

# AGONISTS BAY-K8644 AND CGP-28392 OPEN CALCIUM CHANNELS RECONSTITUTED FROM SKELETAL MUSCLE TRANSVERSE TUBULES

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**ABSTRACT** The recently described calcium channel agonists Bay-K8644 and CGP-28392 have been used to induce long-term opening of calcium channels from purified rat muscle transverse tubules (t-tubules) incorporated into planar phospholipid bilayers. Agonist-open channels are selective for divalent cations (except  $Mg^{++}$ ), display voltage-dependent kinetics, and are blocked by the calcium channel antagonist, nitrendipine. The sensitivity to dihydropyridine agonists and antagonists indicate that a pool of t-tubule calcium channels remain functional after membrane fractionation and purification.

## INTRODUCTION

Presently, there are few membrane preparations in which the increasing pharmacological and biochemical knowledge on calcium channels (1–12) can be combined with reconstitutive techniques to characterize the single channel activity (13, 14, 15–17). In most tissues, calcium channels may be present at surface densities just too low to be considered as sources for biochemical work (2). Fosset et al. (4) recently showed that purified skeletal muscle transverse tubules (t-tubules) contain a remarkable high density of calcium channel markers, as detected by the binding of the antagonist [ $^3H$ ]-nitrendipine (3–6, 9). Both in brain and muscle, the nitrendipine-binding protein has been purified as a large 20S complex that remains associated with binding activity for a variety of antagonists (3, 5). Nitrendipine among other dihydropyridine antagonists, block calcium channels (2, 7, 18–20). A second class of closely related dihydropyridines such as Bay-K8644 and CGP-28392, by virtue of inducing long-term opening of otherwise normally brief events, have been named agonists (1, 8, 19–21). We show here that dihydropyridine agonists and antagonists can be used to identify functional calcium channels from muscle transverse tubule vesicles in an *in vitro* recording system: the planar phospholipid bilayer (22). Following the methods described by Miller (23), we have fused purified t-tubules from rat skeletal muscle into planar bilayers, and developed a protocol using Bay-K8644 or CGP-28392 to chemically open calcium channels. This

has resulted in the spontaneous recording of single channel fluctuations over long periods. Under constant depolarized (0 mV) potentials in 0.1 M  $Ba^{++}$  as the current carrier, channels have an amplitude of 0.5 pA and a slope conductance of 20 pS. We show here that agonist activated t-tubule channels, similar to calcium channels recorded *in vivo* (18–21, 24–29) are voltage-dependent, selective for divalent cations (except  $Mg^{++}$ ), display low permeability for monovalents ( $P_{Ba}/P_{Na} = 25$ ), and are blocked by micromolar concentrations of nitrendipine.

## MATERIALS AND METHODS

T-tubules from rat back and leg muscles were fractionated and purified according to Rosenblatt et al. (30) with the modifications introduced by Moczydlowsky et al. (31). Experiments were carried out in Mueller-Rudin bilayers (22) containing equimolar amounts of brain phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids, Inc., Birmingham, AL). Membranes were formed from 20 mg/ml lipid solutions in decane. Holding potential (HP) is injected into the cup side (*cis* side) and the bath side (*trans* side) is maintained at virtual ground. Divalent salts were analytical grade (JMC, Herts., England). Records were taken on FM tape, filtered at 0.1–0.4 kHz (–3 dB point from eight-pole Bessel filter) and digitized at 0.5–2.0 kHz for computer analysis. Duration of open events was measured using computer programs with two threshold detectors placed between baseline and open peak current. One detector, discriminator 1 (discr 1), was placed at 1 standard deviation (SD) from the mean baseline current and the second detector, discriminator 2 (discr 2), at 1 SD from the mean single channel unitary current. Open events are defined as transitions that cross discr 1 and discr 2 and remain above discr 2 for two or more consecutive points. Open durations were sorted into 100 bins (10 ms/bin) and eye-fitted with the sum of one or two exponential terms. Events <10 ms were not included in the fit. A burst is defined as a sequence of channel fluctuations preceded and followed by a quiescent period of 500 ms or longer.

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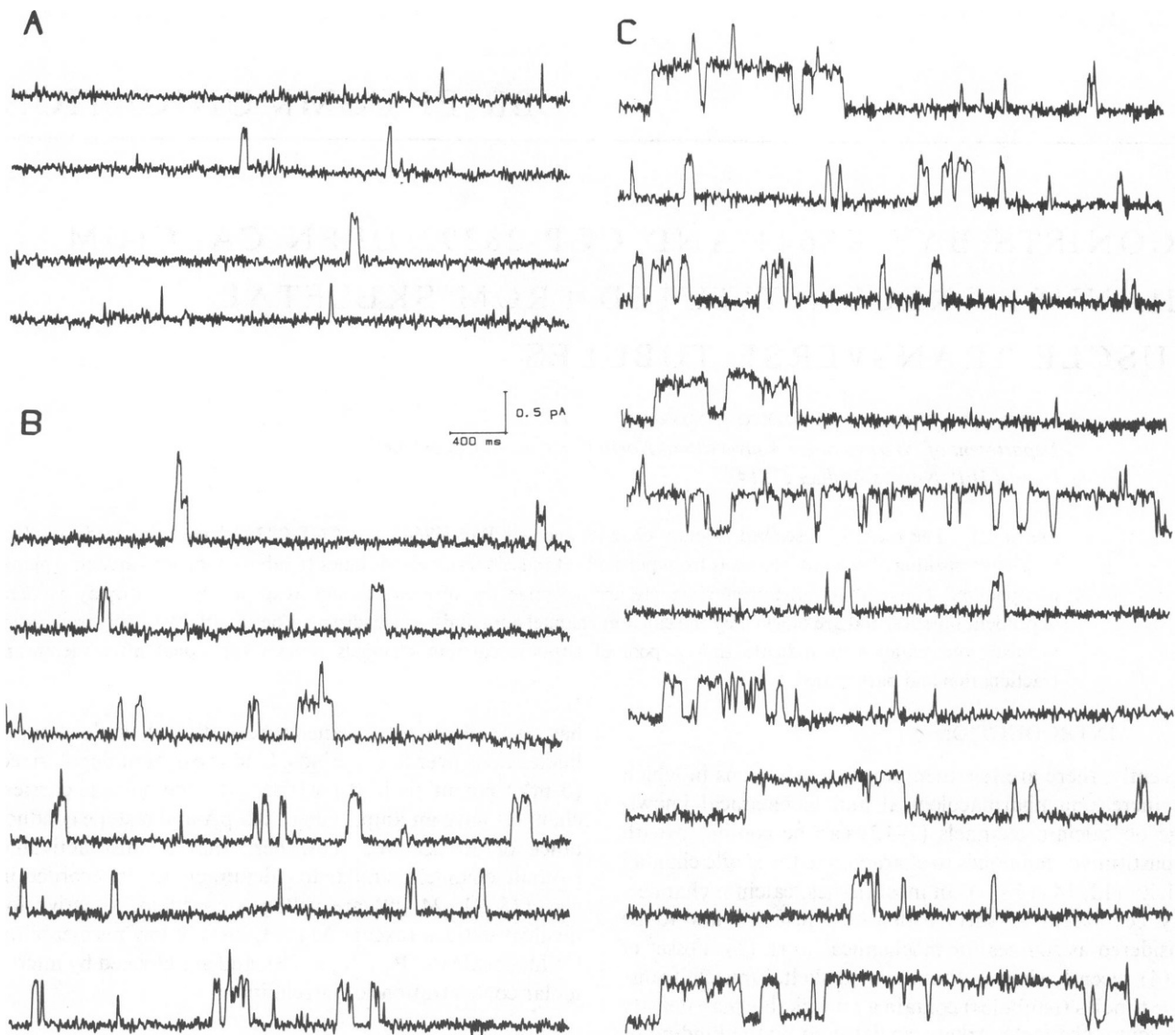


FIGURE 1 Barium current through single calcium channels activated by agonists. (A) Records at HP = 0 mV in the absence of agonists; bilayer was formed in symmetrical *cis-trans* solution containing 0.05 M NaCl, 0.1 mM EGTA, 10 mM Hepes-Tris, pH 7.0. Afterwards, *cis* BaCl<sub>2</sub> was raised to 0.1 M and 100 μg of vesicular protein was added to the *cis* side. (B), (C) Records at HP = 0 mV in 3 μM CGP-28392 (B) or 3 μM Bay-K8644 (C). Using the same solutions described in (A), the bilayer was incubated for 5 min with agonist just prior to addition of t-tubule protein. Agonist was added from a concentrated solution (3 mM) in ethanol. Time mark (400 ms) and current mark (0.5 pA) is the same for all records.

## RESULTS AND DISCUSSION

Fig. 1 shows traces of barium current through single calcium channels at HP = 0 mV after fusion of t-tubule vesicles in *cis* 0.1 M BaCl<sub>2</sub>, 0.05 M NaCl and *trans* (ground) 0.05 M NaCl. To eliminate t-tubule Ca<sup>++</sup>-dependent K<sup>+</sup> channels (32), measurements are done in Na<sup>+</sup> instead of K<sup>+</sup>. For the same reason, we routinely use Ba<sup>++</sup> and not Ca<sup>++</sup> as current carrier. Fusion conditions (23) are met by adding vesicles to the hyperosmotic divalent *cis* solution and by casting planar bilayers from mixtures containing acidic phospholipids. Since under our recording conditions  $E_{Ba^{++}} \ll -200$  mV (nominally infi-

nite),  $E_{Na^{+}} = 0$  mV, and  $E_{Cl^{-}} = +40$  mV, Ba<sup>++</sup>-selective channels can be easily identified at HP = 0 mV as upward deflections (positive current). Fig. 1 A shows barium single channel currents in the absence of agonist. Events without agonist are brief and labile since channels tend to close irreversibly within 30–45 s after recorded for the first time. This spontaneous activity is only present in Ba<sup>++</sup> media and has never been recorded in Ca<sup>++</sup> or Sr<sup>++</sup>. However, when the fusion process is done in the presence of *cis* micromolar CGP-28392 (Fig. 1 B) or Bay-K8644 (Fig. 1 C) channel activity is stabilized in all divalent solutions and recording time can be easily extended over 60–100 min at HP = 0 mV. Whether agonists modify channels present

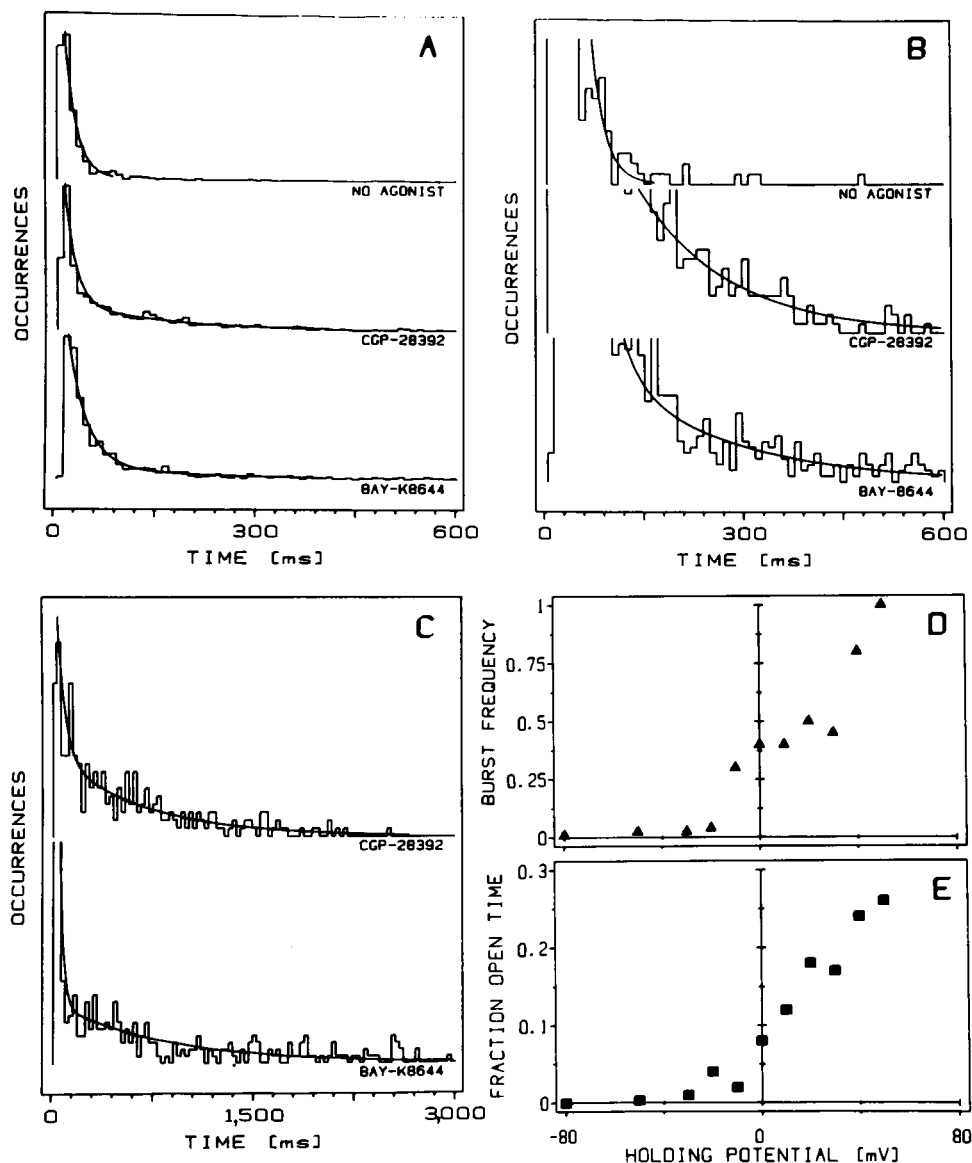


FIGURE 2 Open lifetime, burst distribution, and voltage-dependence of agonist-induced events. (A) Open channel histograms at HP = 0 mV fitted for all events  $\geq 10$  ms. Fitted exponentials are, no agonist:  $t_s = 20$  ms,  $n = 557$  events; CGP-28392:  $t_s = 16$  ms (0.83),  $t_1 = 120$  ms (0.17),  $n = 948$  events; Bay-K8644:  $t_s = 45$  ms (0.85),  $t_1 = 195$  ms (0.15),  $n = 1680$ . In parentheses are given the fraction of the total population fitted by  $t_s$  and  $t_1$ . Agonist concentration was *cis* 3  $\mu$ M. (B) Same histograms described, with y-axis (occurrences) amplified five times to show events at the tail of the distributions. (C) Distribution of identified bursts in *cis* 3  $\mu$ M Bay-K8744 or *cis* 3  $\mu$ M CGP-28392 at HP = 0 mV. Fitted exponentials are, CGP-28392:  $t_s = 56$  ms (0.61),  $t_1 = 670$  ms (0.39),  $n = 272$  bursts; Bay-K8644:  $t_s = 27$  ms (0.90),  $t_1 = 820$  ms (0.10),  $n = 353$  bursts. (D) Frequency of single bursts measured as bursts/seconds (labeled burst frequency), plotted as a function of HP. Each entry is computed from a crude record of 100,000 points digitized at 2 ms/point. (E) The fraction of total digitized points at each voltage (100,000 points at 2 ms/point) that fall under the open peak current (labeled fraction open time), is plotted as a function of HP. All data in (D) and (E) are from a single record of barium current through single calcium channels in *cis* 3  $\mu$ M Bay-K8644.

on native t-tubule vesicles prior to fusion or once channels are inserted into the bilayer is an issue that is difficult to resolve. Dihydropyridines are highly lipophilic molecules that will partition in both vesicular and planar bilayer lipid phases once the compound is added to the aqueous *cis* solution. The most remarkable feature of the agonist-induced activity is the appearance of long open channels that tend to cluster into bursts lasting 1–5 s. Fig. 2 A shows

lifetime distributions of open channels. In the absence of agonist, open events can be fitted by a single exponential with a time constant,  $t_s = 20$  ms. In CGP-28392 and Bay-K8644, distributions are biexponential and have been fitted with time constants,  $t_s = 16$  ms,  $t_1 = 125$  ms and  $t_s = 28$ ,  $t_1 = 186$ , respectively. Thus, the agonist induce a distinctive population of stable open channels,  $o_1$ , which is approximately ten times longer than the population of brief

channels,  $o_3$ , recorded in the absence of agonists. The lack of long events in the absence of agonists is most clearly seen on Fig. 2 B where the tails of the distributions have been expanded to show bin contents for  $t > 100$  ms. Burst formation in the presence of agonist is a significant feature of the channel, as can be observed from the records. This has been confirmed quantitatively since we have found that probability of channel opening is conditioned by the previous occurrence of a closing event (24). Fig. 2 C shows that burst distributions, like open distributions, are biexponential and have been fitted with  $t_s = 56$  ms,  $t_l = 670$  ms for CGP-28392 and  $t_s = 27$  ms,  $t_l = 820$  ms for Bay-K8644. Both the number of bursts formed per unit time (Fig. 2 D) and fraction of time that channels spent open (Fig. 2 E) are a function of voltage, increasing with *cis*-positive holding potentials. In the range HP = -30 mV to HP = +20 mV, burst frequency increases *e*-fold per 15 mV while fraction of open time increases *e*-fold per 25 mV. In spite of the fact that the fraction of open time increases

20-fold from HP = -50 mV to HP = +30 mV, the absolute value is low, seldom larger than  $P = 0.25$  at HP = +50 mV (Fig. 3 C). Thus, in this voltage range, channel opening and bursting are events of low probability, even in the presence of Bay-K8644 agonist.

Fig. 3 A compares the amplitude distribution of open events in the presence and absence of agonist. For Bay-K8644, short events ( $20 \text{ ms} < o_3 < 50 \text{ ms}$ ) and long events ( $200 \text{ ms} < o_3 < 1,000 \text{ ms}$ ), are shown separately. The windows chosen to sample short and long events are justified since we calculate with probability  $P > 0.9$  that events  $< 50$  ms belong to the population fitted by  $t_s$  and events  $> 200$  ms belong to the population fitted by  $t_l$ . Measured under the same conditions, all open channels have the same mean open current of approximately 0.5 pA, at HP = 0 mV. Also, in all cases, reversal occurs at a potential more negative than -50 mV (not shown). Thus, only the channel kinetics is modified by the dihydropyridine agonist, and not the pore selectivity. Shown in Fig. 3 B

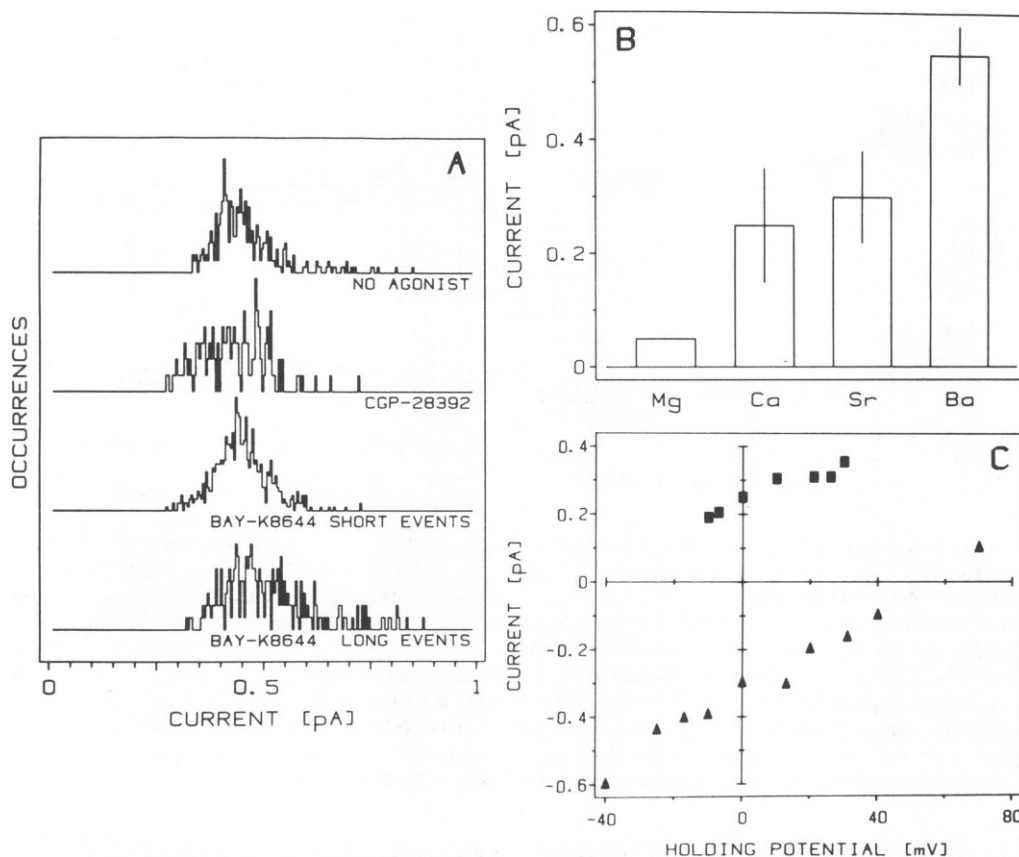


FIGURE 3 Amplitude distribution, divalent cation permeation, and reversal potential of Bay-K8644-induced single calcium channels. (A) Amplitude distribution of open channel currents at HP = 0 mV in *trans* 0.05 M NaCl-*cis* 0.05 M NaCl + 0.1 M BaCl<sub>2</sub> in the absence or presence of *cis* 3  $\mu$ M Bay-K8644 or *cis* 3  $\mu$ M CGP-28392. Events without agonist ( $n = 230$ ) or CGP-28392 ( $n = 140$ ) correspond to total openings  $> 20$  ms. Short events in Bay-K8644 ( $n = 480$ ) are from the duration interval  $20 \text{ ms} < t < 50 \text{ ms}$  and long open events ( $n = 200$ ), from the interval  $200 \text{ ms} < t < 1,000 \text{ ms}$ . (B) Bar histogram of mean and standard deviation of open channel currents (events  $> 20$  ms) at HP = 0 mV measured in *trans* 0.05 M NaCl; *cis* 0.05 M NaCl + 0.1 M XCl<sub>2</sub> (X = Ba<sup>++</sup>, Sr<sup>++</sup>, Ca<sup>++</sup>, or Mg<sup>++</sup>), *cis* 5  $\mu$ M Bay-K8644. Mg<sup>++</sup> currents cannot be measured accurately; the bar (0.05 pA) is an estimated upper limit. (C) reversal potential of single channel currents under bionic *trans* 0.05 M NaCl-*cis* 0.05 M BaCl<sub>2</sub> (filled squares) or *trans* 0.05 M BaCl<sub>2</sub>-*cis* 0.05 M NaCl (filled triangles). Reversal potential for this latter condition is +58 mV. All solutions were buffered with 0.1 mM EGTA and 10 mM Hepes-Tris, pH 7.0.

are means and standard deviations (SD) of single channel currents at  $HP = 0$  mV after *cis*  $Ba^{++}$  is replaced by one of the rest of group IIa divalent cations. Slope conductance at this voltage follows the sequence:  $Ba^{++} = 20$  pS  $>$   $Sr^{++} = 12$  pS  $>$   $Ca^{++} = 10$  pS  $\gg$   $Mg^{++} < 2$  pS. The high  $Ba^{++}$  conductance and lack of any significant  $Mg^{++}$  conductance indicates that the channel is endowed with permeation properties that are characteristic of calcium channels recorded in whole cells (19–21, 25–29). Discrimination against alkali monovalent cations is shown in Fig. 3 C for the biionic pair, 0.05 M  $Na^+$ /0.05 M  $Ba^{++}$ , measured from activity induced by Bay-K8644. By making  $E_{Ba^{++}}$  positive (*trans*  $Ba^{++}$ /*cis*  $Na^+$ ) it is possible to show a genuine reversal potential at +58 mV. This reversal potential corresponds to a permeability ratio  $P_{Ba^{++}}/P_{Na^+} = 25$  from the constant field equation solved with  $P_{Cl^-} = 0$ .

The lack of anion permeation has been shown elsewhere (15–17).

Described in Fig. 4 is the antagonistic action of nitrendipine on the t-tubule calcium channel. Blockade of Bay-K8644-induced activity by nitrendipine was an expected finding given the competitive nature of these two drugs (8). Blockade can also be demonstrated in channels spontaneously open in the absence of agonist (15–17). The overall effect of nitrendipine, shown in Fig. 4 A–C, consists in a decrease in the number of open channels per unit time and consequently, the appearance of long interevent quiescent periods. Surprisingly, Fig. 4 D shows that nitrendipine blockade lacks a marked sidedness inasmuch as prolonged exposure of the channel to either *cis* or *trans* 10  $\mu$ M nitrendipine diminished activity. This could indicate that the drug gains access to the channel after partitioning the

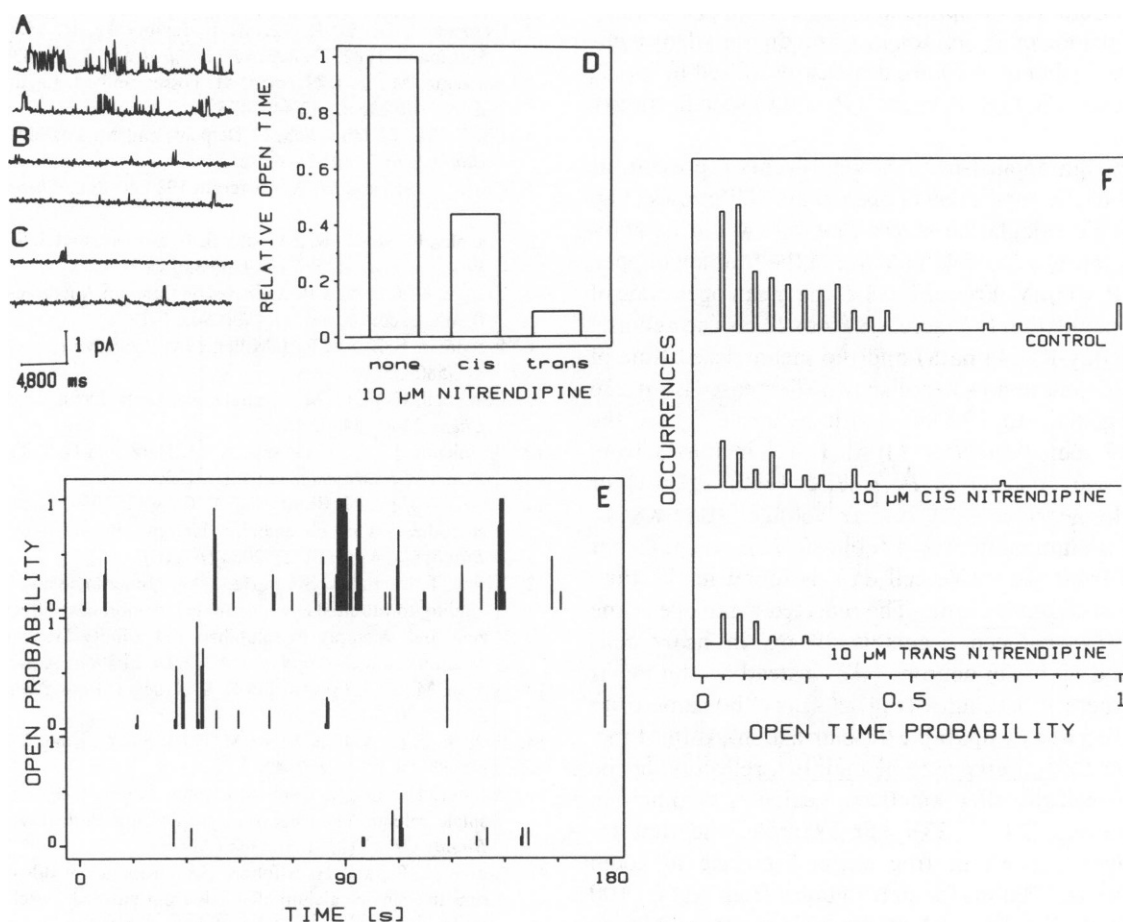


FIGURE 4 Nitrendipine blockade of Bay-K8644-induced barium current through single calcium channels. (A) Control records compressed in time at  $HP = 0$  mV. (B) Representative records 4 min after addition of *trans*-only 10  $\mu$ M nitrendipine. (C) Representative records 12 min after addition of *cis*-only 10  $\mu$ M nitrendipine are the same for all records. Drug was added from concentrated solution in ethanol (5 mM). Ionic solution was *cis* 0.05 M NaCl, 0.1 M  $BaCl_2$ , 1  $\mu$ M Bay-K8644; *trans* 0.05 M NaCl. (D) Fraction of open time,  $f_o$ , calculated from peak current histograms (100,000 points digitized at 10 ms/point) after *cis* or *trans* nitrendipine are shown normalized relative to  $f_o$  of control records prior to *cis* or *trans* drug addition. (E) Fraction of open time,  $P_o$ , was calculated in each consecutive segment of 500 ms along a selected continuous record of 180 s. Amplitude of vertical lines correspond to the value of  $P_o$  in that segment. Absence of a line indicates that no opening occurred in that segment, i.e.,  $P_o = 0$ . From top to bottom records correspond to control (top), 10  $\mu$ M *cis*-only nitrendipine (middle), 10  $\mu$ M *trans*-only nitrendipine (bottom). (F) Frequency distribution of  $P_o$  values for control, *cis*, and *trans* nitrendipine records.  $P_o$  values shown in (E) were sorted into 25 bins of equal width ( $p = 0.04$ ). Entries corresponding to  $P_o = 0$  (segments without openings) are not included. The scale on the y-axis (occurrences) is the same for all histograms.

diffusing through the bilayer lipid phase, similar to the block of calcium channels by aralkylamine-type antagonists (25). The two most significant features of nitrendipine block are shown in Fig. 4 E–F. Control records and records after drug addition are digitized and split into 500 ms segments. For each consecutive segment in time, Fig. 4 E shows the fraction of time that a channel in that segment spent open,  $P_o$ . Blanks correspond to segments without activity. Control record (Fig. 4 e, top) shows open events of high ( $P_o > 0.9$ ) and low ( $P_o < 0.4$ ) probability, which is a typical behavior of cellular calcium channels activated by Bay-K8644 (19–21). In the presence of 10  $\mu\text{M}$  *cis* or *trans* nitrendipine (Fig. 4 E, middle and bottom) there is an increase in the number of empty segments. This indicates that nitrendipine diminishes the pool of active channels. At the same time, Fig. 4 F shows that survivor channels, i.e., channels that are available for opening in 10  $\mu\text{M}$  *cis* or *trans* nitrendipine, are only briefly open. Thus, the distributions of  $P_o$  values in nitrendipine when compared to control (Fig. 4 F) are drastically shifted to low  $P_o$  and do not contain high  $P_o$  events ( $P_o > 0.9$ ) seen in control records.

Even though agonist-induced long events represent, at most, 15% of the population of open channel lifetimes (Fig. 2 A) a simple calculation shows that this would be sufficient to generate a fourfold increase in the fraction of open time at HP = 0 mV. From Fig. 2 A, the mean open time of the whole population ( $t_o$ ) increases from 20 ms (no agonist) to 67 ms (Bay-K8644 data) and the mean closed time of the whole population ( $t_c$ ) (not shown) decreases from 230 ms (no agonist) to 120 ms (with agonist). Thus, the fraction of open time,  $P_o = (1 + t_c/t_o)^{-1}$  increases from  $P_o = 0.08$  (no agonist) to  $P_o = 0.35$  (with agonist) or 4.4-fold. In heart cells at similar voltages, Bay-K8644 increases calcium content 2–4-fold (20, 26). A significant difference from the whole cell data is found in the time scale agonist-induced events. The reported mean open time of Bay-K8644-induced events is 20 ms in heart cells (20, 21) and 60 ms in neurons (19), instead of 186 ms as measured here in t-tubules (Fig. 2 A), at the same drug concentration and comparable depolarization. At this time, we consider this difference to be mainly a reflection of the known intrinsically slow kinetics of calcium channels in skeletal muscle (24, 27, 33). For example, the time-to-peak barium current in frog skeletal muscle at room temperature is ~200 ms for step voltages from rest (–100 mV) to 0 mV (27), thus, 10–40 times slower than calcium channels in heart (19–21). Other calcium channels recorded in vitro from brain microsomes (13) and cilia (14) also seem to share the same slow noninactivating kinetics shown here for t-tubules. However, a detailed comparison with these reconstituted channels does not appear justified here since in these cases there has been no demonstration of sensitivity to dihydropyridines. In conclusion, the behavior of t-tubule calcium channels, including responsiveness to dihydropyridine agonists and antagonists, selectivity for

divalents (but not  $\text{Mg}^{++}$ ), and voltage-dependent kinetics, indicate that channels remain functional, albeit unstable in the absence of agonist after membrane purification. Furthermore, the in vitro demonstration of dihydropyridine agonist action opens several possibilities for the use of these drugs, e.g., in flux assays, to further purify the channel protein.

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