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Isomeric Yohimbine Alkaloids Block Calcium-Activated K^+ Channels in Medullary Thick Ascending Limb Cells of Rabbit Kidney

M. Cornejo, S.E. Guggino, A. Sastre, and W.B. Guggino

Department of Physiology, Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205, and Laboratory of Biological Chemistry, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224

Summary. The alpha$_2$-adrenergic antagonist yohimbine (YOH) and the closely related isomers corynanthine (COR) and rauwolscine (RAU) caused brief interruptions in current characteristic of a fast blocker Ca^{2+}-activated K^+ channels in cultured medullary thick ascending limb (MTAL) cells. The apparent dissociation constants (K_{app}) for COR, YOH, and RAU, respectively, at the intracellular face of the channel in the presence of 200 mM K^+ are 45 ± 1, 98 ± 2, and 310 ± 33 μM. The K_{app} for COR on the extracellular side also in the presence of 200 mM K^+ was much greater at 1.6 ± 0.17 mM. Increasing K^+ on the same side as the blocker relieves the blocking reaction. The K_{app} for the alkaloids varies with K^+ in a manner quantitatively consistent with K^+ and the alkaloids competing for a common binding site. Finally, blocking by the charged form of these alkaloids is voltage dependent with changes in K_{app} of 86 ± 7 and 94 ± 6 μM per *e*-fold change in voltage for blockers applied either from the inside or outside. The alkaloids block at an electrical distance similar to tetraethylammonium, suggesting that the site within the channel pore of these molecules may be similar.

Key Words loop of Henle · potassium conductance · ion channels

Introduction

Yohimbine is a naturally occurring alkaloid (*see* Fig. 1), well known to be an alpha$_2$-adrenergic antagonist (*see* Goldberg & Robertson, 1983). The molecule contains five asymmetric carbon atoms, creating the potential for several isomeric forms. In addition to its effect on alpha receptors, yohimbine at relatively low doses (10^{-5} M) has been shown to block fast Na^+ channel currents in myocardial cells (Azuma et al., 1978). In contrast, the alkaloid has a dual effect on slow cation currents, enhancing them at low doses but inhibiting them at much higher doses (10^{-3} M). Although the exact mechanism of yohimbine action on these currents was not determined, it was postulated that the alkaloid was acting as a local anesthetic because some of the properties of the blocking reaction resembled those of anesthetics such as lidocaine.

Because yohimbine and related isomers contain an ionizable group, we tested the possibility that they block Ca^{2+}-activated K^+ channels in a manner similar to TEA. Several quaternary amines (QA) such as TEA are well known fast blockers of Ca^{2+}-activated K^+ channels in many preparations (*see* Latorre and Miller (1983) and Yellen (1987) for reviews). Because the distance within the electric field of the positively charged amine group can be mapped using a technique first applied by Woodhull (1973) and because amines can be synthesized with various chain lengths, these compounds have been used extensively to model the tertiary structure of the channel's conduction pore (*see* Yellen (1987) for a review). Yohimbine (YOH) and the isomers cor-

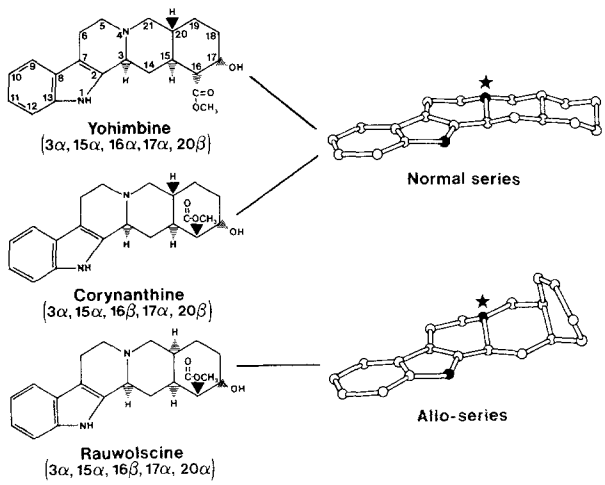


Fig. 1. Structure of the rauwolfia alkaloids

ynanthine (COR) and rauwolscine (RAU) were tested. Yohimbine and COR differ in the orientation of the CH_3COOH group on carbon-16, whereas RAU has this group in the same position as COR but differs in the position of the hydrogen on carbon-20. These differences have an impact on the shape of the molecule. In the first case, the multiring structure is relatively flat, but in RAU ring-5 (containing carbons 15–19) is bent relative to the backbone. Since the nitrogen at position 4 is ionizable, this shape difference has an effect on the pK of the molecules. Thus, with COR and YOH alkaloids in the *normal* series (*see* Fig. 1), have a pK of 7.1 and RAU, in the *allo* series (*see* Fig. 1) has a pK of 6.3 (Beckett & Dwuma-Badu, 1969; Lambert et al., 1978). We show that these alkaloids are fast blockers of Ca^{2+}-activated K^+ channels and that the block is stereo-specific and voltage dependent.

Materials and Methods

Cells used in these studies were identical to those used in several previous reports (Guggino et al., 1987*a*,*b*). The tissue culture conditions, electrophysiological characteristics, patch-clamp technique and data analysis equipment were also described in those studies. Because the blocking reaction of the alkaloids is fast we expressed our data as a fraction of blocking events (F_b). This was determined from amplitude histograms of recordings containing only one channel. The average open-channel current was determined by integrating the total current in the amplitude histogram minus closed-channel current. The latter was determined by integrating the amplitude histogram of a portion of the recording without channel events. The total open-channel current was divided by total recording time to obtain the average. F_b was then the ratio of average open-channel current in the presence and absence of blocker. Data were prefiltered at 3 kHz and digitized at 44 kHz.

K_{app}, the apparent dissociation constant for a blocker in the presence of K^+ is given by

$$F_b = [B] \cdot ([B] + K_{app})^{-1} \quad (1)$$

where $[B]$ is the blocker concentration. If K^+ and the alkaloids act in a manner consistent with competitive kinetics, then Eq. (1) can be written as

$$F_b = [B]/(([B] + K_d) \cdot (1 + [K^+]/K_d^K) \quad (2)$$

where K_d is the true dissociation constant for the blocker (i.e., in the absence of K^+) and K_d^K is the dissociation constant for K^+ (Vergara & Latorre, 1983). From Eqs. (1) and (2) it is clear that K_d and K_d^K can be calculated from

$$K_{app} = K_d \cdot (1 + [K^+]/K_d^K). \quad (3)$$

Finally, the distance of the blocker in the electrical field (δ) was determined from

$$K_{app}(V) = K(0) \exp(-z\delta/RT). \quad (4)$$

Statistics

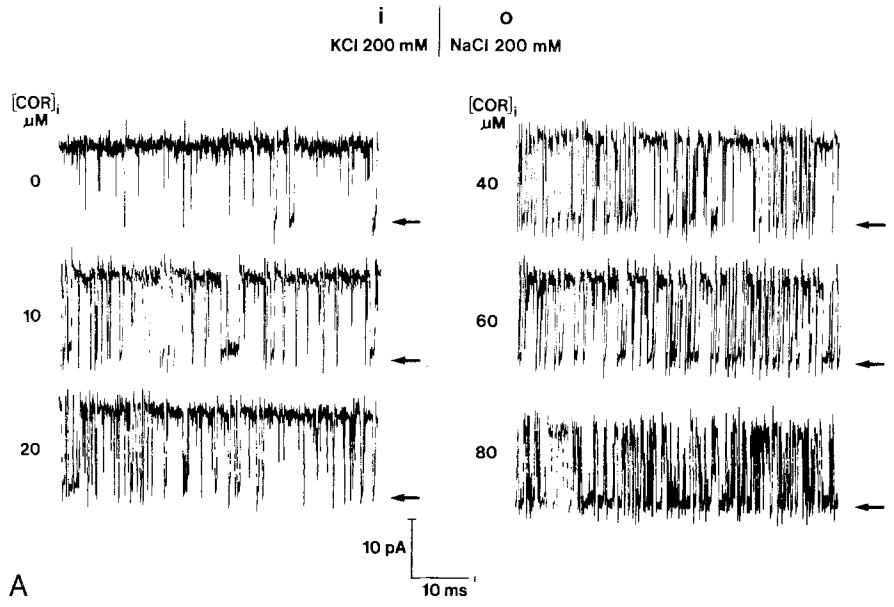
Nonlinear least-squares fitting of data for Eqs. (1) and (2) was carried out using the Quasi-Newton algorithm implemented in the NONLIN module of Systat (Systat, Evanston, IL); it was verified that convergence to the same results was unchanged if a Simplex algorithm was used instead. Although for simplicity linearized plots are presented, the estimates are all from fits of the untransformed data. Estimates are reported as mean ± SE. Although individual experiments are presented, the data represents experiments on 35 patches with single-channel events.

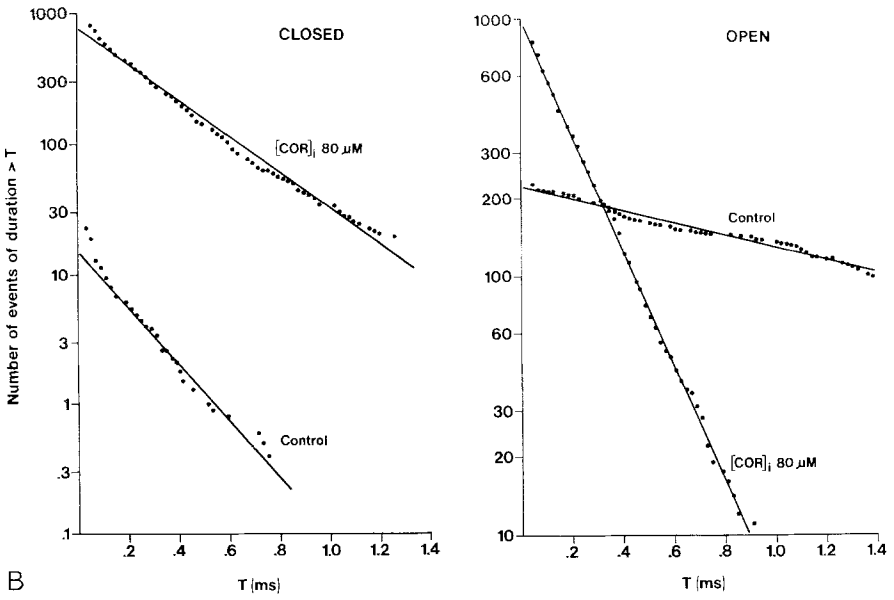
Results

Rauwolfia Alkaloids Block Ca^{2+}-Activated K^+ Channels

Application of the alkaloid COR to the intracellular face of the membrane of an excised patch (Fig. 2*a*) causes short interruptions in current movement through Ca^{2+}-activated K^+ channels characteristic of the fast blockers, quinine, TEA, and Cs^{2+} (Armstrong & Hille, 1972; Coronado & Miller, 1982; Yellen, 1984*a*,*b*; Cukierman, Yellen & Miller, 1985; Cecchi et al., 1987). Figure 2*a* illustrates this phenomenon in a single-channel recording from an inside-out patch. When exposed to 10 μM Ca^{2+} on its intracellular face the channel spends most of the time in the open state (upper left tracing), whereas in the presence of corynanthine brief interruptions in the current occur, which become more frequent with increasing concentrations of the alkaloid. This is confirmed in Fig. 2*b*, which shows that 80 μM COR by producing short current interruptions increases the number of closed events and the number of short openings. Figure 2*c* gives the current amplitude histogram in the absence and in the presence of 80 μM COR. Again consistent with a blocking effect, COR at 80 μM decreases the average open-channel current about five times. Because the blocking events (approximately 0.2 msec) are fast, F_b was used to evaluate blocking kinetics (*see* Materials and Methods).

Similar fast blocking patterns are also produced by YOH and RAU (Fig. 3), with COR being the most potent. The order of potency in outside-out patches with the blocker exposed to the intracellular face of the membrane is corynanthine > yohimbine > rauwolsine. At 60 μM, COR produces about 1.4 times the fraction of blocked events as YOH and about four times that of RAU. This is confirmed in Fig. 4, which shows both linearized double-reciprocal and untransformed plots of F_b *vs.* $[B]$. The K_{app} for the intracellular face measured in the presence of 200 mM $[K^+]_i$ are 45 ± 1 μM for COR, 98 ± 2 μM for YOH, and 310 ± 33 μM for RAU.





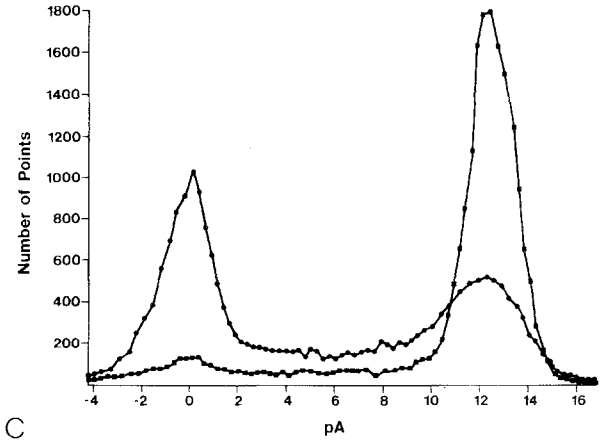


Fig. 2. (*A*) Effect of corynanthine on an inside-out patch of apical cell membrane of rabbit MTAL cells. F_b are as follows: 0 at 0 μM, 0.13 at 10 μM, 0.23 at 20 μM, 0.31 at 40 μM, 0.45 at 60 μM and 0.58 at 80 μM. (*B*) Closed (left) and open (right) channel histograms in the presence and absence of corynanthine. Recording time is 500 msec. (*C*) Amplitude histogram in the presence (circles) and absence (squares) of corynanthine. Note that the blocker diminishes the open-channel current

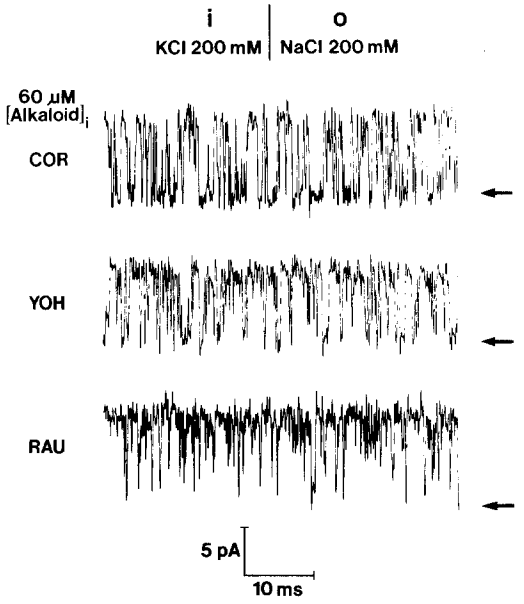


Fig. 3. Inhibitory potency of corynanthine, yohimbine, and rauwolscine. Arrows mark the closed state

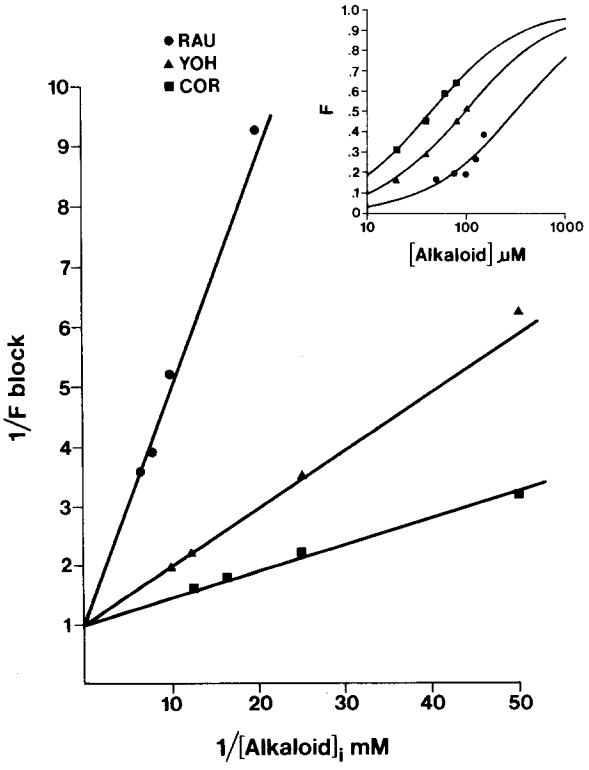


Fig. 4. Double reciprocal plot of fractional block (F) versus blocker concentration [*Alkaloid*]. Inset is an untransformed plot of the same data

These alkaloids are much less potent when applied to the extracellular face. Figure 5 shows that 100 μM COR will produce an order of magnitude greater number of blocking events when applied in

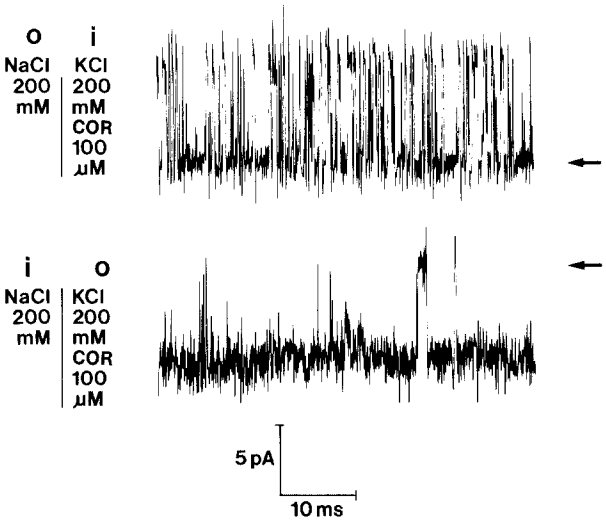


Fig. 5. Comparison of corynanthine blocking on the inside face (upper tracing) and outside face (lower tracing) of a Ca^{2+}-activated K^+ channel

the same conditions to the inside face compared to the drug applied to the outside. The K_{app} calculated from Eq. (1) is 1.6 ± 0.17 mM and the F_h is 0.06 ± 0.17 (n = 3) for 100 μM COR on the outside face.

Influence of K^+ on Rauwolfia Alkaloid Block

Increasing K^+ concentrations on the same side of the membrane enhances the fast blocking effect of ions such as Cs^{2+} and Na^+ on Ca^{2+}-activated K^+ channels (Cecchi et al., 1987; Yellen, 1984*a*,*b*). This phenomenon has been termed "knock on." In contrast, K^+ ions compete directly with the slow blocking process produced by Ca^{2+} and Ba^{2+} (Vergara & Latorre, 1983) such that decreasing concentrations of K^+ enhance the frequency of Ca^{2+}- and Ba^{2+}-induced slow block. In order to test the effects of K^+ on rauwolfia alkaloid block, corynanthine was added to the bath at the intracellular face of the membrane of an inside-out patch and bath K^+ was varied between 100–300 mM. Shown in Fig. 6*A* is that increasing bath K^+ reduces blocking by COR similar to that observed for Ca^{2+} block. Both linearized double reciprocal and untransformed plots (Fig. 6*B*) show that the K_{app} for COR decreases from 61 ± 3, to 45 ± 1, to 26 ± 5 to 16 ± 2 μM, when K^+ is reduced from 300 to 200 to 100 and finally to 50 mM. This suggests that when K^+ and COR enter the lumen of the channel from the intracellular medium both can compete for a common binding site within the channel. Quantitative support for this suggestion is shown in Fig. 6*C*, a plot of K_{app} versus K^+ concentration. The intercept is the true K_d for the alkaloid (in the absence of K^+) and equals

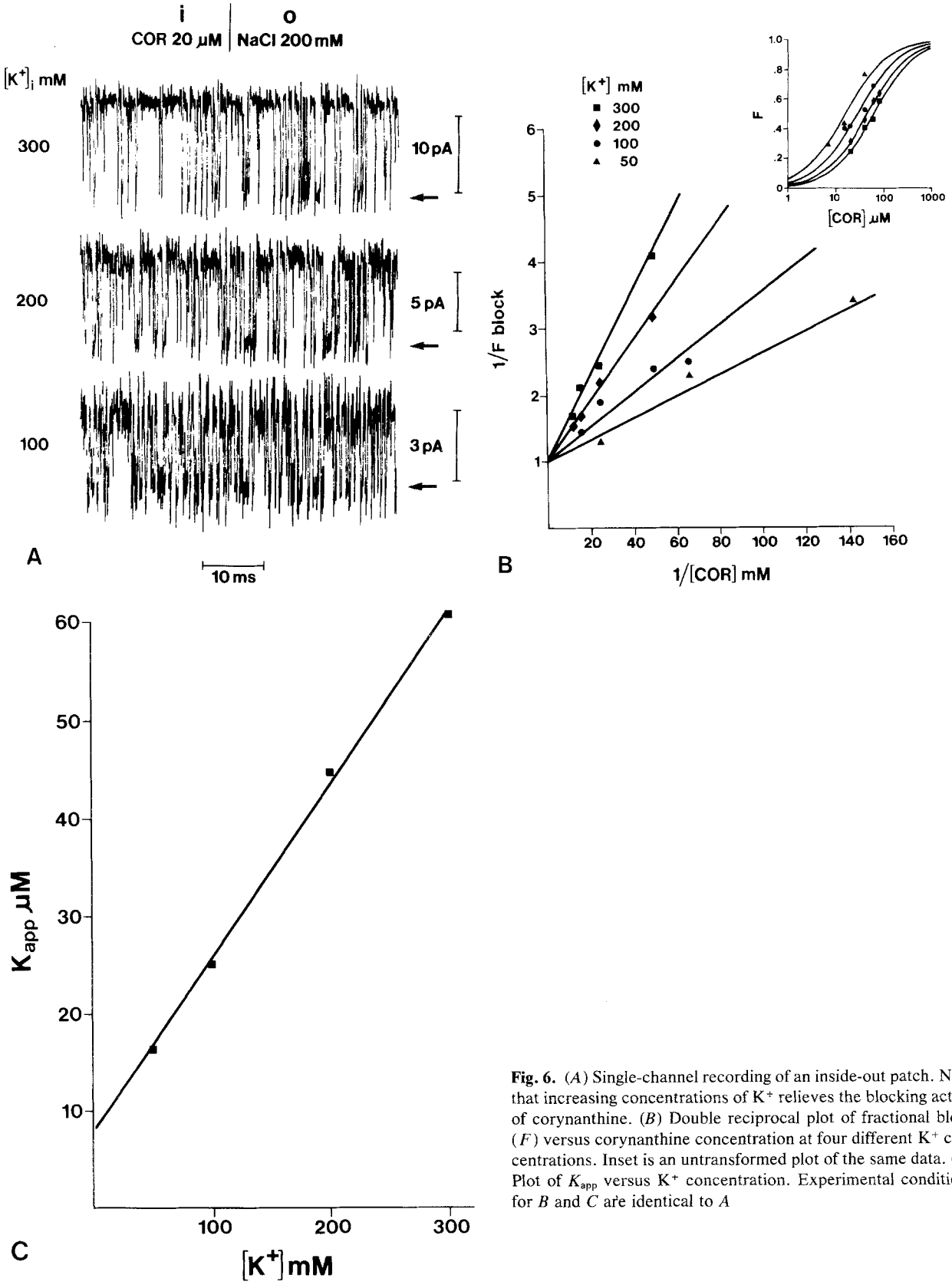


Fig. 6. (*A*) Single-channel recording of an inside-out patch. Note that increasing concentrations of K^+ relieves the blocking action of corynanthine. (*B*) Double reciprocal plot of fractional block (*F*) versus corynanthine concentration at four different K^+ concentrations. Inset is an untransformed plot of the same data. (*C*) Plot of K_{app} versus K^+ concentration. Experimental conditions for *B* and *C* are identical to *A*

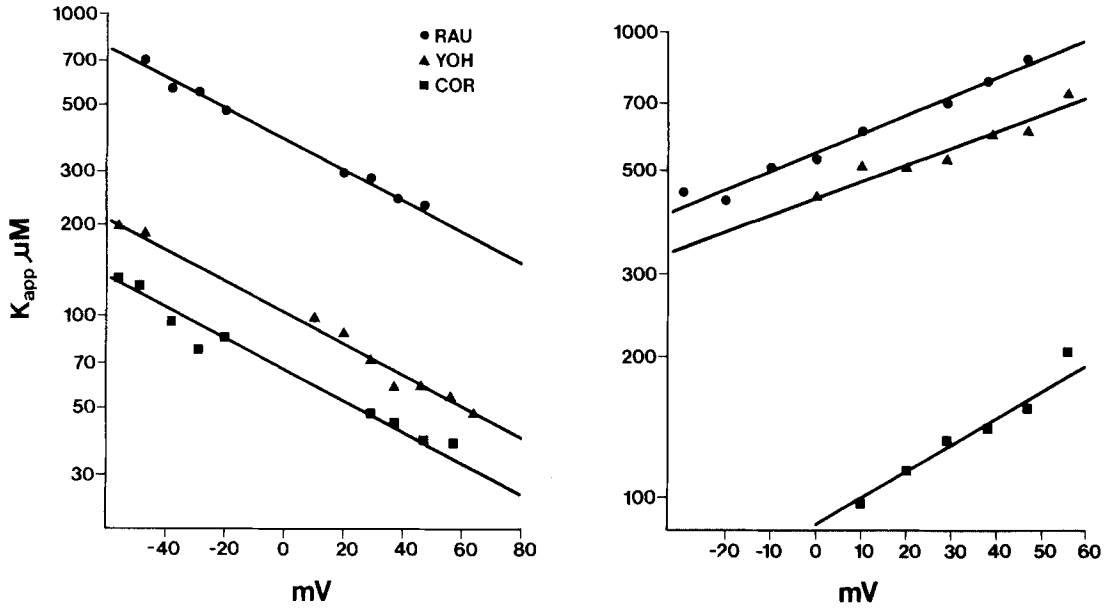


Fig. 7. Voltage dependence of alkaloid block. Left panel is from inside-out patches with the blocker on the inside-facing membrane. K_{app} was determined with 50 μM corynanthine and yohimbine and 100 μM rauwolscine in addition to 200 mM K^+ in the bath and in the pipette. The right panel is from outside-out patches with the blocker on the outside-facing membrane. K_{app} was determined with 100 μM of each alkaloid and with 200 mM Na^+ in the bath and 200 mM K^+ in the pipette

9.0 ± 1.7 μM. The K_d^K is 51 ± 12 mM. The K_d^K, which is the dissociation constant for K^+, is in good agreement to the value of 60 mM determined from the competition of K^+ for Ba^{2+} block of Ca^{2+}-activated K^+ channels in rabbit muscle membranes (Vergara & Latorre, 1983).

Voltage Dependence of Rauwolfia Alkaloid Block

The pK of YOH and COR is 7.1, whereas it is 6.3 for RAU. Thus at the normal pH of our bath, 7.4, 65% of YOH and COR is uncharged, whereas RAU is 93% uncharged. In order to determine the electric distance at which the charged species blocks the channel from the intracellular face, the voltage dependence of the alkaloid block was measured at an internal pH of 4.3. At this pH the change in K_{app} with voltage (Fig. 7) is not significantly different among the three alkaloids (applied to the intracellular face of an inside-out patch) at 86 ± 0.6 μM (n = 3) per e-fold change in voltage. The distance of the binding site is 29 ± 1.7% of the electric field. This value is similar to that measured for TEA (34%) on the intracellular face of the channel (Vergara, Moczydlowski & Latorre, 1984). Thus it appears that the charged amino group at position 4 of these alkaloids is binding at a site similar to that of the quaternary amines. In the outside-out configuration the average voltage dependence of the alkaloids is 94 ± 22 μM (n = 3) per e-fold change in voltage, with a distance of 28 ± 4% of the electric field.

In order to gain more information on the protonated state of the species which is blocking the channel, the effect of COR on the intracellular face of an inside-out patch was compared at pH 4.5 and 9.0. The average K_{app} at pH 4.5 was 80 ± 4 μM and at pH 9 is 558 ± 130 μM (n = 3, measured in 200 mM K^+ on both sides of the membrane). If the effect of corynanthine at the different pH was due solely to the ratio of charged to uncharged species, the values for the K_{app} should vary by a factor of 3×10^4, assuming that the true K_d and the K_d^K remain constant with pH. Since the measured K_{app} are much closer than this, it is likely that both charged and uncharged species can block. Alternatively, it could also indicate that the charged species is indeed the one that is the actual blocking species, but either the K_d or the competition with K^+ for the binding site varies with pH.

Discussion

Characteristics of Rauwolfia Alkaloid Block

We have shown that rauwolfia alkaloids produce fast flicker block of Ca^{2+}-activated K^+ channels. Since several isomers are available with different

conformations, these compounds may be important probes to model the conduction pore. There are several distinct differences in the nature of rauwolfia alkaloid block and that observed for TEA in these same channels (Guggino et al., 1987*b*). First, although we did not measure the on and off rates, examination of previous data on these same channels (Guggino et al., 1987*b*) shows that TEA blocking is much faster than that of the alkaloids. For example, application of 400 μM TEA to the intracellular face of the membrane causes a significant decrease in single-channel amplitude indicative of the inability of the recording system to resolve the rapid flicker produced by the blocker (*see* Yellen, 1984*a*). Rauwolfia alkaloids, on the other hand, produce brief interruptions in single-channel current which are clearly resolvable. Second, TEA blocks with higher affinity (the K_d for the inside is 38 mM and for the outside is 0.29 mM (Vergara et al., 1984)) for the outside face, whereas the rauwolfia alkaloids display a 30-fold greater affinity for the inside compared to the outside face of the channel. This pattern of higher affinity block on the inside of the channel is similar to that of Ba^{2+}, which has about a 1000-fold greater affinity for the inside than for the outside (the K_d is 36 μM for the inside and 2 mM for the outside (*see* Guggino et al., 1987*b*)). Finally, the rauwolfia alkaloids (especially COR) are more potent blockers of these channels that are the QA compounds. For example, the affinity of COR (45 μM) for the inside is about 10 times greater than TEA for the outside face of the channel.

Models of channel structure derived from experiments with QA blockers suggest that there are large mouths on both intracellular and extracellular faces where the large QA molecules block and a tunnel into which only the smaller ions can enter (*see* Latorre & Miller, 1983; Yellen, 1987). This is confirmed by the voltage dependence of rauwolfia alkaloid block (*see* Fig. 7*A*), which predicts that the blockers enter the channel to about 29% of the electric field. This is very similar to TEA, which enters to about 34% of the electric field (Vergara et al., 1984), suggesting that the alkaloids may block at the TEA site. Interestingly, although the affinities vary among the rauwolfia alkaloids used in these experiments the binding site is the same (*see* Fig. 7*A* and *B*). This latter result is significant because it shows that the difference in the conformation in the alkaloids between COR, YOH and RAU does not affect the ability of the molecule from getting to the binding site but only the binding affinity. This has also been observed for several QA blockers, which can vary considerably in their affinity for the inside of the channel but bind at a very similar site within the channel (*see* Villaroel, 1985; Yellen, 1987).

Competition with Potassium

The blocking effect of rauwolfia alkaloids is diminished if the K^+ concentration is increased on the same side of the membrane as the blocker (*see* Fig. 6*B*). This suggests that K^+ and the blockers can compete for a common site within the molecule. This pattern is different for that observed for Cs^{2+} and Na^+ fast block of Ca^{2+}-activated K^+ channels in chromaffin cells (Yellen, 1984*a*). The blocking of both of these ions is enhanced when K^+ is increased on the same side as the blocker. This latter phenomenon has been termed "knock on." It has been hypothesized that knock on occurs because the Ca^{2+}-activated K^+ channel is a multi-ion pore. Both Na^+ and Cs^{2+} are small enough to enter the channel tunnel and block. However, since these ions are not easily conducted and must exit via the same side, an additional K^+ ion entering the tunnel prevents blocker exit, thus enhancing block. The situation is much different with the alkaloids. Because of the larger size of the molecule K^+ does not knock the blocker further into the pore. Instead, binding of K^+ to its site prevents the alkaloid from blocking. Interestingly, the value of K_d^K measured using Eq. (2) and the alkaloids is identical to that measured in Ca^{2+}-activated K^+ channels isolated from muscle. The latter was determined from the competition of Ba^{2+} block with K^+. This is somewhat surprising since Ba^{2+} binds to a site much deeper into the electric field than these alkaloids. What this suggests is that the binding of K^+ to a site within the channel can prevent both Ba^{2+} and the rauwolfia alkaloids from binding.

The Molecular Species

Since the rauwolfia alkaloids occur in solution both as protonated, positively charged and unprotonated, neutral species, it is important to know which species can block the channel. The data at low pH where drug occurs mostly as the uncharged species clearly shows that all three alkaloids block the channel in a voltage-dependent manner (*see* Fig. 7*A* and *B*). In contrast, the block at high pH where the concentration of the uncharged species is high is consistent with some block by the uncharged species. However, the possibility that the K_d or the K_d^K may be pH sensitive makes it difficult to identify the magnitude of the block from the uncharged species.

The stereo isomers of these alkaloids display different affinities for the blocking site with corynanthine blocking with greater affinity than yohimbine and rauwolscine. The difference in blocking could be caused by steric hindrance at the mouth of

the channel but the observation that all three molecules block at a similar distance within the electric field argues against this hypothesis. This latter observation suggests that the mouth of the channel is wide enough to accept all three molecules without steric hindrance. Thus, the affinity sequence appears to be an intrinsic difference in the nature of the channel-blocker interaction. The sequence of affinities of the three alkaloids is consistent with COR and YOH of the *normal* series binding with much greater affinity than RAU of the *allo* series. This suggests that the stereochemistry of carbon-16 is less important than that of carbon-20 (*see* Fig. 1). Molecules with substituents in the *beta* orientation at ^{20}C have much higher affinity than those with *alpha* orientation at that position. The structure at carbon-20 affects the shape of the molecule, which is mostly flat with a 20 *beta*. The pK_a is consequently higher in the *normal* series, as is the binding of the blocker to the channel. Clearly, the alkaloids are binding stereospecifically to Ca^{2+}-activated K^+ channels.

Similar observations have been made for the QA compounds. For example, it is known that several QA molecules bind to Ca^{2+}-activated K^+ channels at a similar site within the pore when exposed to the inside face (Villaroel, 1984; Yellen, 1987). Although the site of binding is similar, the affinities for blocking may vary over several orders of magnitude. The key is the hydrophobicity of the blocker, which suggests that the binding site is a hydrophobic pocket in the channel. It does not appear that hydrophobicity *per se* is the determining factor in affinity differences among the alkaloids, since the octanol/water partition coefficients follow the sequence: corynanthine > rauwolscine > yohimbine; this does not correlate with the sequence of blocking affinities (Beckett et al., 1969).

The effect of QA blockers is different on the outside of Ca^{2+}-activated K^+ channels. This side is very specific for TEA and does not appear to accept the larger QA analogues (Armstrong & Hille, 1972; Villaroel, 1985). The physical representation of this side of the channel is less well known. However, our studies show that rauwolfia alkaloids can indeed enter the pore from the outside and block, suggesting that Ca^{2+}-activated K^+ channels from MTAL cells have large mouths both on the inside and outside. Although the rank order of affinities among the alkaloids is the same, the K_{app} of the blocking is much greater when the drugs are applied on intracellular face than from the outside. This could be caused by an intrinsic difference in the binding site for the inside and outside or, alternatively, a difference in the affinity of K^+ for its site. If the latter is true, the lower K_{app} for the alkaloids may be caused by a higher affinity binding of K^+ to its site on the outside of the channel. The net result would be a weaker competition of the alkaloids against K^+ at the extracellular side of the channel. Evidence for a high affinity site for K^+ on the outside has been observed in human lymphocytes, where as little as 3.5 mM K^+ in the solution bathing the outside face of Ca^{2+}-activated K^+ channels reduces the potency of quinine block (Sarkadi et al., 1985).

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