State-dependent Block Underlies the Tissue Specificity of Lidocaine Action on Batrachotoxin-activated Cardiac Sodium Channels

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ABSTRACT We have identified two kinetically distinct modes of block, by lidocaine, of cardiac sodium channels, activated by batrachotoxin and incorporated into planar lipid bilayers. Here, we analyze the slow blocking mode which appears as a series of nonconducting events that increase in frequency and duration with increasing lidocaine concentrations. This type of block occurred rarely, if at all, for the skeletal muscle sodium channel subtype. Kinetic analysis showed that a linear open-closed-blocked model is sufficient to account for the major features of our data. Slow block occurs from a long closed state that is a distinguishing characteristic of cardiac channels under these conditions. Slow block showed no significant voltage dependence in the range of −60 to −20 mV for which the detailed kinetic analysis was performed, and was not elicited by application of the permanently charged lidocaine derivative QX-314. By contrast, the fast block, described in the companion paper, results from drug binding to the open state, and is similar for cardiac and skeletal muscle sodium channels. Application of trypsin to the cytoplasmic end of the channel eliminates both the spontaneous, long, gating closures and slow block. Thus, the lidocaine-sensitive closed state of batrachotoxin-activated cardiac sodium channels exhibits a protease susceptibility resembling that of the inactivated state of unmodified sodium channels. It is the slow block caused by lidocaine binding to this closed state that underlies the channel-subtype specificity of lidocaine action in our experiments.

INTRODUCTION

Although sharing many characteristics with sodium channels from other tissues, cardiac sodium channels show a unique combination of functional and structural properties, such as gating, voltage sensitivity, and kinetics (1), sensitivity to tetrodotoxin (2), and amino acid sequence (3, 4). The features that distinguish sodium channels in heart from those in other excitable tissues are critical to the choice and development of cardioactive drugs that are most effective and have the least side effects. One group of drugs which is of interest, consists of antiarrhythmic agents, widely used clinically in treatment of patients after cardiac infarction.

Class 1b antiarrhythmics such as lidocaine have been shown to be effective blockers of sodium channels from heart (5), nerve (6, 7), and skeletal muscle (8). A higher sensitivity to lidocaine is considered to be a characteristic of the cardiac sodium channel subtype (9). Recently, this property, and other heart-specific features, were shown to be preserved in sodium channels expressed in *Xenopus* oocytes from cardiac mRNA (10).

We have studied the blocking action of lidocaine on batrachotoxin (BTX)-activated sodium channels from heart and skeletal muscle to investigate the tissue-specific interaction of the drug with these channels. We were able to identify two modes of block which differ in their state dependence and blocking kinetics (an open-state, fast block, and a closed-state, slow block). In the companion paper (11), we have described our experiments on fast block. In this paper we present our study of the slow blocking action of lidocaine on BTX-activated cardiac sodium channels. We demonstrate that lidocaine induces slow block by binding to a long closed state which is characteristic of the cardiac channel subtype.

Lidocaine binds strongly to the inactivated state in unmodified cardiac sodium channels (9). It was reported recently that BTX-activated sodium channels exhibit signs of inactivation (12), in contrast with the common supposition that BTX eliminates both fast and slow inactivation. Our experiments support this conclusion. We suggest that the long-lived closed state observed for BTX-activated cardiac sodium channels may be a modified inactivated state, implying that inactivation is altered by BTX, but is not completely eliminated.

MATERIALS AND METHODS

Experimental protocol and data analysis

A detailed description of the experimental protocol is presented in the companion paper (11). Qualitatively similar slow block of bovine cardiac sodium channels was observed for internally or externally applied lidocaine at the same concentrations. For our quantitative analysis of this slow block we restricted the application to the extracellular side.

Slow blocking events were recorded at each voltage in continuous records of up to 10 min. The block was analyzed by creating event lists with pCLAMP V 5.5 (Axon Instruments Inc., Foster City, CA) software. The data were filtered at 100 Hz and sampled at 250 Hz during transcription and usually digitally filtered at 12.5 Hz while creating the event lists. Open times were studied by ignoring closed events shorter than 70 ms in order to eliminate most of the overlying gating and fast blocking events. No events were ignored for studies of the closed times, however, filtering at 12.5 Hz results in the loss of events shorter than 14 ms (13).

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Dwell time distributions were fitted in pCLAMP, all other fits and preparation of figures were conducted using Sigmaplot (Jandel Scientific, Corte Madera, CA). We have used a linear, three-state model to account for our data. This model is described in detail in the Appendix.

RESULTS

Lidocaine causes long duration block of cardiac channels

The records in Fig. 1 show slow block by external lidocaine. In the absence of the drug, both cardiac and skeletal muscle sodium channel isoforms were open most of the time. Addition of lidocaine results in the appearance of long closures for the cardiac channels (Fig. 1 *A*) that only rarely occur for the rat skeletal muscle channels (Fig. 1 *B*). The reduction in apparent single-channel amplitude is caused by the overlying fast block, which does not produce a visible increase in noise here due to heavy filtering (12.5 Hz). From an examination of longer records, we conclude that lidocaine does induce some long blocked events for skeletal muscle channels of both lamb and rat, but that these events occur with a much lower frequency for the skeletal isochannels. In only one of 12 experiments on skeletal channels (a single channel from a 38-day-old lamb) did the frequency of slow block appear to approach that observed for cardiac channels.

Fig. 2 shows the closed time distribution for a cardiac channel in absence (Fig. 2 *A*) and presence (Fig. 2 *B*) of lidocaine. In absence of the drug at this bandwidth the cardiac channels show at least two different closed time constants ($t_{c1} < 40$ ms; t_{c2}(drug free) $\simeq$ 150–600 ms). The presence of the longer closed-time constant is characteristic of the cardiac subtype (1). Slow lidocaine block results in the appearance of an additional population of closed times (time constant, t_{burst}). Simultaneously, the mean duration of the

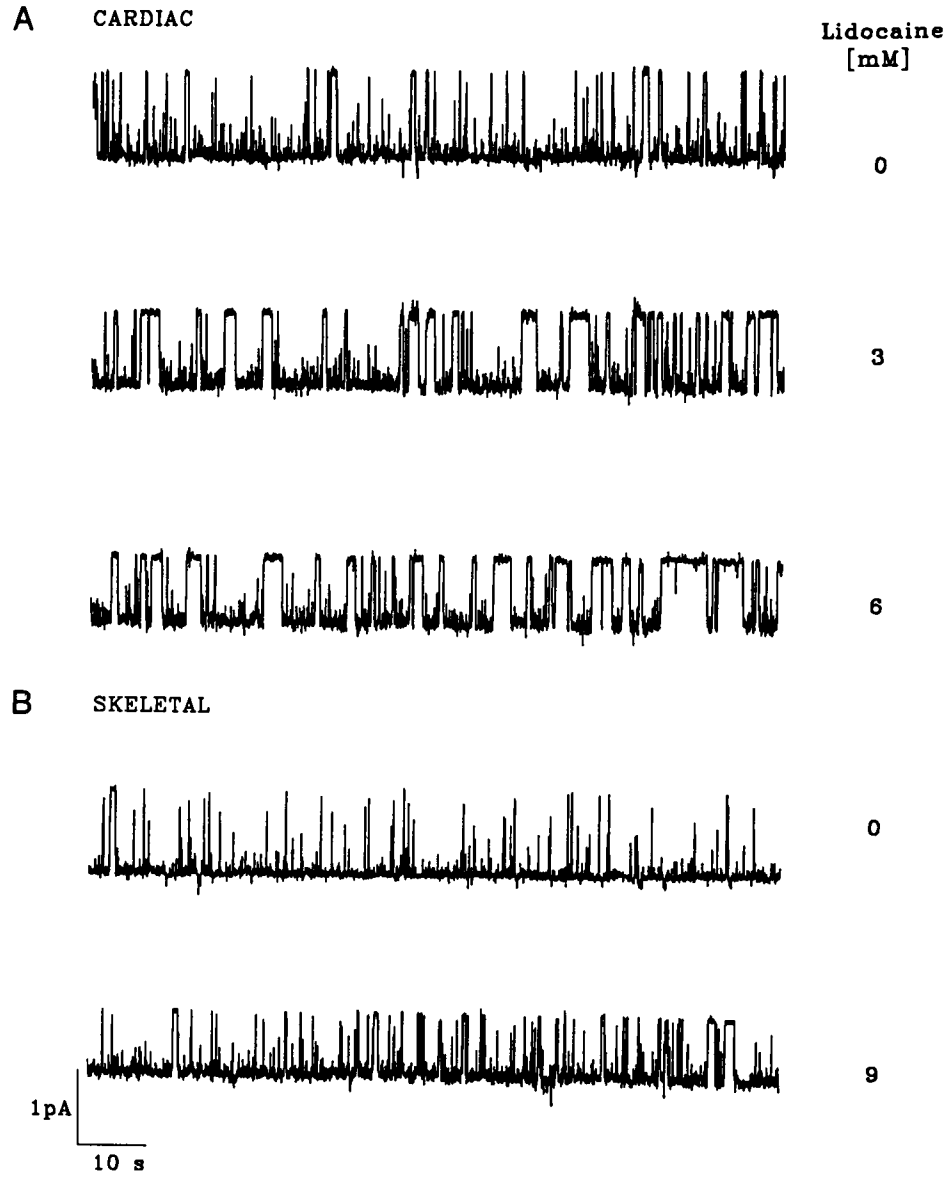


FIGURE 1 Single channel records showing slow block by external lidocaine of batrachotoxin-activated bovine cardiac (*A*) and rat skeletal muscle (*B*) sodium channels. Lidocaine induces slow blocking events for the cardiac subtype and, in this concentration range, hardly affects skeletal muscle channels. The reduction in apparent single channel amplitude at higher lidocaine concentrations is caused by fast block which is not resolved at the chosen filter corner frequency (12.5 Hz). Voltage, −50 mV; upward deflections represent closure or block.

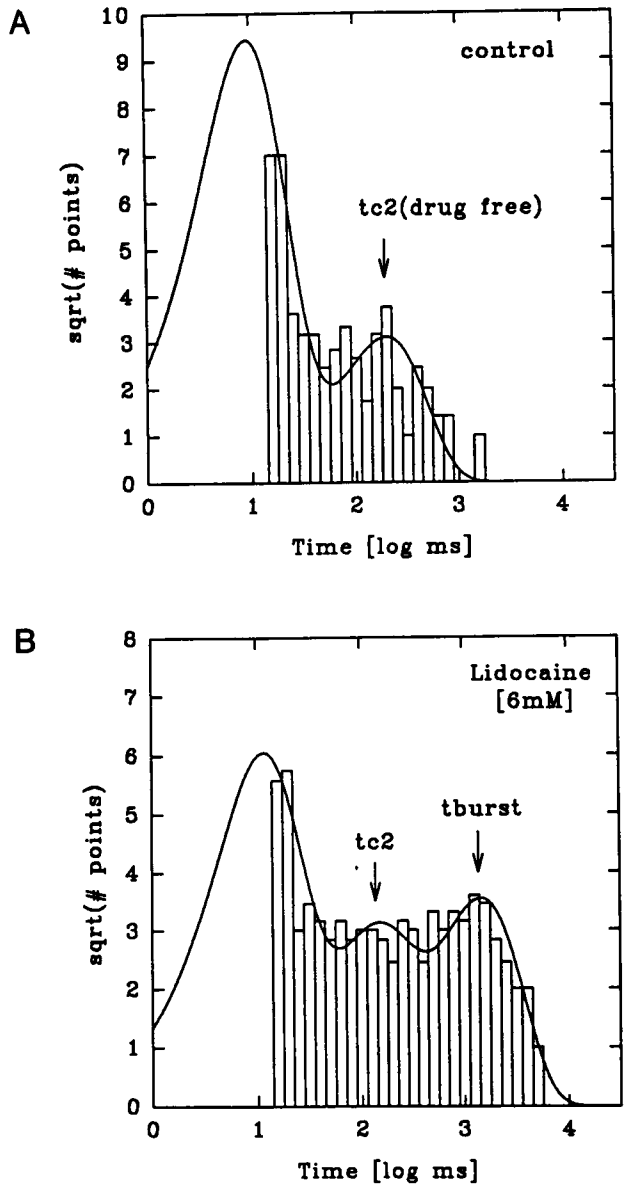


FIGURE 2 Closed time histograms for the bovine cardiac sodium channel, in the form devised by Sigworth and Sine (27), were constructed and fitted as described in the Materials and Methods. The data were fitted in pSTAT, and the fits were recreated in Sigmaplot. (*A*) In the absence of drug, the closed time distribution exhibits at least two distinct time constants for gating closures. The presence of the slower time constant t_{c2}(drug-free) is a characteristic of the cardiac subtype. (*B*) Lidocaine (6 mM) causes the appearance of an additional peak that appears to be due to silent bursts between the long closed and the blocked (lidocaine-bound) state. The time constant, t_{c2}, represents the mean duration for long gating closures which return to the open state without entering into a burst, and it decreases with concentration. With increasing lidocaine concentration, t_{c2} decreases and t_{burst} increases. The time constants (in milliseconds) obtained from the fits were as follows. (*A*) Control: t_{c1}, 9; t_{c2}, 199. (*B*) Lidocaine [6 mM]: t_{c1}, 11; t_{c2}, 111; t_{burst}, 1461.

long gating closures (t_{c2}) appears to be reduced ($t_{c2} < t_{c2}$(drug free), and t_{c2}([L2]) < t_{c2}([L1]) at lidocaine concentrations [L1] < [L2]). Such a concentration-dependent reduction in t_{c2} is to be expected if the presence of the drug were to result in an additional transition from the closed state C2 to a blocked state. This behaviour is described mathematically in the Appendix and is examined in more detail later in the paper (see Fig. 5).

Block depends on channel closure, rather than on voltage

Fig. 3 displays dose-response curves for slow block, by externally applied lidocaine, of a bovine cardiac sodium channel. The reduction in open probability due to slow block can be fitted with a rectangular hyperbola with a Hill coefficient of 1, suggesting a 1:1 interaction between channel and drug. From Fig. 3, we also see that lidocaine shows a higher potency with channels that have a lower open probability (i.e., a higher probability of closure) in the absence of the drug.

In Fig. 4, we examine directly the dependence of lidocaine potency on channel open probability and transmembrane voltage. The concentration at which the channel is blocked half of the time (ED_{50}) correlates with the probability of the channel being open under control conditions as illustrated in Fig. 4 *A* and does not appear to be intrinsically voltage-dependent. The reciprocal of the ED_{50} decreases linearly as the control open probability increases (Fig. 4 *A*), but shows no systematic trend when plotted against voltage (Fig. 4 *B*). This suggests that the drug binds to a nonconducting state of the channel.

As one might intuitively expect under these circumstances, the affinity of the drug for the channel would reach its theoretical maximum when the channel is permanently closed under control conditions ($P_{omax} = 0$) and the receptor

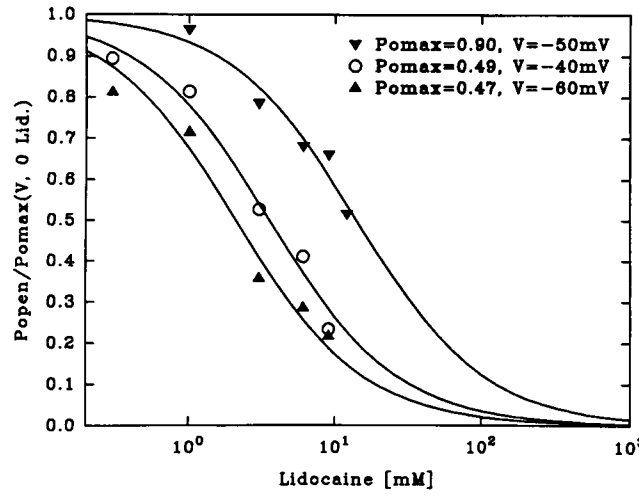
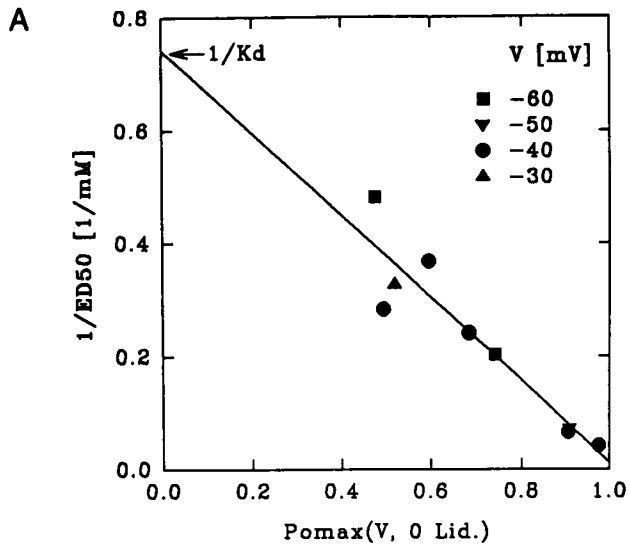


FIGURE 3 Dose-response curves for slow block of three different bovine cardiac sodium channels by externally applied lidocaine. The ratio of the open probabilities in the presence and absence of the drug, at the given membrane potential, is plotted on the ordinate. The open probabilities were determined in pSTAT. The curves were fitted with a Langmuir isotherm, $P_{open}/P_{omax} = 1/(1 + [L]/ED_{50})$. The variables are discussed in the Appendix. Note that the ED_{50} (concentration at $P_{open}/P_{omax} = 0.5$) does not vary systematically with changes in the voltage (see also Fig. 4 *B*).



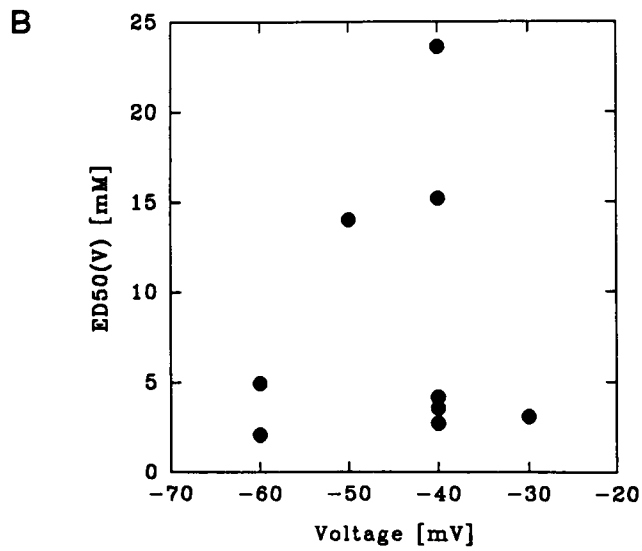
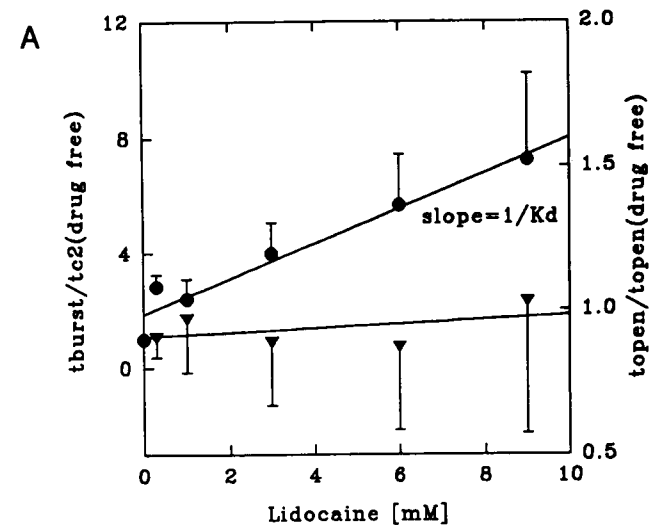


FIGURE 4 (*A*) Slow block of the bovine cardiac sodium channel by externally applied lidocaine is inversely correlated with the open probability, P_{omax}, determined at 0 mM lidocaine. The individual data points represent ED_{50} values determined from fits of dose-response curves like those in Fig. 3, at four different holding potentials. The choice for the ordinate is explained in the Appendix; the intercept represents the inverse of the microscopic equilibrium dissociation constant for binding of lidocaine to the closed state. The solid line is a least squares fit. (*B*) The ED_{50} does not appear to be intrinsically dependent on the membrane potential. The data points are the same as those in Fig. 4 *A*.



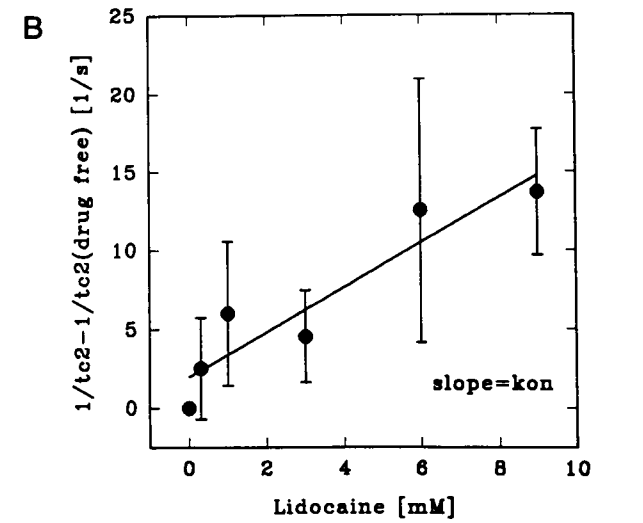


FIGURE 5 (*A*) Concentration dependence of t_{burst} (*circles*) and t_{open} (*triangles*) for slow block of the bovine cardiac sodium channel by external lidocaine. The mean burst time is linearly dependent on lidocaine concentration, the slope of the regression line (r = 0.97, slope = 0.6 ± 0.2 at 95% confidence) represents the inverse of the microscopic equilibrium dissociation constant (see Appendix). The burst times were obtained from fits of dwell time distributions in pSTAT, as in Fig. 2. The mean time between the blocked events is unaffected by lidocaine (*triangular symbols*, r = 0.41, slope = 0.007 ± 0.035 at 95% confidence). Solid lines are least square fits of the data (from six experiments); error bars represent standard deviations. (*B*) The concentration-dependent decrease in t_{c2} can be used to estimate the association rate constant for slow lidocaine block. A rate constant of 1.4/mM-s was determined from the slope of the regression line (r = 0.94, slope = 1.4 ± 0.6 at 95% confidence). Using the K_d from Fig. 5 *A*, the unblocking rate was calculated to be 1.7/s. The choice for the ordinate is explained in the Appendix. Remark: A statistical analysis of the correlation coefficients obtained from the regression lines for t_{burst} and t_{c2} (28) indicates a probability of >99% for a slope different from zero.

is accessible all of the time (see Appendix). Thus, extrapolation of the regression line in Fig. 4 *A* to P_{omax} = 0 provides an estimate of the microscopic dissociation constant (1.3 mM) for lidocaine binding to the closed state. The microscopic equilibrium dissociation constant for slow block can be independently determined from the slope of the regression line in Fig. 5 *A*, and by this method a value of 1.7 mM was obtained.

To further test for possible voltage dependence of slow block, we estimated the apparent K_ds, based on the sequential model described in the Appendix, at voltages between −60 and +60 mV. Data were obtained at both positive and neg-

ative voltages in three of nine experiments analyzed. For negative potentials in the range −60 to −20 mV, the slope of a semilogarithmic plot of K_d against voltage indicated an e-fold change for 165 mV, which, given the scatter in the data, does not differ significantly from zero. This reinforces the suggestion above that block is intrinsically independent of voltage over this range. Block was weaker at positive voltages, indicated by apparent K_d values about threefold greater than those in the negative range. We have recently found that a variety of agents, that cause fast block, inhibit the long gating closures and thus secondarily decrease the probability of slow block (Zamponi et al., manuscript in preparation). For lidocaine, analysis at positive voltages is complicated by the increasing degree of fast block, which precludes the use of concentrations of lidocaine high enough to elicit a sufficient frequency of slow blocked events for detailed analysis.

Closed/blocked durations depend on lidocaine concentration

The duration of the slow blocking events (t_{burst}) increases with increasing lidocaine concentration. In contrast, the apparent open times between the slow events do not appear to be affected by the drug (Fig. 5 *A*). This rules out block of the open channel and confirms the suggestion of drug binding to a closed state of the channel. Block, thus, occurs as a series of transitions (an electrically silent burst) between the closed and the blocked states. Because lidocaine induces a transition from the closed state, the mean time, t_{c2}, spent in this closed state is reduced in the presence of lidocaine. The concentration-dependent decrease in t_{c2} can be used to determine the blocking rate constant for slow lidocaine block (Fig. 5 *B*). The determination relies on the resolution of t_{c2}, and a fit of closed time histograms as in Fig. 2 *B* with three time constants (which was possible for six out of nine experiments; in the remaining three experiments the number of events was insufficient to permit a kinetic analysis). The mean burst times in Fig. 5 *A* were obtained from the same fits. The individual rate constants for association (1.4/mM-s) and dissociation (2.5/s) were determined according to the kinetic analysis described in the Appendix.

Block requires the uncharged form of lidocaine

Fig. 6 demonstrates that degree of slow block from the extracellular side is correlated with the concentration of the uncharged form of lidocaine. Lidocaine-HCl (300 mM, at a pH of 1.7) was added to the extracellular bath to give final concentrations of 0–12 mM. The pH of the bath dropped from 7.0 to 6.5 with increasing total lidocaine concentrations. The pH was measured at each concentration, and the ratio of charged to uncharged form was determined according to the Henderson-Hasselbalch equation. The mean burst duration, which according to our model is linearly dependent on the drug concentration, is correlated with the concentration of the uncharged, but not the charged, form of lidocaine. This,

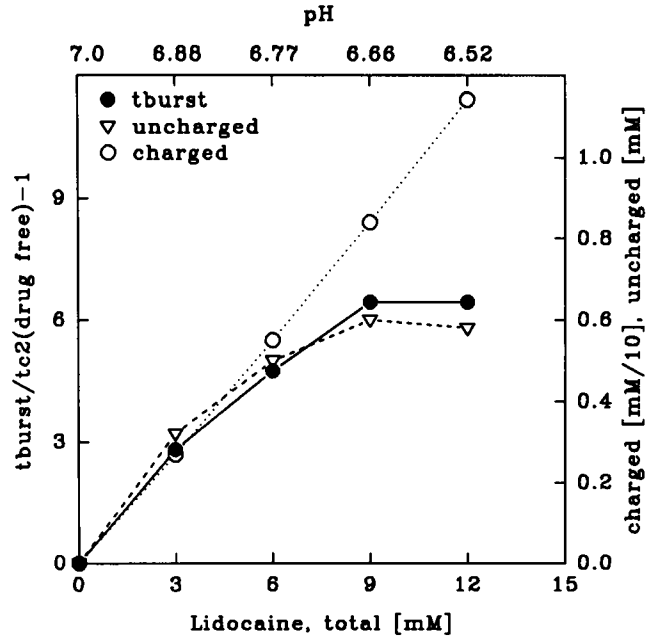


FIGURE 6 Slow block by external lidocaine is correlated with the concentration of the uncharged form. The pH of the bath was allowed to vary from 7.0 to 6.5 by adding a 300 mM lidocaine-HCl stock solution at a pH of 1.7, to give final concentrations ranging from 0 to 12 mM. Thus, the concentration of uncharged drug did not increase linearly with the total drug concentration. The pH of the bath was measured at each concentration, and the ratio of charged to uncharged form of the drug was determined according to the Henderson-Hasselbalch equation. The increase in the blocked burst duration nearly parallels the concentration of the uncharged form, but diverges markedly from the concentration of the charged form.

together with the observation that slow block occurs from either side of the membrane, suggests the involvement of a hydrophobic pathway to the binding site.

Trypsin removes long gating closures and slow lidocaine block

Lidocaine causes a shift in the steady state inactivation curve in non-BTX-activated sodium channels from the node of Ranvier (7) and heart (9). Bean et al. (9) suggested that this shift was caused by lidocaine strongly binding to the inactivated state of the channel. This raised the possibility that the long gating closures observed for the BTX-activated cardiac sodium channels could be a remnant of the inactivated state. To test this hypothesis, we studied the effect of trypsin on gating and slow lidocaine block. Trypsin breaks peptide bonds in proteins whose amino acid sequences contain arginine or lysine residues (14). Addition of trypsin to the intracellular side of unmodified sodium channels results in loss of inactivation, presumably because of removal, or cleavage, of the cytoplasmic loop connecting subunits III and IV which is thought to form the inactivation gate of the channel (15).

Fig. 7 shows traces recorded from a BTX-activated bovine cardiac sodium channel. Under control conditions this particular channel showed unusually frequent long closures and

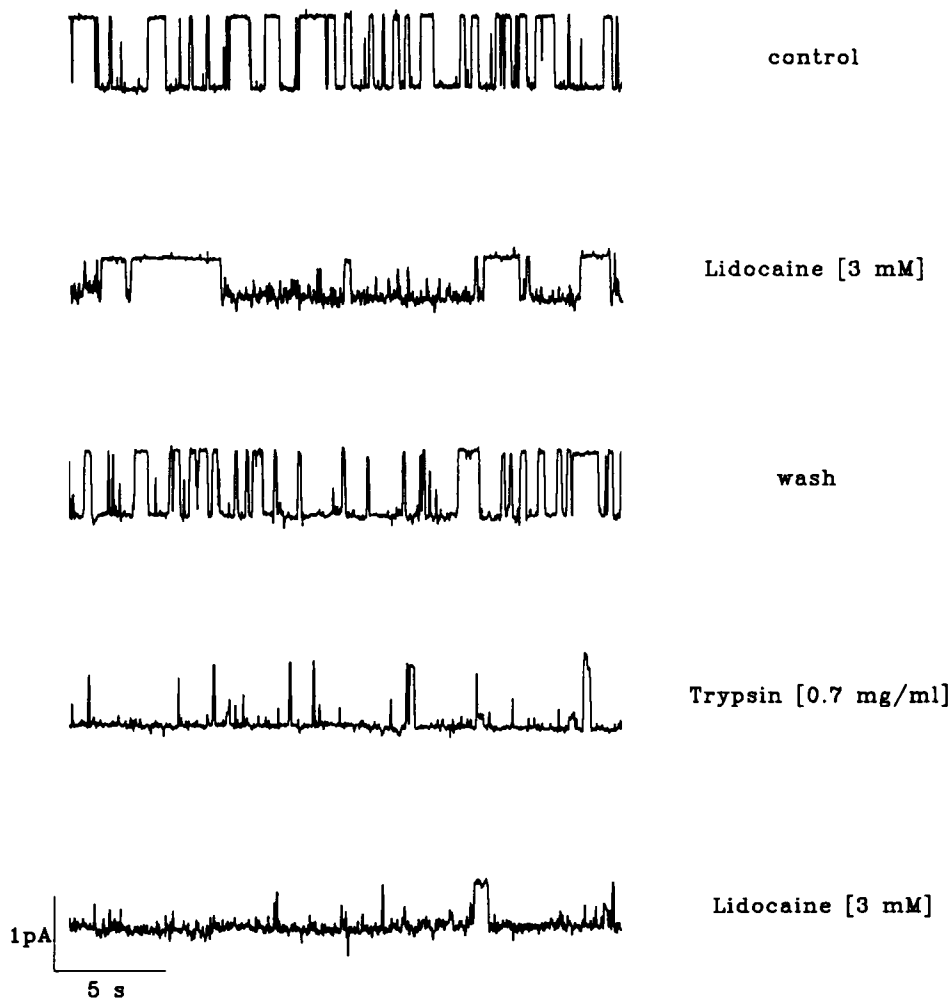


FIGURE 7 Traces recorded from a BTX-activated bovine cardiac sodium channel at a holding potential of −40 mV. The traces were filtered at 12.5 Hz; upward deflections represent closure or block. All the traces were recorded from the same channel and are displayed in the order of the experimental protocol from top to bottom. Under control conditions, this particular channel was open about 50% of the time. Addition of 3 mM lidocaine to the internal side of the channel results in long blocking events. The drug was then washed out by perfusing the chamber with the control solution. Addition of 0.7 mg/ml trypsin (TPCK-treated, from bovine pancreas, Sigma Chemical Co., St. Louis, MO) removed most of the long closures within about 1 min, and subsequent addition of 3 mM lidocaine did not cause slow block. The reduced apparent single channel amplitude in the presence of lidocaine is caused by fast block which is not resolved due to the chosen filter cutoff frequency. Note that fast block was weakly affected by trypsin treatment; mean currents in presence of lidocaine were 0.6 pA before and 0.7 pA after trypsin treatment.

was open only about half of the time. Addition of 3 mM lidocaine to the cytoplasmic side of the channel resulted in pronounced slow block. Note that the slow blocked events for cytoplasmic application of lidocaine are qualitatively similar to the ones observed for external application. The apparent reduction in single channel amplitude is caused by fast block, which is not seen as an increase in noise here due to heavy filtering (12.5 Hz). The drug was subsequently washed out and the control conditions were restored. Addition of trypsin (0.7 mg/ml) to the cytoplasmic side of the channel resulted in a dramatic effect on the gating. The longer closures almost completely disappeared. Subsequent addition of lidocaine did not result in slow block, and fast block was weakly affected by the trypsin treatment (note the reduction in apparent single channel amplitude compared with 0 mM lidocaine before and after trypsin). Because of the lengthy and complex protocol, only relatively few events were collected under each condition in this experiment. Nonetheless, the qualitative result is clear from inspection of the records. Fig. 8 shows closed time distributions for the same experiment. All bins were normalized to the number of events/100 s, and data were sorted into 5 bins/decade. In the absence of lidocaine (Fig. 8 *A*), the channel showed two distinct closed states. When lidocaine was added (Fig. 8 *C*) a peak became defined near 1 s, and, as in Fig. 2, the peak corresponding to the long gating closures shifted to the left and decreased in amplitude. After washout of the lidocaine, and addition of trypsin (Fig. 8 *B*), most of the longer closures (>100 ms) disappeared. Subsequent addition of lidocaine did not lead to slow block, and did not significantly affect the closed time distribution (Fig. 8 *D*). These data suggest that the long gating closures observed for the cardiac subtype might resemble a vestigial inactivation process despite the presence of BTX. Slow lidocaine block thus seems to be caused by lidocaine binding to an "inactivated" state of the channel.

We have observed the effect of trypsin in three additional experiments. In all cases, trypsin treatment resulted in the complete loss of the slow gating component. In two of these experiments, lidocaine was added after trypsin treatment; it did not produce slow block.

Trypsin never eliminated slow block or long gating closures when added to the bath in the presence of lidocaine (four experiments). In one of these experiments, the chamber containing lidocaine and trypsin was perfused to restore control conditions. Subsequent addition of trypsin resulted in the loss of the long gating closures within 1 min after application. Addition of lidocaine to the bath did not result in the ap-

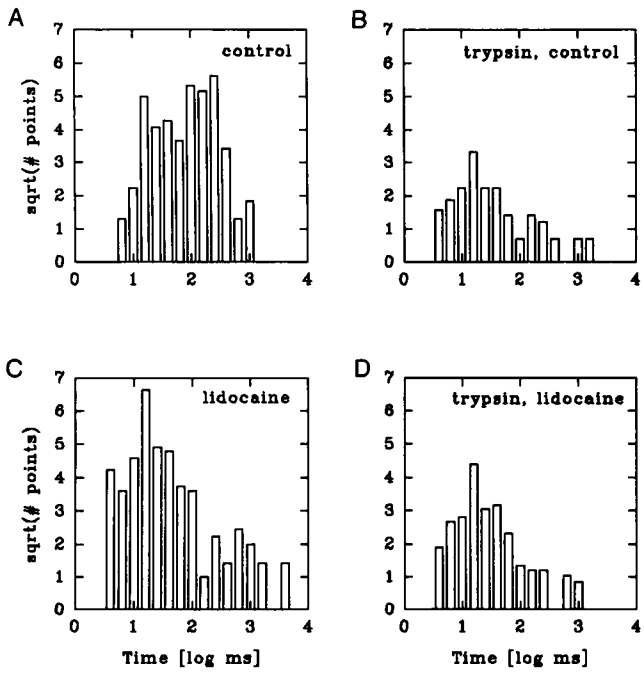


FIGURE 8 Closed time histograms for the experiment described in Fig. 7. The histograms were obtained as described for Fig. 2, except that here 5 bins/decade were used rather than 10 because of the relatively small number of events. The histograms were generated from continuous records between 120 and 280 s; all bins were normalized to events/100 s to facilitate comparison. No fits are shown because of the small number of events. Because of the demanding protocol of solution changes and enzyme treatment it was not possible to take longer recordings in each of the conditions. (*A*) At least two time constants are present under control conditions. (*C*) As described for Fig. 2, lidocaine induces the appearance of additional long events and the longer peak seen in the control moves toward shorter durations. (*B*) Trypsin (0.7 mg/ml, applied in absence of lidocaine) removes most of the longer gating events. (*D*) Addition of lidocaine to the trypsin-treated channel does not significantly affect the closed time distribution. No additional long closures are induced.

pearance of slow blocking events. The observation that trypsin does not remove long gating closures, when lidocaine is present in the bath, suggests that lidocaine might shield the site of proteolysis from trypsin. In five further experiments, the channel ceased to function shortly after addition of trypsin.

DISCUSSION

Slow block is tissue-specific

For bovine heart channels (14 experiments) slow block was clearly identifiable at concentrations as low as 300 μM. For skeletal muscle channels (12 experiments), long lidocaine-induced closures occurred much less frequently and, in general, were apparent only at higher concentrations (>10 mM). The number of events for the skeletal muscle experiments did not justify a quantitative analysis, but in four experiments with each tissue, skeletal muscle channels from lamb seemed to show a somewhat higher susceptibility to slow block than did those from rat. The mechanism of slow block may well be similar for skeletal and cardiac channels, but the events are sufficiently more probable in cardiac channels to be considered a tissue-specific characteristic.

The appearance of slow blocked events in the presence of lidocaine was correlated with the occurrence of long gating closures in the absence of the drug: bovine cardiac sodium channels, in common with those from sheep and dog (1), show a distinct population of voltage-insensitive, long gating closures with mean durations >100 ms. Such long closures occur less frequently for skeletal muscle channels. This provides a clue that differences in gating behavior underly the tissue specificity of slow lidocaine block.

Slow block is closed-state dependent

In order to simplify our analysis of slow block, we reduced the reaction scheme to a linear, three-state model as follows.

$$O \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} C2 \underset{\delta}{\overset{[L]\gamma}{\rightleftharpoons}} Bs \qquad (1)$$

C1, O, and Bf are combined, in our analysis, as a single "open" state, whose duration is independent of lidocaine concentration (Fig. 5 *A*, and see Appendix).

Although slow blocked events become progressively more obvious with increasing lidocaine concentration (Fig. 1), the mean time between slow blocked events is *independent* of lidocaine concentration (Fig. 5 *A*). This excludes the possibility that open-state binding of lidocaine causes slow block. The likelihood of block is inversely related to P_{open} observed in the absence of the drug (Figs. 3 and 4 *A*), confirming that closed-state lidocaine binding is the blocking mechanism. Thus, lidocaine induces a silent burst between the closed and the blocked states. The burst duration (i.e., the mean time, t_{burst}, in the nonconducting state) increases with drug concentration as verified in Fig. 5 *A* (also, see Appendix).

Is slow block caused by binding to an inactivated state?

It has been implied by Wang and Wang (12) that some of the closures (e.g., the longer ones) observed for BTX-activated sodium channnels might be due to inactivation, while others reflect transitions to a resting state. Several proteolytic enzymes, internally applied, have been shown to remove fast inactivation of sodium channels. In particular, trypsin and α-chymotrypsin have been shown to abolish inactivation in unmodified sodium channels of cardiac myocytes (16). We were able to selectively eliminate the long gating closures seen for the BTX-activated bovine cardiac sodium channels by the application of trypsin to the intracellular end of cardiac channels. This is consistent with the idea that these long gating closures might be a remnant of the inactivated state seen in unmodified channels. The observation that trypsin-treated channels do not exhibit slow block supports our kinetic model and suggests that slow block might be caused by

lidocaine binding to the inactivated state of the channel. This would be consistent with reports by Cahalan (17) and Yeh (18) who have demonstrated that the use dependence of local anesthetic block is abolished upon pronase treatment of the channels.

Two independent mechanisms for block of the open and the inactivated states of unmodified cardiac sodium channels have previously been suggested by Matsubara et al. (19) and Clarkson et al. (20). Strong lidocaine binding to the inactivated state has also been proposed by Bean et al. (9). BTX-activated cardiac sodium channels appear to show some residual inactivation in spite of the presence of BTX. BTX-activated rat skeletal muscle sodium channels may inactivate less frequently, or may spend less time in the inactivated state, resulting in a lower susceptibility to slow block.

In five experiments the channels were lost after addition of trypsin. This is consistent with observations by Clarkson (16) who reported a concentration-dependent reduction of the peak amplitude when cardiac myocytes were internally perfused with a solution containing trypsin. He also reported that α-chymotrypsin caused effects on the activation kinetics of the channels. Hence, in interpreting our data one must be aware of a variety of possible trypsin sites on the channel protein. When lidocaine was present in the bath, addition of trypsin had no effect. This suggests that there is a lidocaine binding site close enough to the site of trypsin attack to inhibit proteolysis. This "protection" of the channel from proteolysis deserves further study. A more detailed investigation of trypsin modification of BTX-activated cardiac sodium channels is underway.

Are slow and fast block due to separate sites?

A simple interpetation of the results in Figs. 7 and 8 is that slow and fast block by lidocaine are mediated via different receptors. Trypsin treatment abolishes slow block while causing only a barely noticeable reduction in fast block. It has been previously shown (17, 18) that protease treatment, which removed fast inactivation, not only abolished use dependence of local anesthetic block, but also abolished the voltage dependence of QX-314 block. These multiple effects of proteolysis may result directly from the destruction of the inactivation gate or its receptor, but more complex interpretations are also possible.

Because QX-314 did not cause slow block, we wanted to test whether QX-314 showed any competitive effects on the slow blocking behavior. QX-314, unlike lidocaine, appears not to be able to reach its binding site via a hydrophobic pathway. Hence, QX-314 would probably have to inhibit slow lidocaine block by entering the open channel. QX-314 might then compete for a binding site, which lidocaine reached via a hydrophobic route. In three attempted experiments, channels disappeared from intact bilayers shortly after co-application of lidocaine and QX-314. Hence, we were unable to determine directly whether QX-314 inhibited slow block by lidocaine. This result suggests that lidocaine and QX-314, when co-applied, may interact to promote BTX dissociation from the channel. This would be consistent with the existence of at least two sites at which these closely related drugs interact with the channel.

Finally, preliminary experiments (not shown) with a variety of lidocaine-related compounds suggest that different parts of the lidocaine molecule may be responsible for slow and fast block. Overall, we favor the view that separate sites of interaction, either in close proximity or allosterically linked, are responsible for fast and slow block.

Two components of local anesthetic/antiarrhythmic block are seen in other preparations

Two components for lidocaine block of unmodified cardiac channels have been reported recently by Starmer et al. (21) and Makielski et al. (22). Lowering the pH, and thus the amount of uncharged drug, decreased the slower component. When QX-314 was substituted for lidocaine, only the slower component was present. Alpert et al. (23) have suggested the presence of a low affinity binding site, which is directly accessible from the extracellular side of the channel, and which is capable of binding QX-314 (24). In our study, slow block by lidocaine developed in the same concentration range as internal fast block. We have tested the action of externally applied QX-314 on BTX-modified channels and observed no effect. This suggests that the uncharged form of lidocaine is responsible for slow block. This is supported by the observation that the mean burst times are correlated with the concentration of the uncharged, rather than the charged, form of the drug and that slow block is seen when the drug is applied from either side of the membrane. Thus, our data are consistent with the drug acting on a site located within a hydrophobic part of the channel. Hence, the site responsible for slow block must be distinct from the one proposed by Alpert et al. (23) which suggests that there may be as many as three independent binding sites for lidocaine.

Two modes of block have also been demonstrated for the hydrophobic drugs benzocaine and procaine on BTX-activated rat skeletal muscle sodium channels (8, 25). Procaine, in millimolar concentrations on either side of the membrane, induced a fast block which was favored by depolarization, and a slow block which was enhanced by hyperpolarizing voltages. Moczydlowski et al. (8, 25) suggested that the voltage dependence of slow block could arise from voltage dependence of gating, i.e., that the closed state required for drug binding could be favored by hyperpolarization. Slow block by procaine did not affect open times suggesting that procaine and lidocaine may act via similar mechanisms, but with different affinities. We observed little slow block of rat skeletal muscle channels at millimolar lidocaine concentrations, whereas procaine was more active in this concentration range.

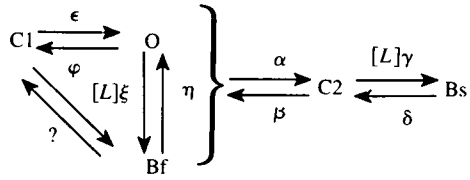
CONCLUSIONS

Lidocaine induces two modes of block of BTX-activated sodium channels: fast block of the open state, and slow block

of a closed state that, in general, is prominent only for cardiac channels. We conclude that slow block involves the uncharged form of the drug gaining access via a hydrophobic pathway to a binding site which can be reached only when the channel is closed. The lidocaine-susceptible closed state is removed by trypsin, suggesting that it is an "inactivated" state that persists after BTX modification of the channel.

APPENDIX: KINETIC ANALYSIS OF SLOW BLOCK

We considered the following model for the action of lidocaine on BTX-activated sodium channels.



(2)

At the bandwidth used to analyze the slow block (12.5 Hz), bovine cardiac sodium channels show at least two closed states. In Fig. 2 *A*, we identify C1, which is short-lived (<40 ms), and C2, a longer-lived closed state (150–600 ms).

Fast block occurs at least from the open state, and slow block occurs when lidocaine binds to the long closed state, C2. The mean duration of the interval between the long "closures" is not affected by the drug. This suggests that long gating closures, and hence, slow lidocaine block, are not influenced by drug binding to the receptor responsible for fast block, and that transitions to C2 can occur from the open and the fast blocked states. We were unable to improve the fit of our data using a linear model with a transition to C1 inserted between the open state and C2.

Given that our analysis was performed at low bandwidth, and for a voltage range at which the channels were mostly open in the absence of the drug, we could not rigorously test the connectivity among the three states C1, Bf, and O depicted above. If transitions from the fast blocked state, Bf, to the short-lived closed state, C1, were not possible at all, the presence of the drug would reduce the frequency of fast gating events. At the K_d for fast block, this reduction would be roughly by a factor 2. We were unable to resolve a change in the frequency of fast gating events in the presence of lidocaine because of the overlying fast block, and hence we do not know whether C1 can be reached from Bf.

Our data are consistent with a model by which transitions to C2 can occur, with equal rate constants, from the open state, the fast blocked state, and possibly the closed state, C1. Hence, the kinetic states in the left portion of the scheme do not influence transitions to C2. This suggests that long closures occur via a mechanism independent of the channel's voltage-dependent gating.

In order to quantify the blocking behavior we consider a simplified model in which the fast blocked (Bf), short closed (C1), and open states are lumped together into one kinetic state, to be called the "open" state in the remainder of the Appendix. Strictly, this is the collection of all states other than the long closed (C2) and slow blocked (Bs) states. Thus, we describe slow block by a linear model (see Equation 1).

The probability of reaching the blocked state varies with the probability of the channel being in the closed state, C2. Thus, the concentration required to block the channel half of the time (ED_{50}) depends on the probability of the channel being closed under drug-free conditions. Under the assumption that the rates α and β are not influenced by the presence of the drug, the model of equation (1) yields a steady state open probability which follows the relation

$$P_{open} = 1/[1 + (\alpha/\beta) + (\alpha\gamma[L]/\beta\delta)]. \quad (3)$$

δ/γ is the microscopic equilibrium dissociation constant, K_d, for binding of the drug to the closed state of the channel and $[L]$ is the drug concentration. This relationship holds only in a range where the voltage-dependent closures (closed states other than the "inactivated" state which is vulnerable to slow block) do not significantly contribute to the closed probability.

The open probability, P_{omax}, in absence of the drug is

$$P_{omax} = \beta/(\alpha + \beta) \quad (4)$$

and thus

$$P_{open}/P_{omax} = 1/[1 + (1 - P_{omax})\gamma[L]/\delta]. \quad (5)$$

This relation has the appearance of a Langmuir isotherm with a correction factor $(1 - P_{omax})$ which reflects the availability of the binding site. This equation provides the basis for fitting the dose-response curves displayed in Fig. 3. The fitting parameter ED_{50} can be expressed as

$$ED_{50} = K_d/(1 - P_{omax}). \quad (6)$$

This is the basis of the plot in Fig. 4 *A*. The other implication of the linear model is that the block of the closed state will occur as a silent burst between the closed and the blocked state. The burst duration is dependent on the drug concentration and is given by the following equation (see Ref. 25)

$$t_{burst} = (n + 1)t_{c2} + (n)t_b \quad (7)$$

where t_{c2} and t_b are the times spent in the closed and blocked states and t_{burst} is the duration of the burst which appears as a long nonconducting event in the single channel trace. The number of events within the burst (n), t_{c2}, and t_b can be expressed as

$$n = \gamma[L]/\beta \quad (8)$$

$$t_{c2} = 1/(\beta+\gamma[L]) \quad (9)$$

$$t_b = 1/\delta \quad (10)$$

and thus

$$t_{burst} = (1 + \gamma[L]/\delta)/\beta. \quad (11)$$

Since t_{c2}(drug free) equals $1/\beta$,

$$t_{burst}/t_{c2}(\text{drug-free}) = 1 + \gamma[L]/\delta \quad (12)$$

which is plotted in Fig. 5 *A* and demonstrates a linear concentration dependence of the burst duration with a slope equivalent to $1/K_d$.

The concentration-dependence of t_{c2} yields information about the blocking rate γ:

$$(1/t_{c2}) - \beta = \gamma[L]. \quad (13)$$

Plotting the above equation delivers a straight line with an intercept at zero and a slope equivalent to the blocking rate constant (Fig. 5 *B*). This requires the ability to resolve t_{c2} which was possible for six out of nine experiments. We can, thus, provide a reasonable estimate for the blocking rate constant.

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