A method for estimation of drug affinity constants to the open conformational state of calcium channels

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ABSTRACT The affinity of D600 to calcium channels in the open state has been examined in isolated smooth muscle cells of the rabbit ear artery. Calcium channel currents were measured in high external barium solution by means of the patch-clamp technique. The current inhibition in various D600 concentrations (3–100 μM) on application of trains of short test pulses (20–80 ms) has been studied in nonmodified calcium channels and in cells where the calcium channels were modified by the agonist dihydropyridine (+) 202 791 (100 nM). The kinetics of the peak current decay has been analyzed with a mathematical model which is based on the experimental finding that D600 interacts primarily with calcium channels in the open conformational state. The model approach allows the estimation of drug affinity constants of D600 to the calcium channel in the open conformation. An association rate constant to the open conformational state of D600 of $6.16 \cdot 10^4$ $M^{-1}s^{-1}$ was estimated. The association rate of the drug was not significantly changed after the calcium channels have been modified with 100 nM (+) 202 791. A method for correction of rate constants for possible drug trapping is discussed.

INTRODUCTION

The inhibition of calcium channels in smooth muscle cells by phenylalkylamines is crucially dependent on the frequency of membrane depolarization (Klöckner and Isenberg, 1986; Hering et al., 1989). In this respect, the mechanism of action of the phenylalkylamines appears to be similar in all excitable cells which have been studied (Ehara and Kaufmann, 1978; Kohlhardt and Haap, 1981; Lee and Tsien, 1983, McDonald et al., 1984; Oyama et al., 1987). In a recent study in smooth muscle cells of the rabbit ear artery, we have shown that the amount of calcium channel block produced by D600 is determined by the channel open time rather than by the change in membrane voltage or channel inactivation (Hering et al., 1989). It was found that drug induced a very similar amount of block on application of a single long pulse or a series of short depolarizing pulses if both pulse protocols produced a similar amount of channel open time (see Fig. 4 in Hering et al., 1989). Furthermore, membrane depolarization to the threshold voltage of calcium channel activation did not induce channel block despite inducing channel inactivation. High affinity binding of D600 to the calcium channels in the open conformation has also been demonstrated in "concentration-jump" experiments when the drug was applied rapidly during a voltage step (Oyama et al., 1987; Hering et al., 1989).

In the present study, we further examine the mechanism of calcium channel block by D600 in arterial smooth muscle cells and propose a method which allows quantification of the open channel block by drugs which have a high affinity for this channel state.

Additional studies were conducted to gain information about possible allosteric regulation of the dihydropyridine and the phenylalkylamine binding site on the calcium channel (see Glossmann and Striessnig, 1989). Therefore, the action of D600 was examined before and after the channels have been modified with the agonist dihydropyridine (+) 202 791.

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METHODS

Single smooth muscle cells were freshly dispersed from rabbit ear artery. Short segments (1–2 mm) of artery were incubated for 50 min in a modified physiological salt solution containing 10 μM calcium, 2 mg/ml bovine serum albumin, 1 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ), 0.5 mg/ml papain (Sigma Chemical Co., St. Louis, MO) and 5 mM dithiothreitol (Sigma), respectively. Cells were dispersed after mild agitation in low-calcium physiological salt solution. After centrifugation (1,000 g for 2 min), the cells were resuspended in normal physiological salt solution, stored on cover slips at 4°C and used within 6 to 9 h. The experiments were performed using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) by means of an EPC-7 (List Electronic, Darmstadt, Germany) patch-clamp amplifier. Patch pipettes had resistances of 3–5 MΩ. The internal (pipette) solution contained (mM): NaCl 126, $MgSO_4$ 3.2, NaH_2PO_4 1, EGTA 2, glucose 11.5, ATP 0.5, TEA 10, and Hepes 5 buffered to pH 7.2 with NaOH. The experiments were carried out in high barium external solution containing (mM): $BaCl_2$ 110 and Hepes 10 buffered to pH 7.4 with TEA-OH. Data was recorded on FM tape and later analyzed off-line using pCLAMP software (Axon Instruments, Inc., Burlingame, CA) on an IBM AT computer with a Labmaster interface. D600 (Knoll AG Ludwigshafen) was applied by bath perfusion. Data are given as mean values ± SD.

RESULTS

Formulation of the model

We have applied the idea of a modulated ionic channel receptor as suggested by Hille (1977) and Hondeghem and Katzung (1977) to the description of the interaction of drug molecules with calcium channels. The mathematical approach to the problem was quite similar to that

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used by Starmer and Grant (1985). In short, the population of unblocked calcium channels can be divided in two:

$$U = U_a + U_p,$$

where U_a is the fraction of channels accessible for the drug, and U_p is the fraction of channels protected from interaction with the drug. In these notations the balance equations are:

$$\frac{dB}{dt} = K_1 \cdot [D] \cdot U_a - K_{-1} \cdot B;$$

$$U_a + U_p + B = 1;$$

where $[D]$ is the drug concentration, B is the fraction of channels which are blocked by the drug, K_1 is the rate constant of drug binding to the channels in the open conformation, and K_{-1} is the rate constant of dissociation of the drug-channel complex.

We have now to introduce some physical assumptions on the mechanisms of drug binding and unbinding. The measured calcium currents, I_{Ca}, reflects the fraction of open channels, thus,

$$U_a = g_{Ca}(t) \cdot U = g_{Ca}(t) \cdot (1 - B),$$

where $g_{Ca}(t)$ is the normalized membrane conductance. Unfortunately, our knowledge about drug trapping and drug dissociation from ionic channels is not sufficient for a detailed quantitative description of this processes, therefore we only assume that the dissociation of drug-channel complexes is proportional to

$$K_{-1} \cdot \beta(t) \cdot B,$$

where $\beta(t)$ is the fraction of drug-channel complexes which are able to dissociate (when the drug molecule is not trapped in the channel).

Finally we obtain (see also Starmer and Grant, 1985):

$$\frac{dB}{dt} = \alpha \cdot g_{Ca}(t) \cdot (1 - B) - K_{-1} \cdot \beta(t) \cdot B, \qquad (1)$$

where $\alpha = K_1 \cdot [D]$.

We have to emphasize that this equation is only an approximation. It is assumed that the interaction of the drug with the channels does not change the kinetics of the current during a pulse train. As we will show later, this assumption is justified under our experimental conditions. The inhibition of calcium channels by D600 was studied during trains of test pulses, Eq. 1 was, therefore, solved for each current in a train of a given number of test pulses under the following assumptions: (a) the binding of D600 to open calcium channels is slow compared to the kinetics of the calcium current, i.e., the shape of current during a train of short test pulses remains unchanged; (b) the tonic block component at a negative membrane holding potential is small compared to the pronounced use-dependent inhibition of the channels (Hering et al., 1989); and (c) in a first approximation, the dissociation rate constant was assumed to be constant ($\beta = 1$). The solution for each period in the train is

$$B_N = \sigma(T) \cdot \{\Phi(T) + B_{N-1}\}, \qquad (2)$$

where B_{N-1} and B_N are fractions of blocked channels in the $(N-1)$th and Nth pulse in the train:

$$\sigma(T) = \exp\left\{-\int_0^T [\alpha \cdot g(t) + K_{-1}]\, dt\right\} \qquad (3)$$

$$\Phi(T) = \alpha \int_0^T g(t) \cdot \exp\left\{\int_0^t [\alpha \cdot g(\tau) + K_{-1}]\, d\tau\right\} dt. \qquad (4)$$

The set of recurrent Eq. (2) can be solved (Starmer and Grant, 1985):

$$B_N = \sigma^N(T) \cdot (B_0 - B_\infty) + B_\infty, \qquad (5)$$

where

$$B_\infty = \frac{\sigma(T) \cdot \Phi(T)}{1 - \sigma(T)}, \qquad (6)$$

with B_∞ as the steady level of the current blockade in train and B_0 as the initial inhibition of the ionic current. The initial inhibition of the inward currents by D600 is negligible compared to the use dependent inhibition during a pulse train (Hering et al., 1989), thus, $B_0 = 0$ and

$$B_N = B_\infty \cdot [1 - \sigma^N(T)]. \qquad (5a)$$

The kinetics of peak current inhibition by D600 during repeated depolarization of the cell membrane of a single smooth muscle cell can be fitted by a single exponential function (see Fig. 4 in Hering et al., 1989). From Eq. 5a, we obtain the parameters of block σ and B_∞. Using the expression (Eq. 6) for B_∞, it is easy to calculate:

$$\Phi(T) = \frac{B_\infty \cdot [1 - \sigma(T)]}{\sigma(T)}. \qquad (7)$$

Eqs. 3 and 4 link the rate constants α and K_{-1} with the parameters of blockade $\sigma(T)$ and $\Phi(T)$. From Eq. 3,

$$\ln\{\sigma(T)\} = -\alpha \cdot \int_0^T g(t)\, dt - K_{-1} \cdot T,$$

taking into account that $g(t) = 0$ when $t > t_{imp}$ (where t_{imp} is the test pulse duration).

$$\ln\{\sigma(T)\} = -K_{-1} \cdot T - \alpha \cdot \int_0^{t_{imp}} g(t)\, dt \qquad (8)$$

Eq. 4 can be reduced to a simple form under the assumptions that (a) $g(t) = 0$ at a negative holding potential, and (b) that the rate constant of dissociation K_{-1} is signif-

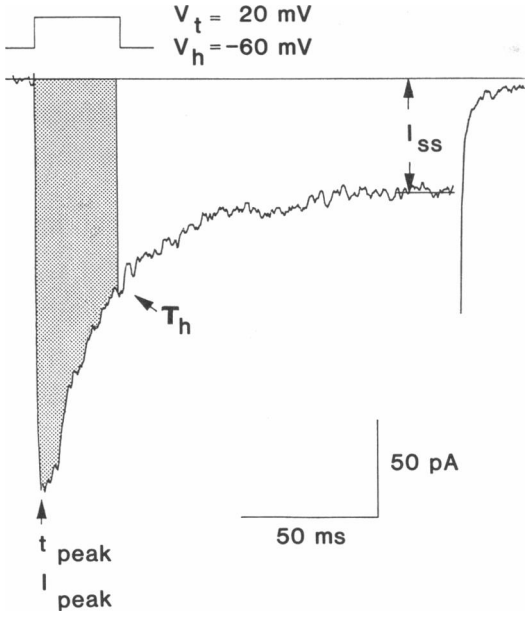

FIGURE 1 Barium inward current recorded from a rabbit ear artery cell in 110 mM extracellular $BaCl_2$ in the presence of 100 nM (+) 202 791. The membrane potential was stepped from a holding potential of −60 to +20 mV. The figure illustrates the procedure of calculating the integral of the current. The activation was approximated as linear current increase to the peak current value I_{peak} at the time t_{peak}. Inactivation kinetics of I_{Ba} during short test pulses were described as an exponential decay with a time constant τ_h to a noninactivating current component I_{ss}.

icantly smaller than the rate constant of association $\alpha \cdot g(t)$ during a depolarizing test pulse:

$$\Phi(T) = \int_0^{t_{imp}} \alpha \cdot g(t) \cdot \left[\exp \int_0^t \alpha \cdot g(\tau)\, d\tau \right] dt$$

$$= \left\{ \exp \int_0^{t_{imp}} \alpha \cdot g(t)\, dt \right\} - 1.$$

This leads immediately to:

$$\alpha = \frac{\ln [1 + \Phi(T)]}{\int_0^{t_{imp}} g(t)\, dt}, \qquad (9)$$

and

$$K_{-1} = -\ln \{\sigma(T) \cdot [1 + \Phi(T)]\}/T \qquad (10)$$

The exponential decay in the peak current amplitudes of calcium channel current in the presence of D600 during a train of test pulses is described by two parameters: B_∞ which determines the steady level of blockade, and σ determining the slope of the decrease in peak current in a train. The estimation of both parameters allowed us to calculate the rate constant of association and apparent rate constant of dissociation of D600 with calcium channels in the open state.

In short, (*a*) to integrate $g(t)$ we approximated the activation kinetics of I_{Ba} as a linear current increase to a peak current value I_{peak} at the time t_{peak}. The current inactivation during short test pulses was described as monoexponential current decay to a noninactivating current level I_{ss} (see schematic representation in Fig. 1). Alternatively, the inward current integral over a given pulse length can be calculated by means of any available software for whole cell current analysis (e.g., pCLAMP CLAMPAN subroutine, Axon Instruments Inc., Burlingame, CA); (*b*) the kinetics of the peak current decay during a pulse train was fitted to a single exponential function Eq. 5a, to estimate the values of σ and B_∞; (*c*) Φ was calculated using Eq. 7; and (*d*) finally we can estimate α and K_{-1} from Eqs. 9 and 10.

Correction for rapid open channel block and changed current kinetics after modification of the calcium channels with (+) 202 791

The chosen short test pulse length in the present study of either 20, 40, or 80 ms (see Figs. 3 *A*, and 4 *A*) minimizes possible errors in the calculation of the open channel time from the current integral. However, to avoid an underestimation of channel open time in the presence of high drug concentrations (100 μM) D600 as a result of a rapid open channel block a single exponential function was fitted to the decay of I_{Ba} at +20 mV under control conditions and in the presence of 100 μM D600 (Fig. 2; see also Hering et al., 1989, Fig. 7). The mean time constants of current decay at a test potential of +20 mV $\tau_{cont.}$ = 163 ± 7 ms (n = 4) and τ_{D600} = 118 ± 8 ms (n = 4) were used for an estimation of the integral of the ionic current in the presence of 100 μM D600. The drug induced changes in inactivation on application of lower drug concentration were too small to affect the estima-

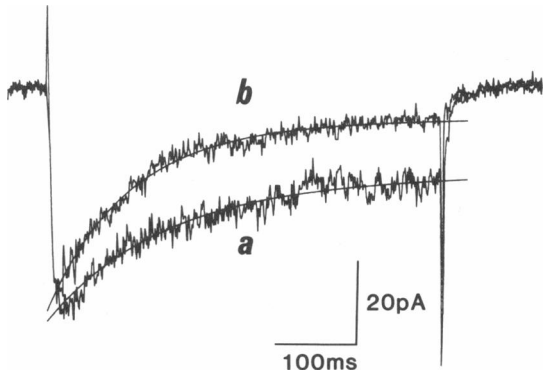

FIGURE 2 Acceleration of I_{Ba} decay in the presence of 100 μM D600. The currents were recorded on depolarizing step to +20 mV for 500 ms from a holding potential of −60 mV before (*a*) and after a 4 min incubation of the cell in 100 μM D600. The decay of both currents were fitted by single exponential functions (continuous curves) with time constants of (*a*) $\tau_{control}$ = 153 ms and (*b*) τ_{D600} = 112 ms.

tion of the channel open time during short depolarizing test pulses.

In a second series of experiments, the action of D600 on the calcium channel current was examined after the channels had been modified by application of the calcium channel agonist (+) 202 791 (100 nM). Agonist dihydropyridines accelerate the current activation and induce a faster decay of the calcium channel current in rabbit ear artery cells (compare current kinetics in Figs. 1 and 2 *a*). The corresponding values for the inactivation time constants after application of 100 nM (+) 202 791 and in the presence of the agonist and 100 μM D600 were $\tau_{(+)\,202\,791} = 54 \pm 6$ ($n = 3$) and $\tau_{(+)\,202\,791/D600} = 51 \pm 9$ ms ($n = 5$), respectively. These (+) 202 791 induced changes in the inactivation kinetics as well as estimated changes in the time to peak in agonist modified I_{Ba} with $t_{peak\,(+)\,292\,791} = 7 \pm 4$ ($n = 4$) compared to $t_{peak\,cont.} = 12 \pm 3$ ($n = 6$) have been taken in to consideration in calculations of the integral of the I_{Ba} during a test pulse train.

A small tonic inhibition of the calcium channel current, which was usually less than 10%, was observed in same cells after a 4 min incubation period with 100 μM D600 (see Fig. 2). The tonic inhibition of the calcium channel current may be the result of an open channel block of calcium channels in the presence of high drug concentrations at −60 mV, where the open probability of the channels is low but is not zero. An interaction with the channels in the closed available conformation is an alternative explanation.

Estimation of association and dissociation rate constants of D600 with open calcium channels

Fig. 3 *A* shows barium currents of a rabbit ear artery cell during a train of 25 test pulses. The 40-ms test pulses were applied at a frequency of 1 Hz in the presence of 50 μM D600, Fig. 3 *B* displays the corresponding peak current values as a function of time. The peak currents were inhibited with a time constant of 6.1 s. The apparent association rate constant $K_1 \cdot [D600] = 2.9\ s^{-1}$ and the corresponding dissociation rate constant $K_{-1} = 0.046\ s^{-1}$ were estimated from the kinetics of peak current decay during the pulse train according to the procedure described above. After a 4 min rest recovery period, 100 nM of (+) 202 791 was added to the bath and 1 Hz trains of 40 or 80 ms pulses were applied to the cell (Fig. 4). As shown in Fig. 4 *B*, the currents decayed with a time constants of $\tau_{block} = 9.55$ s on application of the train of 40 ms pulses compared to a time constant $\tau_{block} = 7.6$ s and on application of the 80-ms pulses. The prolongation of the test pulses from 40 to 80 ms led to a more profound steady level of block. The estimated drug affinity constants in the presence of the calcium agonist were in this cell somewhat lower than the values estimated in the same cell before the calcium channels have been modified with (+) 202 791. We found, however, no significant differences in the mean values of the apparent association and dissociation rate constants in nonmodified and (+) 202 791 modified calcium channels (see Table 1). The action of D600 was studied in a concentration range from 3 to 100 μM in the presence and absence of the agonist dihydropyridine (+) 202 791. To estimate the drug affinity constants for the open channel, we applied test pulses of different length (20–80 ms) at frequencies from 0.8 to 2.4 Hz. Association and dissociation rate constants $K_1 \cdot [D600]$ and K_{-1} were estimated from experiments with the different pulse protocols in the presence of different drug concentrations (see Table 1).

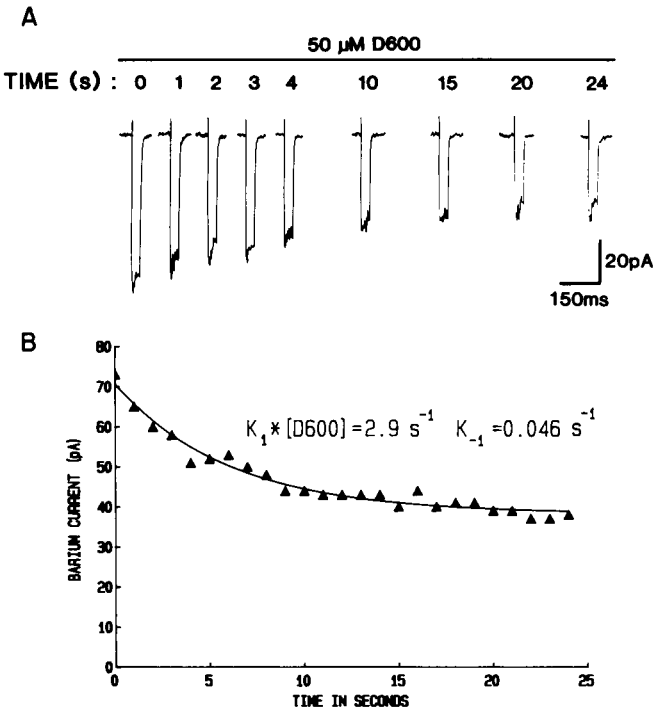

FIGURE 3 (*A*) Barium inward currents during a 1 Hz train of 20 ms test pulses. The cell was incubated with 50 μM D600 at rest for 3 min. (*B*) Time course of I_{Ba} block during the pulse train. The onset of the block was fitted by a single exponential function (*solid line*) with a time constant $\tau_{block} = 6.1$ s. The indicated association and dissociation rate constants were estimated as described in the text.

Fig. 5 shows the development of the I_{Ba}-block in 100 μM D600 on application of 0.8, 1.4, and 2.4 Hz-trains of 20 ms test pulses when 100 nM (+) 202 791 were present in the bath. In the absence of D600 the peak current value of I_{Ba} did not decline during a 2.4 Hz-train of 20 ms test pulses. An increase in the rate of stimulation from 0.8 Hz to 2.4 Hz led to a more rapid decay in the peak current values but at the same time to an increased dissociation rate of the drug from the channel receptor (Fig. 5). As shown in Table 1, an increase in the apparent dissociation rate constant K_{-1} with higher test pulse frequencies was evident in the majority of experiments in the absence as well as in the presence of the agonist

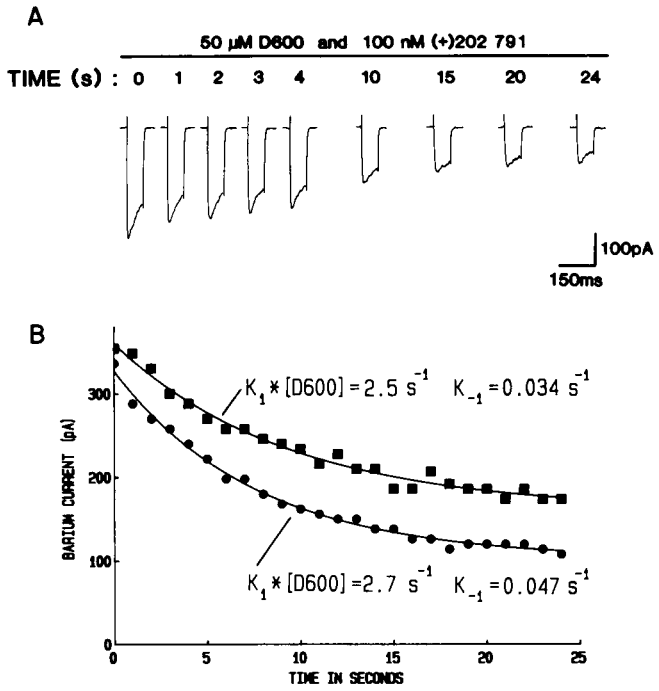

FIGURE 4 (*A*) Barium inward currents during a 1 Hz pulse train of 80 ms test pulses in the presence of 50 μM D600 and 100 μM (+) 202 791. (*B*) Time course of I_{Ba} inhibition during 1 Hz pulse trains of 40 ms (■, τ_{block} = 9.5 s) and 80 ms (●, τ_{block} = 7.6 s) test pulses (same cell as in Fig. 3).

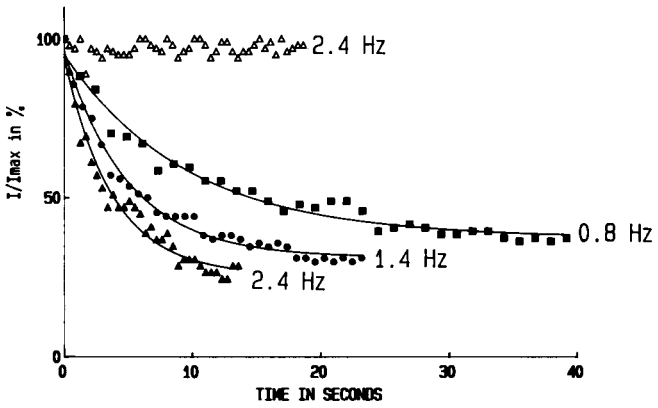

FIGURE 5 Inhibition of I_{Ba} by 100 μM D600 in the presence of 100 nM (+) 202 791 during 20 ms pulse trains applied at (■) 0.8, (●) 1.4 or (▲) 2.4 Hz. No decrease in peak amplitudes was observed during a 2.4-Hz train in the presence of 100 nM (+) 202 791 (△) in the absence of D600. Rest periods of 4 min were allowed between each pulse train where almost complete recovery of peak current was observed. The peak current decayed with time constants of (■) 8.3 s, (●) 4.3 s and (▲) 3.7 s. The estimated values for $K_1 \cdot [D600]$ and K_{-1} were (■) 5.63 s^{-1} and 0,043 s^{-1}; (●) 6.96 and 0.069 s^{-1} and (▲) 6.29 s^{-1} and 0.08 s^{-1} respectively. After a 5 min wash out period, recovery was observed without current decay on repeated stimulation.

dihydropyridine. The estimation of the time course of the recovery of drug bound channels from block is difficult, because any test pulse during the recovery period will induce additional channel block. Thus, the recovery of the current (unblock of the channels after a given time interval) could be measured only ones after maximal inhibition of the calcium channel current during a pulse train. The estimated value of $\tau_{recovery}$ = 63 ± 9 s (n = 4) for an recovery from block after a train of 20 ms test pulses in 30 μM D600 is in good agreement with the value of 4 min for complete recovery from block from Hering et al., 1989.

The apparent association rate constant $K_1 \cdot [D600]$ of D600 can be plotted as a function of the applied drug concentration (Fig. 6). If we fitted a linear regression line to the calculated mean values of the association rate

TABLE 1

D600	Test pulse Length	Frequency	$K_1 \cdot [D600]$	K_{-1}	n
μM	ms	Hz	s^{-1}	s^{-1}	
3	40	1.0	0.2 ± 0.08	0.05 ± 0.03	3
30	20	0.8	2.9 ± 0.8	0.07 ± 0.04	4
30	20	2.4	2.1 ± 0.4	0.09 ± 0.02	3
30	20	0.8	1.7 ± 0.3	0.04 ± 0.02	7*
30	20	1.4	1.7 ± 0.4	0.08 ± 0.06	6*
30	20	2.4	2.3 ± 0.6	0.12 ± 0.05	5*
50	40	1.0	2.9	0.046	1
50	40	1.0	2.5	0.034	1*
50	80	1.0	2.7	0.047	1*
100	20	0.8	5.8 ± 0.4	0.06 ± 0.04	4
100	20	2.4	6.1 ± 0.8	0.06 ± 0.03	4
100	20	0.8	5.6 ± 0.6	0.04 ± 0.02	3*
100	20	1.4	6.9 ± 0.3	0.07 ± 0.04	3*
100	20	2.4	6.2 ± 0.9	0.08 ± 0.07	3*

* Experiments in the presence of 100 nM (+) 202 791; data are mean values ±SD.

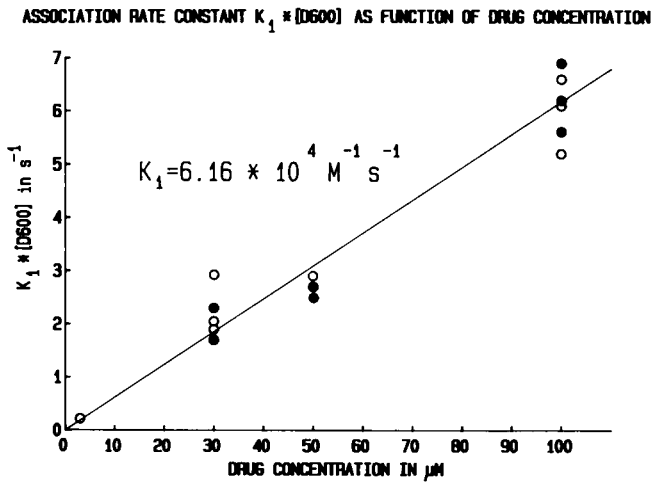

FIGURE 6 Apparent rate constant $K_1 \cdot [D600]$ as function of the applied drug concentration. Open symbols are mean values of apparent association rate constants in the absence of the agonist dihydropyridine where the filled symbols are the corresponding mean values estimated when the channels have been modified with 100 nM (+) 202 791; slope of the regression line is K_1 = 6.16 10^4 $M^{-1}s^{-1}$.

constants of $K_1 \cdot [D600]$ we obtain for the association rate constant K_1 a value of $6.16 \cdot 10^4\ M^{-1}\ s^{-1}$.

DISCUSSION

Interaction of D600 with calcium channels in the open conformational state

The main goal of the present study was to develop and to test a method for estimation of affinity constants for drugs which bind predominantly to the open conformation of calcium channels.

A high affinity of D600 and other phenylalkylamines to the calcium channels in the open conformational state has been found in various cell types (Ehara and Kaufmann, 1978; Lee and Tsien, 1983; McDonald et al., 1984; Oyama et al., 1987; Klöckner and Isenberg, 1985; Hering et al., 1989). In vascular smooth muscle the affinity of D600 to the closed available state is low as documented by the almost compleat recovery of the channels from block at negative membrane potentials (Hering et al., 1989; results of this study).

Comparisons with other approaches

Similar methods for estimation of drug binding rate constants have been developed by Starmer and Grant (1985) and Chernoff (1990). In Starmer and Grant (1985), the authors pointed out that as a first approximation the time course of the open channel fraction during test pulses were replaced by some "mean" quantity. A more complicated formulation by Chernoff (1990) was in fact reduced to the same simplification.

In distinction to the previous approaches, our model and subsequently, the technique for the rate constants estimation account for a more realistic quantification of the open channel fraction.

Strength of the parameter estimation method

The estimated association rate constants from our model which assumes "pure" open channel block by D600 did not differ significantly for different test pulse length as one would expect in the case of a high affinity binding to the inactivated channel state.

Changes in drug binding conditions might also be expected from allosteric regulation between the phenylalcylamine and the dihydropyridine binding site of the calcium channel (Glossmann and Striessnig, 1988). However, the modification of the calcium channels by the agonist dihydropyridine (+) 202 791 did not change the apparent association rate constants of D600. Agonist dihydropyridines are known to increase calcium channel currents by a prolongation of the sojourn of the channel in the open conformational state (Hess et al., 1984). Neither prolongation of pulse length nor modulation of channel open time affected significantly the estimated apparent association rate constant which is an indication for open channel block and the robustness of the parameter estimation.

Limitations of the method

As stated above the rate constant estimation technique is valid only under conditions where the current shape is not significantly affected by drug binding during the test pulse. In other words, the drug binding must be a rate-limiting stage. This is a crucial point which must be clarified. In the frame of the commonly accepted kinetic scheme (Courtney, 1975) every "microscopic" act of channel-drug binding will result not only in an escape of channels from the unbound channel population (see Eq. 1) but also in a redistribution of channels between different states (close, open, inactivated) within both drug-bound and drug-unbound populations. A mathematically correct formulation would have to account for all those transient processes which is, however, in practice not necessary.

APPENDIX

Correction of rate constants for drug trapping

When the experimental data were analyzed (see results in Fig. 5) the rate constant of dissociation appeared to be changing regularly: the more the ratio of pulse length to period of stimulation the more the rate constant of dissociation. This suggested that D600 can dissociate faster from the channel receptor when the channels are in the open (but not conducting) drug-bound conformation. The most likely explanation for an increase in the apparent rate of dissociation on stimulation is a trapping of drug molecules in channel.

The increase in the dissociation rate of D600 on increase in test pulse frequency was not very pronounced (see Table 1). However, taking into account that the proposed method may be also applicable for other channel ligands with high affinity for the open state, a possibility for correction of rate constants for drug trapping has been analyzed.

If the dissociation of drug-channel complexes is faster under depolarizing potential when the drug-channel complex is entering the "open" (but not conducting) conformation the rate of dissociation is assumed to be equal to:

$$[l + b \cdot g(t)] \cdot B,$$

where l is the apparent rate constant of dissociation of "trapped" drug during the interpulse period; and b is the rate constant of dissociation of drug from "open" conformation of channel during pulse. The substitution of the dissociation rate in Eq. 1 by the last expression does not change the model and algorithm of data processing in principle but only leads to new expressions for σ and Φ:

$$\sigma(T) = \exp\left\{-\int_0^T [(\alpha + b) \cdot g(t) + l]\, dt\right\} \qquad (3a)$$

$$\Phi(T) = \alpha \cdot \int_0^T g(t) \cdot \exp\left\{\int_0^t [(\alpha + b) \cdot g(\tau) + l]\, d\tau\right\} dt. \qquad (4a)$$

Thus, we have to calculate three rate constants using only two parameters of the blockade in train. This can be done if different experimental protocols, i.e., different frequencies and pulse length are used for an estimation of the dissociation rate constant (see also Chernoff, 1990). The calculated values for K_{-1} at different pulse frequencies (estimated in Table 1) are higher than the value of $K_{-1} = 0.015\ s^{-1}$ calculated from the time constant of recovery, $\tau_{recovery}$, from a steady-state block (after a pulse train) with a two pulse protocol τ. The second method practically excludes drug trapping. Under this condition (or on application of short test pulses at a very low frequency) it is easy to show that

$$l = -\ln(\sigma_{short})/T.$$

The rate constant of association α is calculated as described previously (see Eq. 9). The rate constant of dissociation of untrapped drug molecules can then be calculated as:

$$b = -\left\{\frac{\ln(\sigma_{long}/\sigma_{short})}{\int_0^{t_{imp}} g(t)\,dt} + \alpha\right\}.$$

E. N. Timin and S. Hering wish to thank Professor T. B. Bolton for his continued support and encouragement of our work.

This work was done during a visit by E. N. Timin at the Department of Pharmacology at St. George's Hospital made possible by a fellowship from the Royal Society of London. S. Hering was a Wellcome Research Fellow at the same laboratory. The experimental work was supported by the Medical Research Council.

Received for publication 1 March 1991 and in final form 9 March 1992.

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