting shift of pK_a to the alkaline side should increase the percentage of single-charged drug molecules at normal pH: this will be evident as a decrease of the apparent dissociation constant for the block. A specific action of quinacrine on a high-threshold Ca channel, therefore, makes acridines suitable for biophysical and biochemical studies of this membrane protein.

We are grateful to Ms S. Engers and Ms H. Tyrlas for isolation and maintenance of cells and making the electrodes.

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(Received August 28, 1991
Revised March 19, 1992
Accepted March 24, 1992)