Mechanism of \( K^+ \) Channel Block by Verapamil and Related Compounds in Rat Alveolar Epithelial Cells

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ABSTRACT The mechanism by which the phenylalkylamines, verapamil and D600, and related compounds, block inactivating delayed rectifier \( K^+ \) currents in rat alveolar epithelial cells, was investigated using whole-cell tight-seal recording. Block by phenylalkylamines added to the bath resembles state-dependent block of squid \( K^+ \) channels by internally applied quarternary ammonium ions (Armstrong, C. M. 1971. Journal of General Physiology. 58:413-437): open channels are blocked preferentially, increased \([K^+]_o\) accelerates recovery from block, and recovery occurs mainly through the open state. Slow recovery from block is attributed to the existence of a blocked-inactivated state, because recovery was faster in three situations where recovery from inactivation is faster: (a) at high \([K^+]_o\), (b) at more negative potentials, and (c) in cells with type 1 \( K^+ \) channels, which recover rapidly from inactivation. The block rate was used as a bioassay to reveal the effective concentration of drug at the block site. When external pH, \( p[H]_o \), was varied, block was much faster at \( p[H]_o \) 10 than \( p[H]_o \) 7.4, and very slow at \( p[H]_o \) 4.5. The block rate was directly proportional to the concentration of neutral drug in the bath, suggesting that externally applied drug must enter the membrane in neutral form to reach the block site. High internal pH (pH, 10) reduced the apparent potency of externally applied phenylalkylamines, suggesting that the cationic form of these drugs blocks \( K^+ \) channels at an internal site. The permanently charged analogue D890 blocked more potently when added to the pipette than to the bath. However, lowering pH, to 5.5 did not enhance block by external drug, and tertiary phenylalkylamines added to the pipette solution blocked weakly. This result can be explained if drug diffuses out of the cell faster than it is delivered from the pipette, the block site is reached preferentially via hydrophobic pathways, or both. Together, the data indicate the neutral membrane-bound drug blocks \( K^+ \) channels more potently than intracellular cationic drug. Neutral drug has rapid access to the receptor, where block is stabilized by protonation of the drug from the internal solution. In summary, externally applied phenylalkylamines block open or inactivated \( K^+ \) channels by partitioning into the cell membrane in neutral form and are stabilized at the block site by protonation.

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INTRODUCTION

Verapamil and D600 are closely related phenylalkylamines which block several types of ion channels. They block Ca\(^{2+}\) channels more potently than K\(^{+}\) channels in cardiac muscle (Hume, 1985), but in nerve (Kostyuk, Krishtal, and Doroshenko, 1975), lymphocytes (DeCoursey, Chandy, Gupta, and Cahalan, 1985), alveolar epithelial cells (Jacobs and DeCoursey, 1990), small-cell lung cancer cells (Pancrazio, Viglione, Kleiman, and Kim 1991), and enterocytes (Tatsuta, Ueda, Morishima, and Okada, 1994) they block K\(^{+}\) currents with about the same affinity as for cardiac Ca\(^{2+}\) currents. Several features of the block of K\(^{+}\) channels resemble block of Ca\(^{2+}\) currents by organic Ca\(^{2+}\) antagonists, as well as the block of Na\(^{+}\) currents by local anesthetics, and block of K\(^{+}\) currents by internal quaternary ammonium ions. Inactivation of squid axon K\(^{+}\) channels is slow enough that any effects on quaternary ammonium ion block can be ignored (Armstrong, 1966), but interactions with inactivation apparently dominate the phenomenology of block of delayed rectifier K\(^{+}\) channels in rat alveolar epithelial cells. Phenylalkylamines produce a time-dependent decay of K\(^{+}\) current in a manner consistent with pure open-channel block, for which we proposed the following model:

![Scheme 1](image)

where C\(_1\) ... C\(_4\) are four sequential closed states used to generate the observed sigmoid activation kinetics of the current by traditional Hodgkin-Huxley n\(^4\) kinetics (1952), O is the open and the only conducting state, OI is the intrinsic inactivated state, OB is the open-blocked state, IB is the inactivated-blocked state, and the transitions shown are the only ones allowed (Jacobs and DeCoursey, 1990). Inactivation of these channels occurs only from the open state, and both inactivation and recovery, \(h\) and \(j\), are independent of voltage at potentials that activate the \(g_K\) (DeCoursey, 1990b). A major assumption in this model is that channel block and inactivation are independent. In contrast, inactivation and block of certain K\(^{+}\) channels by 4-aminopyridine are believed to be mutually exclusive (Thompson, 1982; Castle and Slawsky, 1992; Stephens, Garratt, Robertson, and Owen, 1994). Several types of experiments are therefore described which test (and support) the independence of phenylalkylamine block and inactivation.

The inverse of the time constant of block \(\tau_b\), after correction for intrinsic inactivation, is directly proportional to the concentration of blocker applied. This property is exploited as a bioassay to indicate the (relative) concentration of blocker at the block site. Furthermore, since verapamil and D600 are weak bases, the fraction
of drug which is neutral or positively charged can be controlled by varying the pH. This property is exploited to evaluate whether the drugs block K⁺ channels at a site accessible from the external or internal solution, and whether the cationic or neutral form of these drugs is active. This approach helped to elucidate the site of action and active form of local anesthetics on nerve Na⁺ channels (Narahashi, Frazier, and Yamada, 1970; Hille, 1977a). Calculation of the local concentrations of drug required considering the diffusion of drug between pipette, cell, and bath, as well as the membrane permeability. In the Appendix the diffusion of drugs with various membrane permeability coefficients, $P_m$, is modeled for the situation of the whole-cell configuration of the patch-clamp technique. Preliminary accounts of some of these results have been presented (DeCoursey, Im, and Quandt, 1989; DeCoursey, 1990a).

**METHODS**

**Cells**

Alveolar epithelial cells were isolated from adult male Sprague-Dawley rats following procedures described previously (DeCoursey, Jacobs, and Silver, 1988). Briefly, adult rat lungs were lavaged to remove macrophages, elastase and trypsin were instilled, and then the tissue was minced and forced through fine gauze. Lectin agglutinization and differential adherence further removed contaminating cell types. The preparation at first includes mainly type II alveolar epithelial cells, but after several days in culture, the properties of the cells become more like type I cells. Except for the experiments described in Fig. 4, all experiments were done on cells expressing type $n$ (Kv1.3) inactivating delayed rectifier K⁺ channels. A minority of type II cells express type $l$ (Kv3.1) K⁺ channels shortly after isolation, but most cells express type $n$ K⁺ channels, whose properties remain constant as long as cells are maintained (DeCoursey et al., 1988; Jacobs and DeCoursey, 1990). The criteria used for identification of K⁺ currents as type $l$ or $n$ has been described previously (DeCoursey, 1990b).

**Chemicals**

D890 and D600 (gallopamil hydrochloride) were generously provided by Drs. W. Hechler, H. Grünhagen, and R. Reinhardt of Knoll Pharmaceuticals A.G. (Ludwigshafen, Germany), Dr. Phil Best, University of Illinois at Urbana-Champaign, and by Dr. Robert O'Brien of Knoll Pharmaceuticals (Whippany, NJ). For some experiments, D600 (methoxyverapamil) obtained from Calbiochem Corp. (La Jolla, CA) was used. Verapamil and tetrabutylammonium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Phenylalkylamines were dissolved in ethanol and water to make stock solutions from which final dilutions were made, and were protected from light. Final ethanol concentrations were <1% except for D890, which had as much as 2% ethanol.

**pKa of Verapamil and D600**

Estimates of the $pK_a$ of verapamil in solution range from 8.73 to 8.99 (Mannhold, Rodenkirchen, Bayer, and Haas, 1984; Hasegawa, Fujita, Hayashi, Iwamoto, and Watanabe, 1984; Retzinger, Cohen, Lau, and Kezdy, 1986; personal communication from Grünhagen, H., and R. Reinhardt). The $pK_a$ of D600 is 8.5–8.8 (Dörscheidt-Käfer, 1977; Uehara and Hume, 1985; personal communication from Grünhagen, H., and R. Reinhardt). The slightly higher $pK_a$ of verapamil than D600 may account for its slightly lower apparent affinity for Ca²⁺ channels (Mannhold, Steiner, Haas, and Kaufmann, 1978) and for K⁺ channels (Jacobs and DeCoursey, 1990) in that a smaller fraction would be neutral at any given pH.
Solutions

The standard pipette solution included (millimolar): 100 KCH3SO4, 40 KF, 10 K2EGTA, 5 Hepes, 2 MgCl2, titrated to pH 7.2 with KOH. The pH 10 pipette solution also included 5 CAPS buffer. The pH 5.5 pipette solution included: 100 MES buffer, 92 KF, 10 K2EGTA, and was titrated with KOH. The external bath solution was usually Ringer’s with: 160 NaCl, 4.5 KCl, 2 CaCl2, 1 MgCl2, 5 HEPEs, titrated to pH 7.4 with NaOH. K+ Ringer’s included 160 mM KCl with no NaCl. The pH 10 Ringer’s solution had 3–10 mM TAPS buffer, and the pH 4.5 Ringer’s solution had 3 mM MES buffer.

Patch-Clamp Recording

Conventional whole-cell recording (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used, as described previously (DeCoursey, 1990b; Jacobs and DeCoursey, 1990), at bath temperatures kept near 20°C by Peltier devices. Data are presented without correction for liquid junction potentials or leak current. For cell-attached patch recording, K+ Ringer’s solution in the bath “clamped” the membrane potential to near 0 mV, assessed by reasonable agreement between the voltage-dependence of K+ channel opening and of Vm in the patch and whole-cell behavior in symmetrical high [K+]o solutions.

Simulation of K+ Current Block

To explore various predictions about state-dependent block, K+ currents were simulated using the state diagram described in Scheme 1, and in detail elsewhere (Jacobs and DeCoursey, 1990). The rate constants between adjoining states were assigned the following continuous functions of voltage:

\[ \alpha = 8.6 \frac{V + 25}{1 - \exp(-25 - V)/3.6} \]
\[ \beta = 1.5 \exp((-25 - V)/26) \]
\[ h = \frac{(1 - R)}{\tau_i} \]
\[ j = \frac{R}{\tau_i} \]
\[ k = \frac{(1/\tau_s)}{\exp(V/138)} \]
\[ l = 2.5 \exp(-V/43) \]

where V is the voltage in millivolts, and all rate constants are expressed in units/s\(^{-1}\). Note that the rate of inactivation h and recovery j are assumed to be voltage independent, and are determined in each cell from the observed time constant of inactivation \(\tau_i\) (units/s) and fractional noninactivating K+ current, R, as described previously (DeCoursey, 1990b). The block rate, k, is set from the block time constant, \(\tau_b\), measured at 0 mV, and has a weak voltage dependence. The unblock rate constant, l, was based on the rate of decay of tail currents in K+ Ringer’s solution in the presence of several open-channel blockers, each of which produced slowly decaying tail currents (Fig. 9) with similar absolute voltage dependence (Fig. 11). The rate constants k and l are assigned a uniform exponential voltage dependence, in contrast with the current dependence proposed in early studies (Armstrong and Binstock, 1965; Armstrong, 1966). In symmetrical K+ solutions in which both inward and outward K+ currents were blocked in a time-dependent manner, the voltage dependence of k extracted from the data was qualitatively the same as in Ringer’s solution, in which all currents were outward.

Diffusion Model

A number of theoretical and experimental studies have explored the diffusion of substances from the pipette into cells studied in the whole-cell configuration (Pusch and Neher, 1988; Oliva, Cohen, and Mathias, 1988; Mathias, Cohen, and Oliva, 1990). The calculations here consider the importance of membrane permeability in determining the submembrane concentration of drugs added to the pipette or bath.
Diffusion was computed by dividing the system into compartments, and calculating the diffusion from each compartment to the next in small time increments, following the approach of Hille (1977a). The inset in Fig. 14 B illustrates the arrangement of compartments. The cell is assumed to be spherical, with the tip of the pipette located at its center. This simplification allows the entire membrane to be in the same compartment. The bath is assigned a volume of 300 μl which is essentially infinite for the time scale of these calculations. Starting in the bath, there are 10 concentric spheres 1 μm thick outside the cell, concentric spheres within the cell 0.5 μm thick whose number depends on the cell diameter, with the innermost one being 1 μm in diameter, the pipette tip is assumed to be a 1-μm-long cylinder, 10 compartments 1 μm thick in the taper of the pipette, 19 compartments 10 μm thick also in the taper, 28 compartments 100 μm thick in the taper, and finally 7 cylindrical compartments in the shank of the pipette 1 mm thick and 1.15 mm in diameter. The compartments in the shank of the pipette do not participate significantly in these calculations. Adjustable parameters include the tip diameter, cell diameter, and membrane permeability coefficient, $P_m$. The flux from compartment $n$ to compartment $n + 1$ is:

$$m_{n \rightarrow n+1} = (c_n - c_{n+1}) A_{n,n+1} P_m$$

where $c_n$ is the concentration of drug in compartment $n$ (in moles per cubic centimeter), $P_m$ is the effective permeability which is the diffusion coefficient assumed to be $0.5 \times 10^{-5} \text{cm}^2/\text{s}$ divided by the thickness of the compartment, and $A_{n,n+1}$ is the area between compartments $n$ and $n + 1$. The compartment with the membrane has a permeability $(1/P_m + 1/P_m)^{-1}$. Initially the drug concentration was set at 0 or 1 in the bath and cell compartments, and diffusion was allowed to take place by iterations in 0.2-ms time increments until a quasi-steady state concentration profile was achieved, defined when the concentrations of the intracellular compartments and at the tip of the pipette did not change to seven significant digits in ~100 consecutive iterations. If time increments were made larger, the computation oscillated and crashed; making time increments smaller did not detectably alter the outcome. The real-time elapsed for this quasi-steady state condition ranged from a few seconds to >1 min, depending on $P_m$ and the cell diameter. This result is consistent with the rapid equilibration of pipette ions with the cytoplasmic compartment in whole-cell patch-clamp recording as assessed experimentally for small spherical cells (Fenwick, Marty, and Neher, 1982; Pusch and Neher, 1988). The time constant of equilibration of pipette constituents with the cell will vary directly with pipette tip resistance (Pusch and Neher, 1988; Oliva et al., 1988; Mathias et al., 1990).

**RESULTS**

State-dependent Block of $K^+$ Channels by Phenylalkylamines

$K^+$ currents in alveolar epithelial cells inactivate slowly in the absence of drugs, with a time constant, $\tau_i$, of typically 300–400 ms in Ringer’s solution, and twice that in $K^+$ Ringer’s solution (DeCoursey, 1990b). Adding verapamil to the extracellular solution increases the rate of current decay, with higher concentrations speeding current decay progressively. Whole-cell $K^+$ currents during depolarizing pulses to +20 mV in Ringer’s solution in the presence of several concentrations of verapamil are superimposed in Fig. 1 A. At all concentrations of drug the current was well fitted by a single exponential function. In the model of Scheme I, block is manifested as an increased rate of decay of the $K^+$ current, and the block rate, $k'$, is directly proportional to the concentration of drug applied to the bathing solution, after correction for the decay due to intrinsic inactivation of these channels, from the approximation:
\[ k' = k[\text{drug}] = 1/\tau_b - 1/\tau_i \]  

where \( \tau_b \) is the time constant of current decay in the presence of blocker and \( \tau_i \) is the time constant of intrinsic inactivation. Implicit assumptions which are most valid at high [\text{drug}] are that the rates of channel closing (O \rightarrow C), recovery from inactivation (OI \rightarrow O), and recovery from block (OB \rightarrow O) are slow enough in comparison with \( k[\text{drug}] \) to be negligible. The assumption that the intrinsic inactivation rate is unaltered by drug is tested below (see “Block does not ‘protect’ K+ channels from inactivation”). In summary, the rate of K+ current decay during depolarizing pulses in the presence of blocker directly reflects the concentration of drug at the block site, and will be used to evaluate the blocking potency of phenylalkylamines at various pHo and pHi to determine where block occurs and what form of the drug is active.

**Block in K+ Ringer’s solution.** Fig. 1 B illustrates block by verapamil in the same cell as in Fig. 1 A in isotonic K+ Ringer’s solution. In this cell the apparent block rate constant, \( k \), was \( 1.4 \times 10^6 \text{M}^{-1}\text{s}^{-1} \) in Ringer’s solution and \( 0.8 \times 10^6 \text{M}^{-1}\text{s}^{-1} \) in K+ Ringer’s. A feature of block that was evident in K+ Ringer’s was a slowing of the tail currents, which will be described below. Block was less complete in K+ Ringer’s solution. That a significant fraction of channels remain unblocked at the end of the pulses in Fig. 1 B can be seen from the well-defined inward tail currents after repolarization, even at 100 \( \mu \text{M} \) verapamil. This result suggests that recovery from block was enhanced by high [K+]o, a well-known characteristic of K+ channel block by internal quaternary ammonium ions (Armstrong, 1971; Armstrong and Hille, 1972). Because high [K+]o increased \( \lambda \), the assumption that \( k' >> \lambda \) is no longer valid in K+ Ringer’s and \( k' \) is likely overestimated; the corrected value is \( 0.6 \times 10^6 \text{M}^{-1}\text{s}^{-1} \). The effect of [K+]o on recovery kinetics will be explored below (compare Fig. 3).

**FIGURE 1.** Currents in the presence of various concentrations of verapamil (as indicated [\( \mu \text{M} \)]) in a cell bathed in Ringer’s solution (A), or K+ Ringer’s (B). Pulses are from \( V_{\text{hold}} = -80 \text{mV} \) to +20 mV (A) or +40 mV (B). The time bases have been scaled so that time is uniform in the two parts. The fitted time constant of block, \( \tau_b \), was slower at each concentration of verapamil in K+ Ringer’s solution, by a factor of <2. The tail current in K+ Ringer’s without drug (arrow) decayed most rapidly and crossed over the other tail currents. In both solutions the control K+ current rose slightly more slowly and to a smaller peak than after addition of drug. This phenomenon was not further investigated, but is intriguingly reminiscent of the transient enhancement of Ca2+ currents by D600 which preceded inhibition (McDonald et al., 1989).
**Onset of block is practically voltage independent.** Although the block of open K⁺ channels by phenylalkylamines is only weakly enhanced by depolarization, it is conceivable that closed channel block might be more steeply voltage dependent. The experiment illustrated in Fig. 2 tests whether closed-channel block can be detected at any voltage. The cell was held at −80 mV and a depolarizing pulse to +20 mV applied. The outward K⁺ current rose and then decayed slowly as K⁺ channels became inactivated. In the presence of 100 μM verapamil the current reached a smaller peak and then decayed rapidly, as open channels were blocked. The rate of current decay reflects almost entirely the rate of open-channel block, because intrinsic inactivation proceeds much more slowly. The cell was then clamped at −40 mV for 60 s, a potential just negative to the threshold for activating detectable K⁺ currents in this cell, and the membrane stepped directly from −40 mV to +20 mV. The current superimposed upon the current recorded after a step directly from −80 mV. If verapamil block were strictly voltage-dependent, then closed channels might be blocked to a greater extent at −40 mV than at −80 mV, and the peak K⁺ current would have been reduced. Because there was no block at subthreshold potentials, but nearly complete block at potentials where the gₘ was activated, it is apparent that the state dependence of block predominates over any voltage dependence. In other experiments, test currents superimposed when the holding potential was varied between −40 mV and −100 mV, both at 100 μM and 10 μM verapamil. If any part of the reduced K⁺ current is due to block of closed channels before the test pulse, then this block is not detectably voltage dependent. For the purpose of this study I will assume that K⁺ channels are blocked only when open.

**Are Block and Inactivation Independent?**

The state diagram of Scheme I presumes that block by phenylalkylamines and inactivation are independent processes. In Ringer’s solution at high drug concentra-

![Figure 2](image-url)
tions, $k' \gg l$ and $h \gg j$, and thus during depolarizing pulses open channels will accumulate in the IB state, from which they must both become unblocked and recover from inactivation to reach the O state and then close. This implies that recovery from block by phenylalkylamines will be coupled with recovery from inactivation, and that recovery from block will be slower than recovery from intrinsic inactivation. When fitted with a single exponential, recovery from inactivation in Ringer's solution has a time constant of 9 s, whereas recovery from block by phenylalkylamines has a time constant of 67 s (Jacobs and DeCoursey, 1990). Here I describe three additional experiments to test this model more rigorously.

Recovery from block and from inactivation are faster at high $[K^+]_o$. Compared with Ringer's solution, in K+ Ringer's the onset of inactivation, $h$, is slower and recovery, $j$, is faster, so that $h/j$ is 4 instead of 21 (DeCoursey, 1990b). If block and inactivation are independent, then recovery from block should be faster in K+ Ringer's solution, both because recovery from IB will be faster, and because many channels will recover from OB before inactivating. Fig. 3 shows that this is indeed the case. In Ringer's solution (Fig. 3 A), there was essentially complete block after <100 ms at +20 mV. There was almost no recovery after 1.1 s, and after 60 s recovery was only half complete. The time constant of recovery is ~1 min in Ringer's (Jacobs and DeCoursey, 1990). In contrast, in K+ Ringer's solution in the same cell in 100 μM verapamil (Fig. 3 B), 73% of blocked K+ channels recovered with a time constant of 89 ms, with the rest recovering slowly. Most K+ channels recovered from verapamil block three orders of magnitude faster in K+ Ringer's than in Ringer's solution at −80 mV. Although qualitatively similar, this enhancement of unblock by high [K+]o is much more dramatic than the 4–5-fold effect in squid axon (Armstrong, 1971) or the 10-fold effect in myelinated nerve (Armstrong and Hille, 1972).

Block does not "protect" K+ channels from inactivation. If block and inactivation were mutually exclusive as has been proposed for 4-aminopyridine block of certain K+ channels (Thompson, 1982; Castle and Slawsky, 1992; Stephens et al., 1994),

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![Figure 3](https://example.com/figure3.png)

**Figure 3.** Recovery from verapamil block is slow in Ringer's solution (A), but much more rapid in K+ Ringer's (B). In both experiments, 100 μM verapamil was added to the bath solution, and pulses were applied to +20 mV from $V_{hold} = -80$ mV. Each pulse in A is labeled according to the time since the previous pulse. The first pulse was applied 300 s after addition of verapamil, then the sequence was 30 s, 1.1 s, and 60 s intervals. Note that recovery is barely half complete after 60 s. The large, unlabeled current was after washout of drug. In B, pairs of identical 100-ms pulses applied with various intervals are superimposed. There is a component of rapid recovery, and also a slower component.
then block should protect K⁺ channels from inactivating. Open channel block of K⁺ channels by external TEA⁺ has been shown to fit this scheme:

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  C₁...C₄ ←→ O ←→ O₁
  ↑            ↑
  OB
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**Scheme II**

the rate of inactivation slowing as more channels are blocked (Grissmer and Cahalan, 1989; Jacobs and DeCoursey, 1990). In Scheme II the apparent inactivation rate will be slowed by a factor \( \sim (1 + k[\text{drug}]/k⁻¹) \). In contrast, if inactivation and block are independent, then \( \tau \) should be the same in the presence of blocker. One can estimate progression to inactivated states in the presence of blockers by evaluating the integral of tail currents in K⁺ Ringer’s solution after depolarizing prepulses of varying durations. This assumes that all channels not inactivated will reopen (OB → O) and contribute to the tail current before closing. (The “silent” progression of OB → IB both during the pulse [after \( I_K \) has reached apparent steady state] and after repolarization can be seen in Fig. 10 B.) When this analysis was carried out in three cells exposed to phenylalkylamines, the time constant of the decrease of the integral of tail currents was 252–522 ms, well within the range of control values for intrinsic inactivation \( \tau \) in K⁺ Ringer’s, 736 ± 567 ms (mean ± SD, DeCoursey, 1990b). In the presence of phenylalkylamines then, OB channels progress into an absorbing state, at a rate consistent with this being the IB state. Evidently phenylalkylamines do not impede the inactivation of these K⁺ channels.

**Type l K⁺ channels recover faster.** A fraction of freshly isolated rat type II alveolar epithelial cells express type l K⁺ channels (DeCoursey et al., 1988), which are identical in behavior to those in murine lymphocytes (DeCoursey, Chandy, Gupta, and Cahalan, 1987). One property that distinguishes type l K⁺ channels from the more common type n channels is that inactivation is slower and recovery is faster (DeCoursey et al., 1987; 1988). When relatively brief (~100 ms) depolarizing pulses are applied in rapid trains (e.g., at 1 Hz) substantial inactivation accumulates in type n channels, but not in type l channels. Fig. 4 A illustrates an experiment in a rat type II cell with type l channels. Verapamil produced open-channel block closely resembling that in type n channels: rapidly decaying currents with faster block at higher [drug]. Single-channel currents can be seen in some records, whose amplitude confirms the identity of these channels as type l. The block kinetics are slow enough that the single channel conductance is not reduced.

One striking difference between verapamil block of type l and type n K⁺ channels is that recovery from block was very rapid for type l channels, whereas recovery from block of type n channels in Ringer’s solution is very slow, with a \( \tau = 1 \) min (Jacobs and DeCoursey, 1990; Fig. 3 A). Fig. 4 B illustrates verapamil block at 30 μM in the same experiment as Fig. 4 A. When the pulses were repeated every 30 s there
A single channel conductance (21 pS), clearly >12 pS for type n channels under these ionic conditions (DeCoursey et al., 1987). (A) Superimposed records for pulses to +40 mV from V_{hold} = -80 mV in the presence of 0, 3, 10, 30, or 100 μM verapamil added to Ringer’s solution, as indicated. (B) Currents during pulses to +40 mV in 30 μM verapamil, in the same cell as in A. The three larger currents were recorded with 30-s intervals, which caused no detectable accumulation of block. The three smaller currents were the 12th-14th pulses in a train applied at 1.2 Hz.

was essentially complete block during each pulse within 20-30 ms, and the peak K^+ current was the same as in drug-free Ringer’s solution, indicating no accumulation of block (three larger currents). When the pulses were repeated at 1.2 Hz, there was only minor accumulation of block, with the peak K^+ current during the 12th-14th pulses (smaller currents) reduced by about half. Clearly recovery from verapamil block in Ringer’s solution is much more rapid for type l than for type n K^+ channels. This remarkable parallel between recovery from intrinsic inactivation and recovery from block suggests that the rate-limiting step in recovery from block is recovery from inactivation.

Effects of pH on Delayed Rectifier K^+ Channels

Effects of different pH. Before using pH_o to adjust the relative concentrations of charged and neutral drug, the effects of the large changes in pH_o used on K^+ channel gating in the absence of drugs were explored. The experiment illustrated in Fig. 5 encompasses the most extreme pH_o used in this study. A family of K^+ currents elicited by brief depolarizing pulses at pH_o 7.4 is shown in Fig. 5 A. Lowering pH_o to 4.5 produced a dramatic shift in the voltage dependence of activation to more positive potentials (Fig. 5 B). The g_K-V relationship in this cell was shifted by ~50 mV compared with pH_o 7.4 (Fig. 5 C). The maximum g_K was reduced, but this effect was hard to quantify because the outward current at these large positive potentials had a large instantaneous component, perhaps due to a nonlinear leak conductance. Raising pH_o to 10 shifted the g_K to more negative potentials by ~20 mV (Fig. 5 C), and rapidly destroyed most cells. Similar results were observed in other experiments, with shifts in the midpoint of a Boltzmann function fitted to the g_K-V data (DeCoursey et al., 1988) ranging from +44 to +63 mV for pH_o 4.5 (n = 4) and -13 to -30 mV for pH_o 10 (n = 2), both relative to pH_o 7.4. Activation was slowed markedly at low pH_o (note the slower time base in Fig. 5 B). These changes are in the direction observed for pH_o effects on K^+ channels in a variety of cells (Hille, 1968; Drouin and The, 1969; Carbone, Fioravanti, Prestipino, and Wanke, 1978; Blatz, 1984; Deutsch and Lee, 1989; Arkett, Dixon, and Sims, 1994). In sum-
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**FIGURE 5.** Families of K⁺ currents at pH₀ 7.4 (A) and pH₀ 4.5 (B), recorded at 10-mV increments, as indicated. In addition to shifting activation to more positive potentials, low pH₀ slowed the rate of channel activation. (C) Peak K⁺ chord conductance-voltage relationship including data at pH₀ 10 (up to the point when the cell died), labeled with pH₀. The initial level of current after each voltage step was considered to represent leak current and was subtracted. The holding potential at pH₀ 4.5 was −20 mV, instead of the usual −80 mV at pH₀ 7.4 and 10. The gₓ is almost completely inactivated at −20 mV at pH₀ 7.4 (DeCoursey, 1990b), but at pH₀ 4.5 the gₓ-V relation was shifted to a much more positive voltage range. Inactivation of the K⁺ channels in alveolar epithelium is strictly state dependent, that is, K⁺ channels must first open before they become inactivated (DeCoursey, 1990b). Because no K⁺ channels opened at −20 mV at low pH₀ there was no inactivation, and test currents elicited from Vₜₜₑₜ = −20 mV were the same as from Vₜₜₑₜ = −80 mV (not shown).

mary, when pH₀ is altered substantially, the voltage range over which the gₓ is activated changes dramatically.

**Effects of different pH₀.** Insufficient data were collected to draw quantitative conclusions about the effects of pH₀ on K⁺ currents. The data at pH₀ 5.5 were consistent with a small (<20 mV) shift of the gₓ-V relation to more negative potentials, and outward currents at large positive potentials appeared to be reduced. Cells did not survive very long at pH₀ 10, but no large voltage shift was evident. The impression was that pH₀ produced less dramatic effects on the voltage-activation curve of K⁺ currents than did pH₀, consistent with previous studies (Wanke, Carbone, and Testa, 1979; Deutsch and Lee, 1989).

**Effects of pH₀ and pH₁ on Phenylalkylamine Block**

**Low pH₀ reduces block.** At pH₀ 4.5 100 μM verapamil produced barely detectable block of K⁺ currents, slightly increasing the rate of current decay (Fig. 6 A). The block rate constant corrected for inactivation was ~1.3 × 10⁴ M⁻¹s⁻¹, two orders of magnitude smaller than at pH₀ 7.4 in this cell where k was 1.3 × 10⁶ M⁻¹s⁻¹. At pH₀ 4.5 nearly all the drug is positively charged, thus external cationic drug is a very weak blocker.

**High pH₀ enhances block.** Fig. 6 B illustrates K⁺ currents in a cell at pH₀ 7.4 without blocker, and in the presence of 10 μM verapamil at pH₀ 7.4 and 10. The control K⁺ current at pH₀ 7.4 decays slowly due to intrinsic inactivation. Adding 10 μM verapamil considerably hastened the decay of outward current with complete block about halfway through the pulse. When the verapamil concentration was kept at 10 μM but pH₀ increased to 10, the K⁺ current decayed rapidly. The apparent potency of verapamil was thus much greater at pH₀ 10. The time constant of in-
FIGURE 6. Verapamil block is weak at low pHo and enhanced at high pHo. (A) Currents at pHo 4.5 in the presence and after washout of 100 µM verapamil. The cell was held at −20 mV and 2 s pulses were applied to +80 mV. The small effect of verapamil was reversible. (B) Verapamil appears to be more potent at high pHo. Currents recorded during three identical depolarizing pulses to +20 mV are superimposed. The control current inactivates normally. Addition of 10 µM verapamil increased the decay rate substantially at pHo 7.4. At pHo 10 block by 10 µM verapamil was so rapid that the K+ current looks like a capacity transient. At pHo 10 the K+ current is larger and rises more rapidly during the test pulse than at pHo 7.4, because of the shift of the gK-V relationship to more negative potentials (compare Fig. 5). When depolarizing pulses in pHo 7.4 were made 20 mV larger, the current increased during the pulse rapidly enough to encompass the current shown here at pHo 10 (not shown). The comparison was made at the same potential to avoid the possibility that voltage dependence of block could have produced the effect.

Activation, τa at pHo 7.4 was 512 ms (h = 2 s⁻¹), and τo was 93 ms at pHo 7.4 and 5.4 ms at pH 10 in this experiment. Applying Eq. 1 gives k = 9.9 s⁻¹ at pHo 7.4 and k = 185 s⁻¹ at pHo 10. The simplest interpretation is that the effective concentration of blocker at its site of action was ~19 times greater at pHo 10 than at pHo 7.4. The fraction of drug in neutral form is 19 times larger at pH 10 than at pH 7.4 for a pKa of 8.67, which is within the range of reported values (see Discussion). Apparently extracellular verapamil reaches its block site by the neutral form partitioning into the membrane. This result is consistent with the neutral form of the drug being active, or with the charged form of the drug acting on a block site accessible only from the intracellular solution. To help distinguish between these possibilities, experiments were done at different pHo.

Block at high pHo. If phenylalkylamines block K+ channels at an internally accessible site, then pHo should affect block by externally applied drug. Both the total concentration of drug and the relative proportion of charged and neutral drug molecules inside the cell will depend on pHo. Fig. 7 shows that verapamil was a weak blocker at pHo 10; k was an order of magnitude smaller than at neutral pHo. When pHo was increased from 7.4 to 10 in two cells at pHo 10, k increased by an average of 15-fold, comparable to the effect in cells at pH 7.2. Thus, the enhancement of block by high pHo occurs independently of pHo.

At pHo 10, not only is most of the intracellular drug neutral, but the total intracellular concentration is much lower than the total concentration in the bath at pHo 7.4 (see Appendix). Little block is predicted on this basis if the charged form is active. Because the neutral form should be present in the cell at the same concentration regardless of pHo, the reduced block at pH 10 means that: (a) internal cationic drug contributes to the observed block, (b) neutral drug blocks and block is enhanced by protonation of the drug, or (c) protonation of the receptor enhances block by neutral drug.

Block by verapamil at low pHo. When pHo was 5.5 (data not shown), verapamil added to the external solution produced block which was similar to that at neutral
Mechanism of \( K^+ \) Channel Block by Verapamil

Dr. DeCoursey

FIGURE 7. High pH inhibits block by externally applied verapamil. Block is apparent at 30 or 100 \( \mu M \) verapamil added at pHo 7.4 in a cell with pH 10, but was much weaker than in cells with pH 7.2.

\[ pHi, \text{ with } k = 1.9 \pm 1.0 \times 10^6 \text{ M}^{-1}s^{-1} \text{ (mean \pm SD, } n = 3) \text{, compared with } 1.2 \times 10^6 \text{ M}^{-1}s^{-1} \text{ at } pHi 7.2 \text{ (Jacobs and DeCoursey, 1990). If verapamil were concentrated in the cell according to the pH gradient, the cytoplasmic concentration would be 47 times greater at pHo 7.4//pHi 7.2//5.5 than at pH 7.4//7.2, and almost all internal drug would be cationic. That block was not substantially enhanced at pHi 5.5 suggests that the neutral form of verapamil is more active than the cationic form.} \]

Block by Drugs Added to the Pipette Solution

Block by internal or external D890. The mechanism and location of block by phenylalkylamines was explored further by using a related, permanently charged compound, D890. Block produced by D890 applied internally (or externally at higher concentrations) was indistinguishable from that by externally added phenylalkylamines, consisting of an increased rate of decay of outward current. Block by D890 added to the bath was evident at 3.5 mM, with \( k = 8.5 \times 10^3 \text{ M}^{-1}s^{-1} \) in two experiments. D890 produced distinct time- and state-dependent block when applied to the pipette solution at 0.1–0.5 mM. With 200 \( \mu M \) D890 in the pipette, inactivation was faster than in most cells at the start of the experiment and within a half hour \( \tau \) settled at \( \sim 35 \text{ ms} \), much faster than in any control cell. Measured at least 30 min after establishing whole-cell configuration in this and two other experiments, \( k \) averaged \( 2.0 \times 10^5 \text{ M}^{-1}s^{-1} \), five times lower than for externally applied D600, \( 1.0 \times 10^6 \text{ M}^{-1}s^{-1} \) (Jacobs and DeCoursey, 1990). Although this value could not be corrected for intrinsic inactivation, correction would reduce it by \( \approx 10\% \). Although the affinity of D890 for \( K^+ \) channels might simply be lower than that of D600 or verapamil, D890 is about equally effective as D600 in blocking Ca\(^{2+}\) channels when pressure-injected into cardiac myocytes (Hescheler, Pelzer, Trube, and Trautwein, 1982). The slow and rather weak block by D890 in the pipette solution suggests that charged drug molecules do not have as rapid access to the block site as neutral molecules. This would help to explain the weak enhancement of verapamil block by low pH\(_i\) (previous paragraph). That the apparent relative potency of D890 was 24 times greater when applied to the pipette than to the bath solution, however, shows that cationic blockers act at an internally accessible site.

Internal D600 blocks \( K^+ \) channels only at high concentrations. Given the block by internal D890, it was surprising that 30 \( \mu M \) D600 added to the pipette solution had no detectable effect. Because block is manifested as an increase in the rate of \( K^+ \) current decay, and \( K^+ \) currents in alveolar epithelial cells exhibit wide variability in
their rates of intrinsic inactivation—τi ranges 100–800 ms with mean 369 ms (DeCoursey, 1990b)—it was not possible to be certain whether weak block may have occurred in a given cell. In three experiments with 400–500 μM D600 in the pipette the current decayed with τ 332, 398, and 388 ms ~15–20 min after establishing whole-cell configuration. In one of these cells τ fell to 183 ms after 116 min in whole-cell configuration. These τ’s are not faster than τi in drug-free cells. The maximum block rate extractable from these experiments, ignoring the decay due to intrinsic inactivation, is \(7.7 \times 10^3 \text{ M}^{-1}\text{s}^{-1}\), which is 27 times weaker than block by internal D890, and 130 times weaker than the apparent block rate when D600 is added to the external solution. Clearly, tertiary phenylalkylamines block at best only weakly when added to the pipette solution.

**Effect of low pH on block by internal verapamil.** Block by tertiary phenylalkylamines added to the pipette solution might appear weak because the drugs diffuse through the cell membrane and into the bath faster than they diffuse from the pipette to the membrane. If so, then block ought to be enhanced by inhibiting membrane permeation. Since partitioning of drug into the membrane is much more likely for the neutral form of the drugs, low pHi ought to reduce drug efflux by reducing the formation of membrane-permeant neutral drug. Nevertheless, verapamil in the pipette solution at pHi 5.5 was found to have relatively weak blocking ability, although some presumed block was detectable at 100–500 μM. Without correcting for inactivation, the block rate was \(~5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}\) in two experiments, ~20 times weaker than block by external phenylalkylamines at neutral pH. Thus, although low pHi may have enhanced block over that described above for pHi 7.2, the effect was not dramatic. Fig. 8 illustrates that the K⁺ currents in these experiments decayed more rapidly at pHo 10 and more slowly at pHo 6, consistent with the idea that the drug escapes into the bath only after it is protonated at the extra-cellular face of the membrane, and consequently that this escape is retarded by high pHo (see also Fig. 13 below). In summary, either charged drug inside the cell

**Figure 8.** Apparent block by internally applied verapamil is enhanced at high pHo. The pipette solution included 100 μM verapamil at pHi 5.5, at which most of the drug should be charged. The decay of outward current at 0 mV is shown for pHo 6.0, 7.4, and 10, as indicated. Note that the decay becomes much faster at high pHo. The late rising phase at pHo 10 is due to proton currents, which are activated preferentially at low pHi and at high pHo (DeCoursey, 1991; Cherny, Markin, and DeCoursey, 1995). The decaying component of outward current was due to delayed rectifier K⁺ channels because it was abolished by 20 nM charybdotoxin, with no effect on the slowly rising H⁺ currents, which are insensitive to this peptide blocker (DeCoursey and Cherny, 1994). It is not possible to be sure how much of the decay is due to block and how much to intrinsic inactivation, but the data clearly indicate that verapamil applied internally via the pipette solution is a very weak blocker, and are consistent with the idea that such block that exists is enhanced at high pHo.
blocks weakly, or even the extreme pH used was not sufficient to prevent substantial loss of drug from the cell.

**Block by Internal D890 Resembles Block by Externally Applied D600 or Tetrabutylammonium (TBuA⁺)**

The kinetics and state dependence of phenylalkylamine block of K⁺ channels in alveolar epithelial cells are so strikingly similar to the block of nerve K⁺ channels by internal quaternary ammonium ions (Armstrong, 1969, 1971; Armstrong and Hille, 1972), that it seems reasonable to suggest that block occurs at the same site. Several experiments were done to compare block by TBuA⁺ ions in alveolar epithelial cells. TBuA⁺ produced qualitatively similar block applied either internally or externally, but was about five times more potent applied via the pipette. Determined as for verapamil, k averaged $3.3 \times 10^6$ M⁻¹s⁻¹ in three experiments with 10–100 μM TBuA⁺ added to the bath, while k for internal application of 1–10 μM TBuA⁺ averaged $1.6 \times 10^7$ M⁻¹s⁻¹ in two cells. Evidently, this permanently charged drug reaches the same block site regardless of the side of addition (DeCoursey et al., 1989; Quandt and Im, 1992).

Fig. 9 shows the striking similarity of state-dependent block in K⁺ Ringer’s solution by externally applied verapamil (A), internally applied D890 (B), or externally applied TBuA⁺ (C). In each experiment, the membrane was held at −80 mV and depolarizing pulses of various durations were applied. During these pulses the outward K⁺ current rapidly reaches a peak and then decays to a nonzero value, as open channels are blocked. Upon repolarization, the K⁺ channels close with a time course defined by the inward tail currents, which are large at −80 mV because $E_K$ is near 0 mV. After a short depolarization, the tail current decays exponentially with a time course similar to that in a drug-free cell. As the depolarizing pulse becomes long enough for substantial block to occur, the tail current becomes markedly slower. In several records distinct “hooks” are evident, that is, the tail currents increase in amplitude for a short period before eventually decaying. The hooked tail currents in K⁺ Ringer’s solution are qualitatively different from anything ever seen in the absence of blockers, and demonstrate state-dependent block unequivocally.

Armstrong (1966; 1969; 1971) proposed that K⁺ channel block by internal quaternary ammonium ions is state dependent, that channels must open to allow entry of the blocking ion, and blocked K⁺ channels recover upon repolarization by first

![Figure 9](https://example.com/fig9.png)
returning to the open state, as though the channel cannot close with the blocking ion inside. This behavior is consistent with a linear model of block:

\[
\begin{align*}
\cdots & \overset{C}{\longrightarrow} O \overset{\text{OB}}{\longrightarrow} \cdots \\
\end{align*}
\]

**Scheme III**

in which the return of blocked channels directly to the closed state is forbidden. The decay of tail currents is slowed, and given favorable kinetic circumstances, hooks or nonmonotonically decaying tail currents may occur. The tail current is small at first because most channels are blocked, but as channels become unblocked the inward tail current amplitude increases. Finally the current decays as unblocked channels close.

In Scheme III the integral of the tail current should be the same in the absence or presence of blocker, because blocked channels eventually become unblocked and then close at their normal rate. In two cells studied with 100 \( \mu \text{M} \) TBuA\(^+\) and prepulses long enough to block most channels (100 ms), the tail current integrals over a voltage range \(-40\) to \(-120\) mV were identical within \(\sim 10\%\) in the presence or absence of drug. In one cell studied with 100 \( \mu \text{M} \) verapamil, the tail current integrals were reduced by \(\sim 30\%\) over a wide voltage range. As the pulse duration was made longer, the integral of the tail current became smaller. This result requires that a fraction of channels enter an absorbing state, a nonconducting state from which recovery is very slow. Armstrong and Hille (1972) proposed that this absorbing state was a closed-blocked state; for the K\(^+\) channels studied here I suggest that this is the IB state (Scheme I).

Fig. 10 A illustrates the behavior predicted in K\(^+\) Ringer’s by Scheme I. The calculated tail currents decay rapidly after depolarizing pulses which open K\(^+\) channels but are short enough that few channels are blocked. A longer pulse resulting in partial block generates a biexponential tail current decay. Finally, a long pulse produces profound block and the tail current decay is nonmonotonic, i.e., hooked. This pattern closely resembles that illustrated in Fig. 9. Fig. 10 B illustrates the oc-
cupancy of various states during a 100-ms depolarizing pulse. During the pulse, channels that open rapidly enter the OB state. These progress slowly into the IB state, even after repolarization, because the presence of blocker holds the channel open, and inactivation occurs from the open state at all potentials (DeCoursey, 1990b). After repolarization OB channels pass through the open state before closing, resulting in a hooked tail current.

Voltage dependence of recovery from block. Information on the voltage dependence of recovery from block can be obtained from $\tau_{\text{tail}}$ in the presence of blockers. In Fig. 11, $\tau_{\text{tail}}$ is plotted for several cells studied in K$^+$ Ringer's solution with externally applied verapamil, D600, or TBuA$^+$, and with internally applied D890. The $\tau_{\text{tail}}$ in cells studied in K$^+$ Ringer's without drugs (X) is also plotted for comparison. All of these drugs increased $\tau_{\text{tail}}$ by a factor of $\sim 5-10$, and weakened the voltage dependence compared with deactivation in K$^+$ Ringer's without drugs. Because $\tau_{\text{tail}}$ was much slower in the presence of drugs, it reflects mainly the rate-determining process of unblock (OB $\rightarrow$ O). As a first approximation, the empirical relationship in Fig. 11 was used to define the voltage dependence of $i$ ($i \approx 1/\tau_{\text{tail}}$) in the model. In a few experiments pulse pairs were applied in the presence of phenylalkylamines. When the potential between the pulses was made more negative, more recovery occurred, as assessed by the peak K$^+$ current during the second pulse. This result provides additional support for the conclusion that unblock is faster at more negative potentials.

Phenylalkylamine block of single K$^+$ channels. The normal behavior of K$^+$ channels in cell-attached patches is illustrated in Fig. 12 A. At least one K$^+$ channel opened during most pulses. When the channel was open at the end of the pulse (Fig. 12 A, top and bottom records), an inward tail current was seen upon repolarization. When the K$^+$ channel closed before the end of the pulse, openings were de-
Block of K⁺ channels in cell-attached patches is manifest as reopenings after repolarization. In both experiments the pipette contained KMeSO₄+KF, and the bath contained K⁺ Ringer's solution to depolarize the cell. Potentials given assume that the membrane potential was clamped to 0 mV. The dashed lines show the closed-channel current level during each pulse. (A) Representative currents in a control patch. The patch was held at -100 mV and 500-ms pulses applied, with 250 ms of baseline before and after the pulses. The open probability was clearly higher at -40 mV than at -50 mV. In 66 of 93 pulses, at least one K⁺ channel opened during the test pulse. Of these, a K⁺ channel was open at the end of the pulse and immediately after repolarization in 24 sweeps, in 40 there were openings during the pulse but not after repolarization, and in one sweep each there was an opening before the pulse or an opening after the pulse without an opening during the pulse. (B) Representative currents in a patch with 400 µM D600 in the pipette solution. In each record a K⁺ channel opened briefly during the test pulse, was closed at the end of the pulse, and reopened after repolarization. The stored records include 100 ms at V_{hold} = -90 mV before and after each 300-ms pulse. In 80 out of 93 sweeps at least one channel opened during the test pulse. Only three times was a channel open at the end of the pulse and immediately after repolarization. In 31 sweeps there were openings during the test pulse but not after repolarization, and in 47 there were openings during the pulse as well as after repolarization, even though the channels were closed at the end of the pulse. There were no openings preceding the pulses and one opening after a pulse in which no opening was detected.

Washout of phenylalkylamine is also pH₀ dependent. The time course of drug removal from the vicinity of the block site after washout from the bath could be monitored precisely by measuring the time constant of K⁺ current decay. Removal of drug was found to be a separate process from recovery from block. Fig. 13 illustrates an experiment in which verapamil was washed out of the bath, and test pulses applied periodically. With the bath solution at pH₀ 10 substantial block remained after 11 min (record 3). Within 2 min after the bath solution was changed to pH₀ 7.4, recovery was nearly complete (4). Evidently verapamil accumulates in the
membrane in uncharged form. The partition coefficients in 0.1 N HCl are ~3 and 30 for verapamil and D600, respectively, while in 0.1 N NaOH the partition coefficient of both drugs increases to >10^3 (personal communication from Grünhagen, H., and R. Reinhardt). Thus, phenylalkylamines leave the membrane more readily when they become positively charged by accepting a proton from the external solution. At high pHo, the protonation rate is small and verapamil remains in the membrane mainly in uncharged form. At neutral pHo, most of the drug is charged and it more readily dissociates from the membrane.

An intriguing point in Fig. 13 is that after washout, the drug is nominally absent from the solutions on both sides of the membrane, yet block persists for >10 min. The block retains its distinctive state dependence, in that essentially complete block of all channels occurs during depolarizing pulses of sufficient duration, and complete recovery or unblock occurs upon repolarization, with the channels then able to open and again be blocked during a subsequent depolarization. Apparently the drug blocks the channel directly from a site accessible from within the lipid membrane phase, rather than via the internal (or external) solution. If block required the drug to leave the membrane to reach a site accessible only via hydrophilic pathways, then one would expect there to be a continual and fairly rapid loss of drug into the pipette (compare Appendix Fig. 17). A comparable mechanism for the access of local anesthetics to Na⁺ channels was suggested by Hille (1977b) on the basis of analogous experiments in which pHo was varied (Hille, 1977a). In conclusion, there appears to be a large pool of drug in the membrane, which can block open K⁺ channels repeatedly.

DISCUSSION

State-dependent Vs Voltage-dependent Block

The predominant factor determining the affinity of phenylalkylamines for their receptor is the gating state of the K⁺ channel. There was not detectable block at po-

![Figure 13. Dependence of the rate of removal of verapamil on pHo. Four currents during identical depolarizing pulses are plotted. Record 1 was recorded in the presence of 10 µM verapamil at pHo 10, pH 7.2. Verapamil was then washed out of the bath, with the same pHo 10 solution. 2 and 3 were recorded 4 min and 11 min after washout, and reflect a very slow removal of drug. The bath was then changed to pHo 7.4 Ringer's and after 2 min record 4 was recorded.](image-url)
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... potentials where $K^+$ channels are closed (at concentrations up to 100 $\mu$M), and nearly complete block at potentials where $K^+$ channels open (e.g., $-40$ and $+20$ mV, respectively, in Fig. 2). The open-channel block rate increased with depolarization only $e$-fold/100–200 mV, consistent with the cationic form of the blocker crossing 0.16 of the membrane field, measured from the inside, to reach the block site (Jacobs and DeCoursey, 1990). The unblock rate deduced from $\tau_{tail}$ in the presence of blockers was more steeply voltage dependent, with $\delta = 0.59$. Accordingly, steady state open-channel block would vary only from 0.18 to 0.82 over a 100-mV range centered at $K_D$. Thus, most of the apparent voltage dependence of block by phenylalkylamines is due to its state dependence.

Recovery from block is more voltage dependent than onset of block. The weak voltage dependence of block raises the possibility that block itself (i.e., $k$ and $\delta$) might be voltage independent, but coupled with voltage-dependent gating. However, several types of evidence indicate that the recovery from block is facilitated by hyperpolarization. Blockers slowed $\tau_{tail}$ in $K^+$ Ringer’s solution by roughly an order of magnitude, indicating that the rate-determining step is unblock, so that to a first approximation $l \approx 1/\tau_{tail}$. In the presence of blockers $\tau_{tail}$ was clearly voltage dependent, although less so than deactivation. More direct measurements of the extent of recovery at various potentials between pulse pairs also indicated that recovery was faster at more negative potentials. Finally, the presence of hooks on the tail currents can only be explained if block has some voltage dependence. If the $O \leftrightarrow OB$ relaxation is at equilibrium during a depolarizing pulse, then upon repolarization only a monotonically decaying tail current is possible if block is voltage independent. Hooks are possible when $l > 4\delta$ (Armstrong, 1966), but only when in addition, the steady state probability of block decreases with hyperpolarization. Coupling of unblock with the steeply voltage-dependent deactivation ($O \rightarrow C$) process will impart some voltage dependence to $\tau_{tail}$ calculated using Scheme I, even if $l$ were intrinsically voltage independent, but not as much as was observed experimentally.

The voltage dependence of $l$ for phenylalkylamines ($e$-fold/43 mV or $\delta = 0.59$) was comparable to that for internal quaternary ammonium ions on squid $K^+$ channels (twofold/40 mV or $\delta \approx 0.43$) (Armstrong, 1971), and was similar among internally applied D890, and externally applied phenylalkylamines or TBuA$^+$ (Fig. 11). One interpretation is that all of these molecules act at the same site, that each must cross approximately half the membrane field to exit the channel toward the interior of the cell, and each dissociates from the block site at about the same rate. An alternative possibility is that hyperpolarization reduces the affinity of the block site for any blocker. Such a mechanism might involve the voltage-dependent entry of $K^+$ into the channel (Armstrong, 1971) or a conformational change in the protein.

The nature of the absorbing state. One distinct difference between the proposed mechanism of block by phenylalkylamines, and the classical mechanism proposed for block of $K^+$ channels by internal quaternary ammonium ions, is the identity of the absorbing blocked state. Armstrong (1971) identified the necessity of this state, and suggested that it represented channels which closed while containing a blocking ion:
The main difference between Schemes I and IV is the existence of an additional, inactivated (OI) state, and the deep-blocked state is IB rather than CB. This distinction is not trivial because it was clear long before the advent of molecular biology that K⁺ channel closing and inactivation were distinct molecular processes. Scheme I predicts specific interactions between block and inactivation, many of which are supported experimentally. Several types of evidence support the idea that the absorbing state is related to K⁺ channel inactivation:

(a) The progression of blocked channels into the absorbing state during long depolarizing pulses occurs at a rate consistent with the rate of inactivation, whereas the normal rate of channel closing (O → C₄) at depolarized potentials is negligible. An alternative possibility is suggested by the demonstration of closed states adjacent to the open state which are neither voltage dependent nor in the “normal” opening pathway (Hoshi, Zagotta, and Aldrich, 1994). Conceivably the OB channel might close into one of these states with the blocker present. However, the nature of these “flicker” states is of rapid opening and closing, so the presence of blocker would have to greatly reduce the reopening rate to produce an absorbing state. The simpler interpretation is that blocked channels can inactivate.

(b) Recovery from block by externally applied phenylalkylamines, internally applied D890, or TBuA⁺ applied to either side of the membrane was much more rapid in high [K⁺]₀ than in Ringer’s solution. High [K⁺]₀ reduces the onset of inactivation and speeds recovery (DeCoursey, 1990b). The very slow recovery in Ringer’s solution is mainly due to the existence of the inactivated-blocked (IB) state; channels must recover from both inactivation and block. Because the blocked channel (OB) is prevented from closing by the presence of the blocker, the likelihood of inactivation (OB → IB) is greatly enhanced, even at negative holding potentials because inactivation is voltage independent (DeCoursey, 1990b).

(c) Recovery from block was much more rapid in cells with type I K⁺ channels, which also recover rapidly from inactivation even in Ringer’s solution.

(d) Recovery from block and from inactivation are both faster at more negative potentials. Estimated values for l increased e-fold/−43 mV for phenylalkylamines and −57 mV for TEA⁺ (Armstrong, 1971).

In summary, inactivation has profound effects on the recovery from phenylalkylamine block of K⁺ channels in alveolar epithelial cells, consistent with the existence of an IB state. This conclusion does not rule out the possibility of a CB state, especially in other K⁺ channels which inactivate more slowly, such as squid axon (Armstrong, 1971) or node of Ranvier (Armstrong and Hille, 1972). Removal of inactivation of Na⁺ channels by pronase greatly accelerates recovery from analogous state-dependent 9-aminoacridine block, due to elimination of a blocked-inactivated state (Cahalan, 1978).
How Do Phenylalkylamines Reach the Block Site?

Charged drugs can reach the block site on K⁺ channels from the intracellular solution. The quaternary D890 blocked K⁺ currents much more potently when added to the pipette than to the bath solution. However, quaternary ammonium ions such as TBA⁺ added externally can permeate the membrane and block K⁺ channels, presumably at the same "internal" site.

Tertiary phenylalkylamines reach the block site via hydrophobic pathways. Values of k for bath application of verapamil at various pH₀ and pHi are summarized in Table I. Changing pH changed k by a factor of ~500. The relative concentrations of extracellular and intracellular cationic verapamil, [V⁺]₀ and [V⁺]ᵢ, are given for a nominal total concentration in the bath of 100. These values assume that neutral verapamil, V₀, rapidly equilibrates across the membrane and is thus present at the same concentration on either side, and is present in the membrane, [Vᵢ]ᵢ, at a concentration proportional to the neutral form in the bath. The membrane permeability of charged phenylalkylamines probably is negligible near neutral pH due to the much higher Pₘ of the neutral form.

Tertiary phenylalkylamines added to the bath blocked much more potently at

<table>
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<th>pH₀</th>
<th>pHᵢ</th>
<th>k*</th>
<th>[V⁺]₀</th>
<th>[Vᵢ]ᵢ</th>
<th>[V⁺]ᵢ</th>
<th>[Vᵢ]ᵢ × percent charged</th>
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</table>

*Estimates of the block rate constant k have been corrected for inactivation and the total [drug] added to the external solution (Eq. 1) and are expressed in units (× 10⁻⁵ M⁻¹·s⁻¹).

**Values given in parentheses indicate the concentrations if pHi were 9 instead of 10.

Adsoption to an amphiphilic surface favors deprotonation of verapamil, thus its pKa decreases from 8.99 to 8.34 after adsorption to membranes (Retzinger et al., 1986). Concentrations of charged, [V⁺], and neutral, [V₀], drug are calculated based on the "interfacial" pKa of 8.34 for a total bath concentration of 100, because the concentration of membrane-bound drug presumably is most relevant. It is assumed that verapamil in uncharged form permeates the membrane rapidly compared with the rate at which internalized drug diffuses up the pipette and is therefore present at the same concentration on both sides of the membrane. The actual concentration of drug in the membrane will be in the two orders of magnitude higher according to the partition coefficient 94–200 for verapamil and D600 at neutral pH (Mannhold et al., 1978; Schaufler et al., 1996; personal communication from Grünhagen, H., and R. Reinhardt); the value given here, [Vᵢ]ᵢ, simply indicates relative concentrations for different pH. The total intracellular concentration will depend strongly on pH, because once in the cell, neutral drug will become protonated to the extent determined by pH, and neutral drug will replace protonated drug until equilibrium is achieved. This result would be expected to apply to drug accumulation in an intact cell, but not strictly in the whole-cell configuration.
Mechanism of K+ Channel Block by Verapamil

high pHo, and much less potently at low pHo, with the block rate directly proportional to the concentration of neutral drug in the bath (Table I). Increasing pHo from 7.2 to 10 increased $k$ by 10–15-fold, comparable to the calculated concentration of neutral verapamil (fifth column). Increasing pHi to 10 greatly reduced the block rate, but even here $k$ increased 15-fold when pHo was increased from 7.4 to 10. When pHo was lowered from 7.4 to 4.5, $k$ decreased by $\sim$100-fold. The calculated concentration of neutral drug decreases by a factor greater than this; however, block was barely detectable at pHo 4.5, and finite permeability of the cationic form might account for this small discrepancy. Within the error of the measurements, the apparent potency was directly proportional to the concentration of neutral drug in the membrane over a wide range of pHo. Externally applied phenylalkylamines evidently permeate the membrane in neutral form to reach the block site.

Which form is active? That pHi affects block by externally applied phenylalkylamines strongly suggests that block is determined by the concentration of drug at a site accessible to the intracellular solution. The concentration of intracellular neutral drug will be the same at pHi 10 as at pHi 7.2, but the concentration of internal charged drug will be greatly reduced. The lower block rate at pHi 10 than pHi 7.2 is thus qualitatively consistent with the idea that charged internal drug is the main determinant of block, as has been concluded in nearly all previous studies of weak base K+ channel blockers. Also, D890 was much more potent added internally than externally. However, if intracellular cationic drug, [V+]i, either free or adsorbed to the membrane, equilibrated directly with the receptor, the effective concentration of drug should depend on the pH gradient as in column 6, a prediction clearly at odds with the data. Increasing pHi from 7.2 to 10 decreased the apparent potency of externally applied verapamil by a factor of 8–12 (Table I). The concentration of charged drug in the cell should decrease much more, by a factor >600. At least part of this discrepancy may be ascribed to the true pHi being lower than 10. The buffer concentration was only 5 mM, which is not adequate to control completely the pH in alveolar epithelial cells (DeCoursey, 1991), and the life-span of cells studied with the pH 10 pipette solution may have been too short to allow full equilibration. Calculations for pHi 9 are also given in parentheses in Table I. However, it is also possible that the neutral form of verapamil can block K+ channels. Cohen, Vereault, Wasserstrom, Retzinger, and Kezdy (1987) found that the concentration of neutral verapamil or congeners adsorbed to the membrane correlated most closely with pharmacological activity. Neutral nifedipine produces block with qualitatively similar time and state dependence as by phenylalkylamines (Jacobs and DeCoursey, 1990). In experiments with pHi 5.5 an even more severe discrepancy arose. Phenylalkylamines added to the bath blocked at a rate at most slightly (less than twofold) greater than when pH was 7.2. If verapamil were concentrated in the cell according to the pH gradient, the cytoplasmic concentration would be 1.5 times greater than the bath concentration at pHo 7.4//pHi 7.2, but 71 times greater at pH 7.4//5.5. Calculations in the Appendix show that diffusion into the pipette is unlikely to account for the weak enhancement by pHi 5.5, unless desorption from the membrane occurs very slowly, and therefore, the concentration in-
side the cell remains low. In general, the effects of changes in pH, were orders of magnitude smaller than predicted if $[V^+]$ were the active form.

Another possibility is that the main active form of verapamil is neutral drug in the membrane which reaches its receptor and is stabilized there by protonation via the internal solution. The last column in Table I gives the relative concentrations calculated under these assumptions, which agree well with the values for $k$. Block is determined by the concentration of neutral drug in the membrane, not the total intracellular concentration, and hence is not dependent on the pH gradient per se. The effect of pH, is due to protonation of some fraction of receptor-bound drug molecules. A similar suggestion was made for local anesthetic block of Na⁺ channels in muscle, with the exception that the proposed protonation site was accessible from the external solution (Schwarz, Palade, and Hille, 1977). This idea may be difficult to distinguish experimentally from the possibility that the block site can be protonated via the internal solution, and that protonation of the receptor increases its affinity for neutral drug.

Why are phenylalkylamines in the pipette solution ineffective? D600 added to the pH 7.2 pipette solution produced block only at very high concentration, with an apparent block rate >100-fold lower than that for bath addition. This result is ascribed in the Appendix to rapid dissipation of intracellular drug by diffusion of the neutral form across the cell membrane. Surprisingly, verapamil added to a pH 5.5 pipette solution was only slightly more effective ($k$ was >20 times smaller than for bath addition), in spite of nearly all the drug molecules being charged at this pH. That significant drug escapes through the membrane even at pH 5.5 is suggested by the enhancement of block at high pHo (Fig. 8). However, block was still weak. This paradox can be explained if internal protonated drug reaches the receptor only slowly, whereas the neutral form reaches the block site rapidly. Block by D890 in the pipette solution reached a steady state level only after ~30 min, whereas externally applied phenylalkylamines which reach the receptor in neutral form act rapidly.

Washout is slow. Recovery from block can occur by drug entering the bulk solution or simply exiting the block site in the channel laterally into the membrane. The washout of quaternary ammonium ions is rather slow (data not shown) in spite of their permanent charge, consistent with the presence of a significant energetic barrier to removal of cationic blockers from the vicinity of the block site. After washout of verapamil from the external solution, open channels can be blocked, recover completely, and then become blocked again during repeated depolarizing pulses (Fig. 13). Evidently the drug remains in the membrane but at a site distinct from the block site. The effects of pHo in the experiment in Fig. 13 suggest that protonation of the drug at the membrane surface greatly accelerates its removal from the membrane. This idea is supported by the finding that the $pK_a$ of membrane-bound verapamil is lower than in free solution, 8.34 vs. 8.99, respectively (Retzinger et al., 1986).

Other drugs may have analogous blocking mechanisms. A variety of weak bases produce block of K⁺ channels in alveolar epithelium and other cells similar to that of phenylalkylamines. Time-dependent block consistent with an open-channel block mechanism has been reported for a variety of blockers, including strychnine (Sha-
picro, 1977), quinidine (Hermann and Gorman, 1984), diltiazem and nifedipine (DeCoursey et al., 1985), phenycyclidine, tetrahydroaminoacridine, chlorpromazine, and capsacain (Jacobs and DeCoursey, 1990). It appears that many different blockers enhance inactivation of K+ currents in a variety of cells. Many of these drugs may act at the same site, namely the internal quaternary ammonium ion receptor. The idea that weak bases permeate the membrane in neutral form, are protonated internally, and block K+ channels at an internal site has been suggested for strychnine (Shapiro, 1977), flurazepam (Swenson, 1982) phencyclidine (D’Amico, Kline, Maayani, Weinstein, and Kupersmith, 1983), 4-aminopyridine (Gillespie and Hutter, 1975; Choquet and Korn, 1991), and for several other drugs (Jacobs and DeCoursey, 1990).

Aside from general similarities, there are some differences in the block mechanisms of some of these drugs. Varying degrees of closed channel block were noted for 9-aminoacridine, phenycyclidine, tetrahydroaminoacridine, chlorpromazine, quinidine, and capsacain, in addition to open channel block, which was seen in pure form only for phenylalkylamines (Jacobs and DeCoursey, 1990). The block mechanism of 4-AP appears qualitatively different in different preparations. In many preparations, e.g., squid (Yeh, Oxford, Wu, and Narahashi, 1976), block is relieved by depolarization. Furthermore, neither hooked tail currents nor any changes in deactivation kinetics were observed for 4-aminopyridine in GH3 cell K+ currents (Wagoner and Oxford, 1990). The recent report that 4-aminopyridine block inhibits inactivation (Stephens et al., 1994) suggests that 4-aminopyridine blocks some K+ channels at a different site or by a different mechanism than phenylalkylamines.

Verapamil and D600 may act in a similar manner in other cells and on other channels. Verapamil speeds inactivation of K+ current in snail neurons (Kostyuk et al., 1975), human T lymphocytes (DeCoursey et al., 1985), cardiac myocytes (Lefevre, Coulombe, and Coraboeuf, 1991), and in human tracheal cells (Galietta, Rasola, Barone, Gruenert, and Romeo, 1991). Quite similar state-dependent block of K+ currents by externally applied verapamil occurs in small-cell lung cancer cells in which 100 μM verapamil added to the pipette solution had no detectable effect (Pancrazio et al., 1991).

Block of Ca2+ channels by phenylalkylamines has been extensively studied, and is generally believed to be state dependent. Evidence has been presented supporting preferential block of open Ca2+ channels (Pelzer, Trautwein, and McDonald, 1982; Lee and Tsien, 1983; McDonald, Pelzer, and Trautwein, 1984; Oyama, Hori, Tokutomi, and Akaike, 1987), inactivated Ca2+ channels (Kanaya, Arlock, Katzung, and Hondeghehm, 1983; McDonald, Pelzer, and Trautwein, 1989), or both (Uehara and Hum, 1985). The idea that block is compatible with inactivation in Ca2+ channels is analogous with the proposed existence of an IB state for K+ channels. The site of action of phenylalkylamines on Ca2+ channels has been proposed to be internal but accessible for external application by neutral drug crossing the membrane, based on a comparison of the sidedness of D890 action (Hescheler et al., 1982), and the demonstration that bath addition of D600 blocked Ca2+ channels in cell-attached patches (McDonald et al., 1989). Although most studies conclude that the
internal charged form of these drugs blocks $\text{Ca}^{2+}$ channels, Cohen et al. (1987) found that the concentration of neutral membrane-bound drug correlates better with efficacy than other forms.

Regardless of block mechanism, the evidence presented here illustrates that an intracellular site of action for verapamil and D600 is not only possible, but likely in cells under whole-cell patch-clamp, in spite of perfusion by the pipette, and certainly must occur in intact cells in which the concentration of drug within the cell will proceed to equilibrium.

**APPENDIX**

In the whole-cell configuration of the patch-clamp technique, small molecules in the pipette solution rapidly exchange with cytoplasmic molecules (Hamill et al., 1981; Pusch and Neher, 1988). Thus, it is often assumed that any constituent of the pipette solution will equilibrate rapidly in the cytoplasmic compartment. Similarly, one might assume that substances added to the bathing solution exert their effects at the extracellular side of the membrane, and that even a membrane-permeant compound would rapidly disappear into the pipette, so that cytoplasmic concentrations would be negligible. For many pharmacological agents, however, these assumptions are incorrect. We were surprised that phencyclidine, presumed to block open K$^+$ channels at an internally accessible site, was ineffective when added to the pipette solution (Jacobs and DeCoursey, 1990). The explanation that is examined quantitatively here is that the rate-limiting step in the diffusion of membrane-permeant drugs present in the pipette solution is at the tip of the pipette, and not at the cell membrane. Thus, blockers which act at the inner side of the membrane may paradoxically have a much greater effect when added to the bath than when added to the pipette solution.

**Predictions of the Model**

Fig. 14 shows the quasi-steady state concentration profiles for a drug added to the bath (A) or to the pipette solution (B) in the whole-cell configuration, assuming a 1-$\mu$m pipette tip diameter, a 10-$\mu$m cell diameter, and with the membrane permeability $P_m$ set at $10^{-5}$ to $10^{-6}$ cm/s. The x-axis represents the compartments in the model, the first 10 being extracellular, the cell membrane indicated by a vertical dashed line, the next 10 compartments intracellular with the tip of the pipette indicated by another vertical dashed line, and all remaining compartments inside the pipette. The ordinates give the quasi-steady state concentration of drug, in arbitrary units. At low $P_m$ ($\ll 10^{-7}$ cm/s, not illustrated) little drug added to the bath permeates the membrane and the concentration in the cell is low, but as $P_m$ is increased to $10^{-6}$ cm/s significant levels of drug begin to appear inside the cell (Fig. 14 A). At $P_m$ $10^{-2}$ cm/s the concentration inside the membrane is 98% of the bath concentration. The membrane and the pipette tip comprise the main obstacles to diffusion (Mathias et al., 1990), and the membrane essentially “disappears” when $P_m$ is large.

Analogous calculations for inclusion of drug in the pipette solution are illustrated in Fig. 14 B. At a low $P_m$ of $10^{-7}$ cm/s (not shown) the drug is present just in-
side the membrane at practically the same concentration as in the pipette. As $P_m$ is increased, the concentration decreases, because transmembrane efflux becomes significant. At $P_m = 10^{-2}$ cm/s the concentration of drug near the membrane is only 2% of that in the pipette. When $P_m$ is high the drug concentration drops mainly at the tip of the pipette, as reported previously for ions transported across cell membranes (Mathias et al., 1990). For $P_m = 1$ cm/s the membrane is essentially invisible and the concentration profile approaches that for simple diffusion from the pipette into the bath, with the concentration near the membrane determined by its distance from the tip of the pipette.

*Apparent relative potency.* Because the main question is the concentration of drug in the vicinity of its hypothetical receptor facing the intracellular solution, the calculated data can be expressed as apparent relative potency, defined as the concentration of drug at the block site as a fraction of the concentration added to the bath or pipette. The relative potency is plotted as a function of $P_m$ both for bath and for pipette addition of drug in Fig. 15. The clear conclusion of these calculations is that depending on $P_m$, a drug which acts at an internal blocking site may appear to be more effective when added to the bath or to the pipette, or may have detectable blocking potency for either route of delivery. The crossover point, i.e., the $P_m$ at which internal or external application produces equivalent block, depends on the specific geometry, in this case a 1-$\mu$m diameter pipette and a 10-$\mu$m diam-
The fractional concentration of drug at the block site, assumed to be at the inner side of the cell membrane, is plotted for a range of $P_m$ values, for drug added to the pipette solution (○) or to the bath (■). The calculation is for a 1-μm tip diameter, a 10-μm diameter cell, and a $D$ of $5 \times 10^{-6}$ cm/s.

**FIGURE 15.**

The calculation is for a 1-μm tip diameter, a 10-μm diameter cell, and a $D$ of $5 \times 10^{-6}$ cm/s.

The fractional concentration of drug at the block site, assumed to be at the inner side of the cell membrane, is plotted for a range of $P_m$ values, for drug added to the pipette solution (○) or to the bath (■). The calculation is for a 1-μm tip diameter, a 10-μm diameter cell, and a $D$ of $5 \times 10^{-6}$ cm/s.

**FIGURE 16.**

The ratio of calculated relative potency for addition of drug to the external/internal solutions, assuming that the active site for the drug is at the intracellular side of the membrane. The pipette tip diameter is 1 μm, the diffusion coefficient is $5 \times 10^{-6}$ cm/s, and other model parameters are described in Methods. Ratios for three cell diameters are plotted. For example, for the case of a 10-μm diameter cell, addition of drug with $P_m = 10^{-5}$ cm/s to the bath results in a relative concentration just inside the membrane 25.1% of the bath concentration, and addition to the pipette solution results in a concentration near the membrane 74.9% of that in the pipette, for a potency ratio of $0.251/0.749 = 0.335$. At low $P_m$, the potency ratio decreases in direct proportion to $P_m$.
the drug added to the bath or to the pipette solution. The possibility that charged ions can permeate membranes at a significant rate is generally dismissed. However, tetraalkylammonium ions with alkyl chains of at least four carbons permeate cell membranes and block at an internal site when applied to the external solution during whole-cell recording (DeCoursey et al., 1989; Quandt and Im, 1992). The quaternary ammonium ions, dodecyltriethylammonium, and to a lesser extent, nonyltriethylammonium, produced detectable block after external application to squid axon, but much more weakly than when applied internally, consistent with an internal block site (Armstrong, 1971). TBuA⁺ was about five times more potent for pipette than for bath addition in cells 15-18 μm in diameter, a potency ratio of ~0.2 consistent with $P_m \sim 2 \times 10^{-6}$ cm/s estimated from Fig. 16. $P_m$ of TBuA⁺ was estimated to be $1.2 \times 10^{-7}$ cm/s in planar lipid bilayers (Quandt and Im, 1992). This reasonable agreement supports the interpretation that externally applied TBuA⁺ is sufficiently permeant to block at an internal site.

The model shows that, given the same geometry (cell size, pipette tip), the potency ratio is directly proportional to $P_m$. D890 was 24 times more potent when added to the pipette solution, for an apparent potency ratio of ~0.04. From Fig. 16, $P_m$ can be estimated at ~4 × 10⁻⁷ cm/s, because the cells studied were 13–18 μm in diameter. Because of their chemical similarity, this estimate might also apply to the charged form of D600 and verapamil. This $P_m$ is not negligible, but under most conditions the flux of neutral drug is so rapid that permeation of the charged form can be ignored.

Application of the model to weak bases (or acids). Verapamil, D600, and a host of other K⁺ channel blockers are weak bases which exist at neutral pH both in charged, poorly permeant form and in neutral, highly permeant form. Analogous calculations were made with the additional step that at the membrane compartment, the total drug concentration was divided according to the Henderson-Hasselbalch equation into charged and neutral drug forms, and each was allowed to permeate the membrane according to its own $P_m$. The value for $P_m$ for neutral verapamil was taken to be 424 cm/s, using Hille’s (1977a) adaptation of Finkelstein’s (1976) empirical relationship between partition coefficient (141, Mannhold et al., 1978), diffusion coefficient (0.5 × 10⁻⁵ cm²/s assumed), and $P_m$ for lecithin-cholesterol membranes. (A similar calculation gives 395 cm/s for $P_m$ for D600.) This $P_m$ is arbitrary but is large enough that the diffusion of neutral verapamil would not be impeded by the presence of the membrane even if $P_m$ were two orders of magnitude lower. Some of the results of these calculations were quantitatively predictable. Because $P_m$ is large for neutral verapamil, and many orders of magnitude lower for the charged form (4 × 10⁻⁷ cm/s, by analogy with the above estimate for D890), the concentration of verapamil in the cell is directly proportional to the concentration of neutral drug added to the external solution. Verapamil added to a pH 7.2 pipette solution should block poorly (as was found experimentally), because even the small fraction of uncharged drug at neutral pH leaves the cell rapidly enough to deplete the cytoplasmic compartment. For a 15-μm diameter cell, the calculated concentration of drug near the membrane was only 0.4% of the concentration in the pipette solution. Consistent with this result, Hescheler et al. (1982) found that D600 or D890 injected directly into cardiac myocytes blocked...
Ca$^{2+}$ currents, and that the effects of D600 but not D890 were transient, presumably because D600 diffused out of the cell.

Verapamil added to a pH 5.5 pipette solution is mostly protonated, and therefore the net efflux is slower than at neutral pH. Some retention of drug in the cytoplasmic compartments was calculated in this situation, which was enhanced by raising pH$_o$ to 10. The concentration in the cell at pH 7.4//5.5 was calculated to be 15% of the pipette concentration for a 15-μm diameter cell; in fair agreement with the 20-fold lower potency for pipette addition estimated from real data. At low pH$_i$ 5.5, the efflux of protonated verapamil only slightly enhanced the net efflux. A higher $P_m$ would bring the model closer to the data.

If pH$_o$ and pH$_i$ are different, then drug added to the bath will accumulate in the cell if pH$_i$ < pH$_o$. The neutral drug concentration is determined by pH$_o$ and equilibrates rapidly across the membrane so that it is the same on both sides. Inside the cell, the drug is redistributed between charged and neutral forms according to pH$_i$. If pH$_i$ is low, more neutral drug will enter the cell and become protonated until the concentrations of neutral drug are equal on both sides of the membrane. This principle is identical to the well-known situation in intact cells (Narahashi et al., 1970; McLaughlin and Dilger, 1980; Roos and Boron, 1981). If pH$_i$ = pH$_o$, the cell will act as an ideal pH meter. However, if the pH gradient is highly asymmetrical, it is unlikely that extreme levels of concentration will be achieved because diffusion into the pipette will become significant. At pH 7.4//5.5 verapamil ideally would be concentrated in the cell by a factor of 71, and at pH 10//5.5 by 680. The calculated concentration factor is lower due to diffusion into the pipette, and decreases as cell diameter decreases, at pH 7.4//5.5 for example, from 61 in a 15-μm cell to 40 in a 6-μm cell. Substantial accumulation is predicted in any case, and that block was only slightly enhanced at pH$_i$ 5.5 compared with pH$_i$ 7.2 rules out the possibility that intracellular cationic drug is the main active species.

High pH$_o$ slows washout of blockers. Although the onset of block by phenylalkylamines was rapid, after long continuous exposure (tens of minutes) the block rate increased by a factor of approximately two (Jacobs and DeCoursey, 1990). In Fig. 17 recovery from a moderately long exposure to verapamil is simulated at two different pH$_o$. The source of drug from which recovery takes place is assumed to be that which accumulated in the tip of the pipette after 10-min exposure at pH$_o$ 7.4, based on the flux rate through the tip of the pipette under these conditions. One would expect that most drug in the pipette tip would rapidly diffuse up the pipette and disappear. Nevertheless, there may be a finite effect of drug which accumulates in the pipette tip during long bath exposure. To simulate recovery when pH$_o$ was changed, the pipette was “filled” at a rate appropriate to pH 7.4//7.2 and then allowed to “recover” at pH 10//7.2. At pH 7.4//7.2 (◆) much of the drug diffused out through the cell membrane, but at pH 10//7.2 (■) most of the drug diffused into the pipette, and recovery was greatly slowed. It was assumed that the drug must become protonated at the external face of the membrane to exit the membrane to the bath. Although this calculation predicts a 50-fold slowing of washout at high pH$_o$, qualitatively consistent with the results shown in Fig. 13, the actual time constants are much faster than the time scale of minutes observed experimentally. This discrepancy may reflect that drug adsorption and desorption occur instantaneously.
FIGURE 17. Simulation of washout of drug to assess the extent to which drug accumulation in the pipette tip during long bath exposure might slow recovery. The limiting flux into the pipette at pHo/pHi 7.4/7.2, multiplied by the total exposure time, would "fill" a volume equivalent to the first ~11 μm of the pipette tip in 1 s, and after 10 min would fill the pipette to a distance 113 μm from the tip. To simplify the calculation, this volume at the tip of the pipette was filled at uniform concentration and then allowed to dissipate. The concentration of drug in the compartment just inside the membrane is plotted. Washout of drug is greatly slowed at pHo 10 (■) compared with pHo 7.4 (●), due entirely to the requirement that drug must be protonated at the external face of the membrane to leave the membrane. The curves show the best fit of a double exponential which limits to 0, although the calculated values did not fit this model well. At pH 7.4/7.2 the two time constants were 33 and 100 ms (0.73 and 0.27 relative amplitudes), and at pHo 10 the time constants were 0.93 and 5.2 s (0.66 and 0.34 amplitudes).

in the model, which is unlikely. In addition, the calculations ignore the membrane and intracellular proteins (Scheufler, Vogelgesang, Wilffert, Pegram, Hunter, Wermelskirchen, and Peters, 1990) as reservoirs for drug accumulation. The hypothesis that protonation of membrane-bound phenylalkylamines greatly facilitates desorption from the membrane at least qualitatively accounts for the data.

The manuscript was improved by thoughtful discussions with Drs. Elizabeth R. Jacobs, Fred N. Quandt, and Vladimir V. Cherny. Drs. H. Grünhagen and R. Reinhardt of Knoll Pharmaceuticals, Ludwigshafen, Germany, generously provided chemicals and relevant information. I appreciate the able technical assistance of Mary Grover and Donald Anderson.

Parts of this study were supported by National Institutes of Health grant HL-37500, Research Career Development Award K04-1928, and by a grant-in-aid from the American Heart Association.

Original version received 19 December 1994 and accepted version received 4 May 1995.

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