SIDEDNESS OF RECONSTITUTED CALCIUM CHANNELS FROM MUSCLE TRANSVERSE TUBULES AS DETERMINED BY D600 AND D890 BLOCKADE

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ABSTRACT The verapamil-type calcium antagonist, D600, and its charged quaternary derivative, D890, were used to assess the sidedness of blockade in single calcium channels reconstituted from purified transverse tubules of skeletal muscle. Spontaneous single channel openings were induced with the agonist Bay-K8644 and recordings were made in a two-chamber planar bilayer setup so that drugs could be delivered to either side of the channel. Micromolar drug addition resulted in a >10-fold decrease in probability of open channel events (p_o) without a significant change in single channel currents. Changes in p_o occurred in parallel with changes in mean open time and both parameters could be titrated with a similar IC_{50}. At pH 7.2, *cis* or *trans* D600 blocked with an IC_{50} of 5 μM but for D890 the IC_{50} was *cis* 3 μM and *trans* >75 μM (*cis* is the intracellular-equivalent side as defined by the voltage-dependent activation). The asymmetry of D890 blockade indicates that the drug can readily gain access to the blocking site from the aqueous phase adjacent to the inner but not extracellular end of the channel.

INTRODUCTION

One class of calcium channels, usually referred to as the dihydropyridine sensitive channels, is at present the target of an intense physiological, biochemical, and clinical research (1, 2). Nevertheless, little is known about their biochemical properties and their regulation, e.g., by neurotransmitter and hormones (3, 4). Attempts to isolate and purify calcium channel-related proteins have made use of their dihydropyridine binding characteristics. In transverse tubules (t-tubules) of skeletal muscle, the nitrendipine binding protein forms part of a large >200,000 MW complex which remains associated with binding activity for a variety of antagonists (5, 6). Furthermore, a pool of calcium channels from purified t-tubules has been shown to remain functional after reconstitution of native t-tubules into planar phospholipid bilayers (7, 8). Calcium channels were identified as such based on (*a*) a sensitivity to dihydropyridine agonists and antagonists; (*b*) a selectivity for divalent cations, with the exclusion of Mg^{++}; and (*c*) a steady-state voltage-dependent kinetics. In bilayers, the reconstituted t-tubular calcium channel shows a definite orientation that can be verified by its voltage dependence of activation (7). In many other experimental situations, however, a voltage dependence cannot be easily determined, e.g., when studying ion fluxes through channels incorporated in vesicles. In these cases, the asymmetric functions of channels must be deduced by other means. If available, specific toxins, such as the recently described atrotoxin for the calcium channel (9), are the most suitable tools. Another potentially useful group of calcium blockers is the quaternary derivatives of verapamil, D890 (10), D575 (11). D890 is the membrane impermeable *N*-methyl derivative of D600 (gallopramil), and has been described to block calcium channels only when injected into the cells (10) by a mechanism similar to the action of local anesthetics (12).

In this paper we present the blocking effects of D890 and D600 on reconstituted single calcium channels from t-tubules of rat skeletal muscle. In agreement with the results of Hescheler et al. (10) in whole heart cells, we find that D600 reduces single calcium channel activity when added to either side of the channel. However, D890 blocks only when added to the *cis* side, the side where the cytoplasmic end of the channel is located. Thus, sidedness of reconstituted channels is completely preserved upon insertion of t-tubule vesicles into planar bilayers. This sidedness is most probably rendered by the preferential fusion to the bilayer of native vesicles that have pinched-off from the transverse tubules with an inside-out orientation.

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MATERIALS AND METHODS

Divalent salts were of analytical grade (JMC, Herts, England). D890 was a kind gift of W. Trautwein (Universitaet des Saarlandes, Homburg Saar, Federal Republic of Germany). T-tubules from rat back and leg muscles were fractionated and purified according to Rosemblatt et al. (13) with the modifications introduced by Moczydlowski et al. (14). Experiments were carried out as described previously (7, 8). Briefly, Mueller-Rudin bilayers (15) were formed from a 20 mg/ml lipid solution in decane having equimolar concentrations of brain phosphatidylethanolamine and phosphatidylserine (Avanti, Birmingham, AL) in symmetrical *cis-trans* solutions containing 50 mM NaCl, 0.1 mM EGTA, 10 mM Hepes-Tris, pH 7.2. Afterwards, fusion of t-tubule vesicles was induced by raising *cis* $BaCl_2$ to 100 mM and adding 100 μg of vesicular protein to the *cis* side. Holding potential (HP, 0 mV in all experiments) was injected into the cup side (*cis* side) and the bath side (*trans* side) was maintained at virtual ground. Records, filtered at 0.1 kHz (−3dB point from 8 pole Bessel filter) were acquired online on an IBM-XT at a sampling rate of 0.3 kHz. Duration of open events was determined using two threshold detectors placed between baseline and open peak current. The first discriminator was placed at 1 SD from the mean baseline current and the second at 1 SD from the mean single channel unitary current. Open events are defined as transitions that cross both discriminators and remain above the open discriminator for two or more consecutive sampled points. To stabilize the channel activity for the prolonged time essential for the measurements, all experiments were performed in the presence of 3 μM Bay-K8644 (7). Control records were sampled for 3–5 min, collecting an average of 423 open events (but never <220 events). After, drug addition recordings were continued for at least 5 min. Due to the variant number of incorporated single calcium channels per bilayer (from one to three open channels recorded simultaneously) the fraction of open time in control experiments varied from 4.5% to 21%. Accordingly, records in the presence of drugs are always compared with the corresponding controls.

RESULTS AND DISCUSSION

Fig. 1 shows traces of barium current through Bay-K8644 activated single calcium channels at HP = 0 mV after fusion of t-tubule vesicles in *cis* 0.1 M $BaCl_2$, 0.05 M NaCl and *trans* (ground) 0.05 M NaCl. To eliminate t-tubule Ca^{2+}-dependent K^+ channels (16), measurements are done in barium instead of calcium as current carrier. Under the chosen recording conditions, $E(Ba^{2+}) << -200$ mV (nominally infinite). $E(Na^+) = 0$ mV, and $E(Cl^-) = +40$ mV. Thus, Ba^{2+}-selective calcium channels can be easily identified at HP = 0 mV as positive-going currents (shown as upward deflections), which revert at HP's more negative than −50 mV. In addition, Bay-K8644 agonist induces open events that are characteristically clustered in bursts lasting 1–5 s. Fig. 1 shows control records (*A*, *B*) and the activity that follows after 10 μM D890 added to the *cis* side only (*C*, *D*). Several findings are evident. First, the frequency of open events per unit time is markedly decreased while the remaining events are much shorter than in the control record. Second, after drug addition, channels have a lesser tendency to form bursts; and third, the probability of opening per single channel, albeit unknown, is apparently decreased given that after drug addition there is no incidence of two (or more) simultaneously open channels. When D890 was added on the *trans* side, however, little if any changes were detected at concentrations as high as 50 μM.

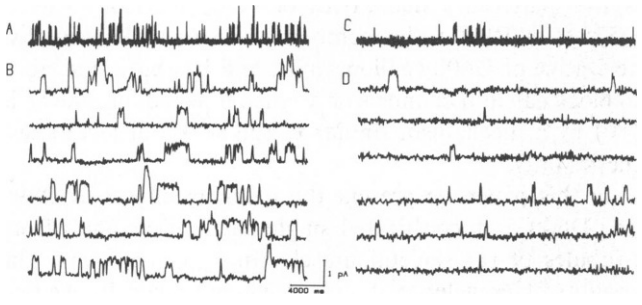

FIGURE 1 Barium current through single calcium channels recorded in absence and presence of D890 on the *cis* side. (*A*, *B*) Control records at HP = 0 mV in the presence of 3 μM Bay-K8644; bilayer formed in symmetrical *cis-trans* solutions containing 50 mM NaCl, 0.1 mM EGTA, 10 mM Hepes-Tris, pH 7.2. Afterwards, *cis* $BaCl_2$ was raised to 100 mM and 100 μg of vesicular protein added to the *cis* side. (*C*, *D*) Records after addition of *cis* 10 μM D890. Drug was delivered from a 10-mM stock solution in ethanol. Records in (*A*) and (*C*) have been compressed sevenfold.

A quantitative measure of *cis* and *trans* blockade is shown in the peak current histograms of Fig. 2(*A–D*). Histograms were constructed by sorting all the sampled current points into bins, each of a width of 0.011 pA. Histograms like these, taken for sufficiently long periods to capture a significant number of events ($n > 200$, see Methods), served to estimate the stationary fraction of time that channels spent open. In each histogram, the left peak (marked with a cross) corresponds to the incidence of the baseline current. This peak has been truncated to show the small but measurable unitary current peaks due to one or two (or more) open channels. Figs. 2 *A* and *C* correspond to control activity before 10 μM D890 added *cis* or *trans*, respectively. The open current peak (*right* peak in Fig. 2 *A*) is practically eliminated by *cis* 10 μM D890 (Fig. 2 *B*) but histograms are unaltered when drug was added on the *trans* side (Fig. 2 *D*). Fig. 2 (*E–G*) shows amplitude distributions of the unitary channel current obtained after open events are separated from the baseline current using the dual threshold criteria (see Methods). Distributions remain centered around the same mean as control amplitudes, despite the fact that at 5 μM D890 the fraction of open time has decreased by 50% (Fig. 4). Thus, drug blockade does not modify divalent ion conduction.

Fig. 3 shows the various kinetic modifications induced by D890. Upper trace (Fig. 3, left panel) shows lifetime distributions of open events in the absence of antagonist. The time constants of the two fitted exponential distributions (7) decreased with increasing *cis* D890 concentrations. Concommitant with the shorter lifetime the silent periods were prolonged (Fig. 3, right panel), as demonstrated by the increased time constants of the fitted exponentials. The observation that D890 reduces the open channel lifetime, but at the same time makes bursts infrequent, tends to rule out a simple flickering-block of

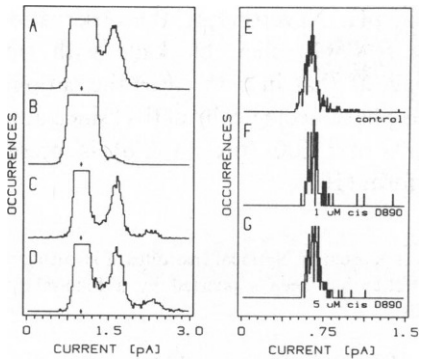

FIGURE 2 Current histograms and amplitude distributions in absence and presence of D890. (*A*, *B*) Peak current histograms from control records (*A*, 667 events) and after *cis* addition of 10 μM D890 (*B*, 115 events). The *Y*-axis indicates the number of sampled data points and current amplitude is indicated on the *X*-axis. The baseline current peak (left peak) has been truncated to expand the unitary open current peak (right peak); after drug delivery, the area under the unitary current peak decreased from 8.7% in (*A*) to 0.8% in (*B*). (*C*, *D*) Current histogram from control experiment (*C*, 233 events) and after *trans* addition of 15 μM D890 (*D*, 338 events). The small peak at the right of the open current peak corresponds to the current flowing through two simultaneously open channels. The sum of the areas under one or two open channels is 21.2% in control and 23.1% after *trans* drug delivery. (*E*, *F*, *G*) Amplitude distributions of single channels after separation of unitary currents and baseline current using a dual threshold criteria. Events due to the simultaneous opening of more than one channel were discarded. From top to bottom distributions correspond to control (*E*, 667 events); 1 μM *cis* D890 (*F*, 396 events) and 5 μM *cis* D890 (*G*, 170 events).

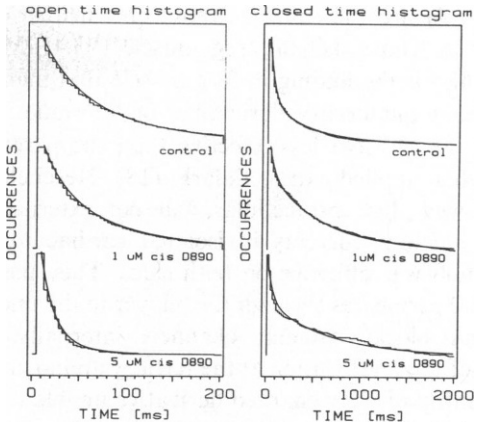

FIGURE 3 Lifetime distributions of open events (*left panel*) and closed periods (*right panel*) in absence and presence of *cis* D890. Histograms correspond to cummulative distributions of events (labeled occurrences) lasting a time, *t*, or longer than *t* shown on the *X*-axis. The concentration of *cis* D890 was none (*upper* traces, 667 open events), 1 μM (*middle* traces, 396 open events), and 5 μM (*bottom* traces, 170 open events). The distributions can be fitted by two exponentials (by minimizing the sum of squared deviations) with the time constants t_{short} = 35 ms, 25.9 ms and 13.7 ms, and t_{long} = 94 ms, 84.7 ms, and 35.5 ms for control, *cis* 1 μM and *cis* 5 μM D890, respectively. The fitted time constants of the closed periods were t_{short} = 49.0 ms, 52.5 ms, and 62.5 ms, and t_{long} = 435 ms, 563 ms, and 649.5 ms, in the presence of no antagonist (*upper* trace), *cis* 1 μM D890 (*middle* trace) and *cis* 5 μM D890 (*bottom* trace), respectively.

open channels as the underlying mechanism. As described above, both the fast and slow components of the open distribution are quickened, those of the closed periods are lengthened, in a concentration-dependent manner. Thus, we argue that these drugs act not only on open channels, as indicated by the reduction of the mean lifetime, but also on closed channels, by markedly prolonging the silent periods. These two effects are fully compatible with observation of D600 blockade in whole cardiac cells (17). For example, the observed long blocked times would make the recovery from blockade a slow process, thus inhibition would tend to accumulate during repetitive activation of channels. In vivo, this has been referred to as frequency-dependent block (17). At the same time, the shorter lifetime of channels would decrease the relaxation time of the inward calcium currents, as observed in D600 blockade during long depolarizations (17). Fig. 4 summarizes the dose-response curves for the effects of D890 and D600 on either side of the calcium channel. D600 was equally active in reducing the fraction of open time on *cis* and *trans* side with an IC_{50} (concentration inducing half maximal inhibition) of 5 μM. Similarly, this drug reduced the mean open time on both sides with an IC_{50} of ~5 μM (data not shown). In contrast, D890 blocked single calcium channels with markedly different potency, depending on which side this agent was added. The IC_{50} on the *cis* side was 3 μM, while on the *trans* side it exceeded 75 μM for both, diminishing the fraction of open time as well as reducing mean open time.

There are several points of agreement between the results shown here in bilayers and calcium channels in vivo. The IC_{50} of D600 blockade in the reconstituted channel (5

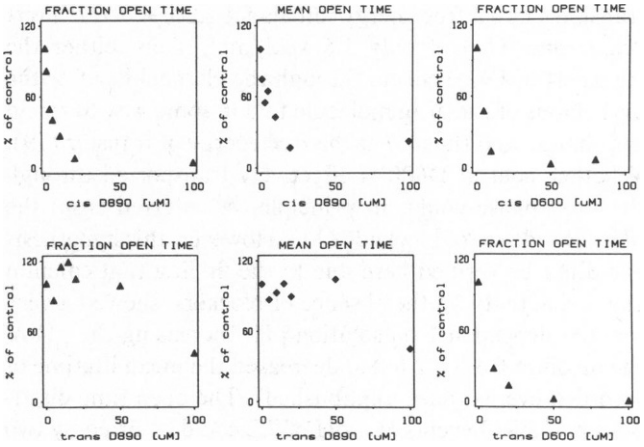

FIGURE 4 Dose response curves of D890 and D600 on *cis* and *trans* side. Each point represents the mean of two to four independent experiments; each experiment consisted of a control record lasting at least 3 min (with an average of 423 open events), and a second record taken afterwards for at least 5 min in the presence of drug. Data is presented as % open time or % mean open time relative to control period. The mean open time is the arithmetic mean of the open event durations during a record. In *cis* D890 experiments, the number of events taken during the control period were 1,225, 799, 1,268, 1,569, 731, and 1,095 at drug concentrations of 1, 3, 5, 10, 20, and 100 μM, respectively.

μM, Fig. 4) are the same as those obtained in calcium currents in whole skeletal frog muscle (13.4 μM) (18). Similar also is the finding in frog muscle that membrane-impermeant quaternary derivatives of lidocaine, such as QX-314, are 30-fold less effective than the parent compound when applied extracellularly (18). Hescheler et al. (10) showed that intracellular, but not external D890, blocked calcium currents in isolated cardiac myocytes, while D600 was effective on both sides. They postulated that D600 permeates through the bilayer in the uncharged form and blocks calcium channels internally. D890, because of the methylation at the tertiary amino group is a permanent, positively charged derivative unable to diffuse through the lipid phase. Thus, D890 is inactive when present on the extracellular solution (10). We find that IC_{50}s for *cis* and *trans* D890 blockade, measured independently on both sides, differ by 25-fold while those for D600 are the same regardless of the side of addition. Qualitatively, these results confirm that reconstituted t-tubule calcium channels insert into bilayers with the cytoplasmic end on the *cis* chamber, as previously argued, on the basis that the frequency of open channels and the fraction of open time increases with *cis*-positive potentials (7). Quantitatively, however, a difference in apparent dissociation constants of 25-fold between *cis* and *trans* D890 is at first sight too small to be accounted for by the simple transfer of a naked positive charge across the bilayer phase, as suggested by Hescheler et al. (10). For example, the electrostatic work of transferring a K^+ ion from the aqueous medium of high dielectric constant to the interior of a low dielectric constant membrane is of the order of 60 kcal/mol (19). The difference between *cis* and *trans* D890 IC_{50} translates into a free energy imbalance ($\Delta G = -RT \ln(cis\ IC_{50}/trans\ IC_{50})$ of only 1.6 kcal/mol. Thus, either the transport of D890 occurs through the channel itself or the aryl chains of the drug molecule fold in some way to shield the charge, and thus lower the free energy of transfer (19). Whether neutral D600 is effectively transported through the lipid phase could, in principle, be inferred from the pH-dependence of blockade (11). However, this hypothesis could not be verified here due to the finding that calcium channel activity, in the absence of blockers, showed a very marked dependence on solution pH. Increasing the pH of the medium from 7.2 to 8.0 decreased the mean lifetime of recorded events (data unpublished). The open time distribution of 5,873 events at a pH of 7.2 could be fitted by two exponentials with the time constants t_{short} = 45.1 ms and t_{long} = 186.3 ms. At a pH of 8.0 the fitted time constants of 694 events were t_{short} = 10.0 ms and t_{long} = 38.6 ms. As our experimental protocol requires the presence of an agonist, there are two likely explanations: either the agonistic action of BAY-K8644 decreases with increasing pH or the calcium channel gating itself is pH-dependent. At the moment we are not able to resolve this question and, therefore, we could not verify the dependence of D600 inhibition on pH. Nevertheless, the observation that *cis* D600 and *cis* D890 elicit blockade with similar IC_{50} strongly suggests that in both cases the cationic form of this amine is pharmacologically active, since at neutral pH less than 5% of D600 (pK_a = 8.6) is present in the uncharged form (10).

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