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DUAL ACTION (STIMULATION, INHIBITION) OF D600 ON CONTRACTILITY AND CALCIUM CHANNELS IN GUINEA-PIG AND CAT HEART CELLS

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SUMMARY

1. We examined the effects of D600 (0·2–40 μM, generally 2 μM) on the following: (i) developed tension in guinea-pig papillary muscles, (ii) calcium current (I_{Ca}) and tension in cat ventricular muscle strands, (iii) I_{Ca} in guinea-pig and cat ventricular myocytes, (iv) single Ca^{2+} channel currents carried by Ba^{2+} in cell-attached membrane patches of guinea-pig ventricular myocytes, and (v) Ba^{2+} currents through dihydropyridine (DHP)-binding sites (skeletal muscle) reconstituted into single functional Ca^{2+} channels in lipid bilayers.

2. In 27 of 140 preparations studied, D600 elicited a transient stimulation that preceded marked inhibition. The stimulation was normally of short duration (< 5 min) and moderate strength ($< 50\%$ increase).

3. D600 had no effect on the unit conductance of single cardiac Ca^{2+} channels. Stimulation was characterized by a decrease in the number of records with no openings (blanks) and an increase in the open-state probability of non-blanks (longer open times, shorter closed times). Inhibition began with an increase in the number of blanks and later included a curtailment of open times and a prolongation of closed times. The net effect after 9 min D600 was a 75% reduction in average current amplitude.

4. A similar pattern of changes in channel open and closed times produced enhancement and then depression of time-averaged open-state probability in single reconstituted channels.

5. Single Ca^{2+} channel current that was stimulated by adrenaline was only slightly depressed after 2 μM-D600 for 30 min. It may be that channel phosphorylation or G_s-protein activation following β-receptor stimulation reduces channel affinity for D600.

6. Short-lived binding of D600 to a single inhibitory site may enhance association/activation of G_s-protein and thereby cause transient up-regulation prior to increased drug occupancy and inhibition. Alternatively, there may be separate stimulatory and inhibitory sites. One aspect of inhibition, the increased frequency of blanks, is attributed to a stabilization of the inactivated state; the other aspect, changes in fast kinetics, seems to require a different explanation.

INTRODUCTION

Phenylalkylamines such as verapamil and D600, dihydropyridines (DHP) such as nitrendipine and nifedipine, and Ca^{2+}–calmodulin inhibitors such as fendiline, are well-known inhibitors of contractility and calcium current (I_{Ca}) in cardiac preparations (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Nawrath, Ten Eick, McDonald & Trautwein, 1977; Pelzer, Trautwein & McDonald, 1982; Sanguinetti & Kass, 1984; Fleckenstein, 1985; Pott & Lipp, 1987). Some of these compounds have a dual action on cardiac Ca^{2+} channels. For example, DHP inhibitors can provoke a small transient stimulation prior to inhibition (Hess, Lansman & Tsien, 1984; Brown, Kunze & Yatani, 1984, 1986; Reuter, Porzig, Kokubun & Prod'hom, 1985). It is likely that dual activity can reside in each enantiomer of a DHP compound (Kokubun, Prod'hom, Becker, Porzig & Reuter, 1986; Kass, 1987), and that stimulation is favoured by negative holding potentials (Kokubun *et al.* 1986; Kass, 1987; Scott & Dolphin, 1987) and low drug concentration (Brown *et al.* 1986). Fendiline is also reported to have a concentration- and voltage-dependent dual action on I_{Ca} in atrial cardioballs (Pott & Lipp, 1987).

Phenylalkylamine inhibitors can also have stimulatory actions. Marwaha & Treffers (1980), as well as Frank (1984), found that sub-blocking concentrations of D600 ($1–5 \times 10^{-5}$ M) produced a small stable potentiation of twitch tension in frog skeletal muscle. Frank (1984) also found that submaximum, 20 mM-K^+-induced contractures were abolished by 3 μM-D600 but increased in amplitude by 0·1 μM-D600. A related finding is that D600 can lower mechanical threshold in skeletal muscle (Dörrscheidt-Käfer, 1977). Berwe, Gottschalk & Lüttgau (1987) examined this phenomenon and proposed that D600 stabilizes the 'active state' of 'potential sensors' connected with excitation–contraction coupling (cf. Schneider & Chandler, 1973). An inhibitory action of the drug, 'paralysis' of skeletal muscle (Eisenberg, McCarthy & Milton, 1983), was attributed by Berwe *et al.* (1987) to 'inactivation' of the coupling sensors. These concepts are analogous to those developed for the dual action of DHP inhibitors (stimulation due to a 'stabilization' of the open state of the Ca^{2+} channel, inhibition due to a stabilization of the inactivated state; Hess *et al.* 1984).

A more direct example of the dual action of D600 on Ca^{2+} channels has been provided by Scott & Dolphin (1987). They found that 10 μM-D600 increased whole-cell Ba^{2+} current by 10–15% in three of five cultured dorsal root ganglion (DRG) cells. This stimulation after 30 s with drug gave way to a 40% inhibition over the next 4 min. Both diltiazem (30 μM) and nifedipine (5 μM) also produced transient stimulation in the DRG neurones. By contrast, Iijima, Yanagisawa, & Taira (1984) reported that I_{Ca} in guinea-pig ventricular myocytes was not stimulated when superfusates contained nifedipine, nicardipine or D600. However, there was a transient stimulation after intracellular application of DHP. They attributed this stimulation to phosphodiesterase inhibition, and also concluded that the absence of a similar response with intracellular D600 was due to a lack of D600 action on the enzyme.

We have found that D600 can elicit a dual action on contractility and I_{Ca} in cardiac preparations. Drug-induced stimulation and inhibition of Ba^{2+}-carried currents were also recorded from single Ca^{2+} channels in myocardial cell patches and from skeletal

muscle DHP-binding sites incorporated in lipid bilayers. A brief communication has been published (Pelzer, Cavalié, Hofmann, Trautwein & McDonald, 1988).

METHODS

Ventricular muscle strands. Papillary muscles and ventricular trabeculae (diameter, 0·15–0·4 mm; length, 3–5 mm) were isolated from the right ventricles of adult guinea-pig and cat hearts. Tissue was removed from animals under nembutal or ether anaesthesia. Guinea-pig preparations were mounted in a small-volume bath and superfused with Krebs solution containing (in mM): NaCl, 113·1; KCl, 4·6; $CaCl_2$, 2·45; $MgCl_2$, 1·2; NaH_2PO_4, 3·5; $NaHCO_3$, 21·9; and glucose, 5; equilibrated with 95% O_2–5% CO_2 (pH 7·4, 36 °C). Muscles stimulated with 1 ms pulses at 1 Hz had resting potentials between −85 and −90 mV. Developed tension was monitored with a Statham UC2 force transducer. Further experimental details are given in Watanabe, Rautaharju & McDonald (1985).

Cat ventricular preparations were mounted in a single sucrose gap for voltage-clamp experiments. The Tyrode solution perfusing the right (test) compartment of the bath contained (in mM): NaCl, 140; KCl, 5·4; $MgCl_2$, 1·0; $CaCl_2$, 1·8; $NaHCO_3$, 12·0; NaH_2PO_4,0·4; and glucose, 5·0. Tetrodotoxin (TTX, 5×10^{-5} M) was added to suppress sodium current. The solution in the middle compartment contained 304 mM-sucrose, 5 mM-glucose and 0·01 mM-$CaCl_2$, and the solution in the left compartment was KCl–Tyrode solution (NaCl substituted by KCl). Tyrode and sucrose solutions were gassed with 95% O_2–5% CO_2 and 100% O_2, respectively, and warmed to 36 °C. The amplitude of I_{Ca} was estimated as the difference between the early inward-going peak and the late current after 200 ms (McDonald & Trautwein, 1978). Further experimental details are given in McDonald, Pelzer & Trautwein (1984*a*, *b*).

Single ventricular cells. Single ventricular myocytes were obtained from the hearts of adult guinea-pigs and cats by enzymatic dispersion. After the cells had settled on the culture dish bottom of the recording chamber, the bath was perfused (2–4 ml/min) with saline containing (in mM): NcCl, 131; KCl, 10·8; $CaCl_2$, 3·6; $MgCl_2$, 1; glucose, 10; and HEPES, 5 (pH 7·3–7·4; 35 + 1 °C). Whole-cell currents were recorded with a two-microelectrode method after the addition of 5×10^{-5} M-TTX to the superfusate. Further experimental details are given in Cavalié, Ochi, Pelzer & Trautwein (1983).

Cell-attached patches. Elementary Ca^{2+} channel currents were recorded from cell-attached membrane patches of guinea-pig ventricular cells superfused as described above. The recording pipettes contained (in mM): $BaCl_2$, 90; NaCl, 2; KCl, 4; TTX, 0·02; and HEPES, 5; the pH was adjusted to 7·4. Depolarizing voltage pulses (75–85 mV amplitude, 300 ms duration) were applied from the resting potential (estimated as −50 mV; McDonald, Cavalié, Trautwein & Pelzer, 1986). The membrane patch currents were recorded on a RACAL FM tape-recorder and subsequently digitized (sampling rate 6 kHz, low-pass filtering at 2 kHz). Further experimental details are given in Cavalié *et al.* (1983) and Cavalié, Pelzer & Trautwein (1986).

Reconstituted Ca^{2+} channel. The dihydropyridine–receptor complex purified from rabbit skeletal muscle T-tubules was incorporated into phospholipid bilayer membranes at the tip of glass patch pipettes. The solution on both sides of the bilayer contained 90 mM-$BaCl_2$, 5 mM-HEPES and 0·02 mM-TTX (pH 7·3, 20–23 °C). Currents were digitized at 1 kHz and low-pass filtered at 0·3 kHz. Further experimental details are described in Flockerzi, Oeken, Hofmann, Pelzer, Cavalié & Trautwein (1986).

Drugs. Tetrodotoxin and adrenaline were purchased from Sigma (St Louis, MO, USA); D600 was a kind gift from Knoll AG (Ludwigshafen, FRG). Drugs were dissolved in water, or water containing ascorbic acid (adrenaline), and appropriate volumes were added to the bathing solutions.

RESULTS

Dual action of D600 on contractility and I_{Ca} in cardiac tissues and myocytes

Developed tension was measured in eighty-six guinea-pig papillary muscles driven at 1 Hz and treated with 0·2–40 μM-D600 (Fig. 1). In the majority of the muscles, tension declined with time (○ with standard deviation bars). However, in three of

ten muscles treated with 0·2 μM-D600, four of thirty-two with 2 μM-D600, six of thirty-six with 10 μM-D600, and two of eight with 40 μM-D600, drug-induced depression was preceded by a positive inotropy that lasted for 0·5–3 min (●). The mean increases in tension were 12% (0·2 μM-D600), 11% (2 μM), 14% (10 μM) and 11% (40 μM).

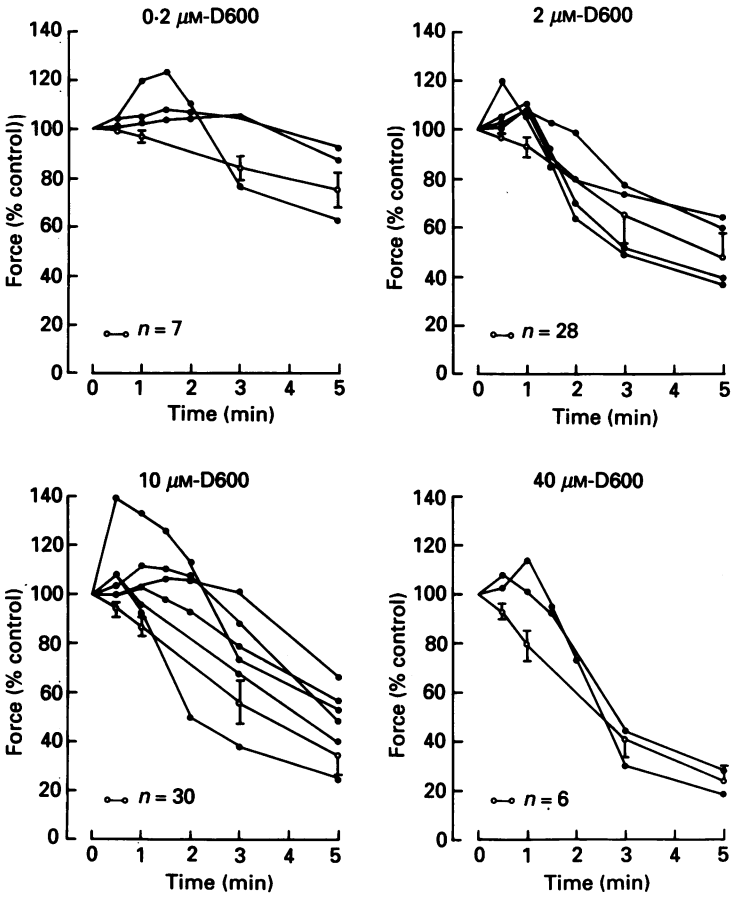

Fig. 1. The dual effect (stimulation, inhibition) of D600 on the contractile force of guinea-pig papillary muscles. D600 (0·2, 2, 10 or 40 μM) depressed developed tension (force) in most muscles (○; vertical bars, mean ± S.D.); in each of the others (●; three of ten, 0·2 μM-D600; four of thirty-two, 2 μM; six of thirty-six, 10 μM; two of eight, 40 μM), a short-lasting positive inotropy preceded inhibition. Stimulation rate, 1 Hz.

D600 (2 μM) caused a transient stimulation of I_{Ca} and tension in five of twenty-four cat ventricular muscles clamped at −55 mV and stimulated with 300 ms pulses to 0 mV at 0·33 Hz. In the example experiment (Fig. 2), D600 increased I_{Ca} and tension by 20–25% after 2 min (middle panel); after an additional 2 min, I_{Ca} and developed tension had declined to about 60% control (right-hand panel). In the five muscles that exhibited clear-cut stimulation, the mean increases in I_{Ca} and tension after 2 min D600 were 32% (range 18–47%) and 31% (range, 14–41%), respectively. During the next 2 min, mean I_{Ca} declined to 59% control (range, 34–72%) and mean tension to 56% control (range, 32–78%).

Isolated ventricular myocytes (eight guinea-pig, five cat) were clamped at −55 mV and pulsed with 300 ms steps to 0 mV at 0·33 Hz. In three of thirteen myocytes, the application of 2 μM-D600 solution caused an early transient stimulation of I_{Ca}. The two examples shown in Fig. 3 illustrate that the stimulation at 2 min and inhibition at 4 min were similar to the responses recorded from the multicellular preparations.

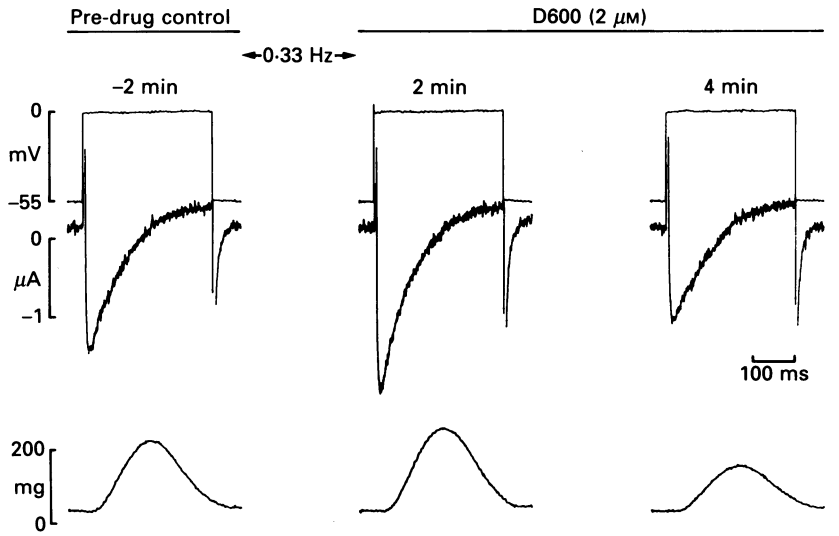

Fig. 2. The dual effect of D600 on contractile force and I_{Ca} in a cat ventricular muscle. Top to bottom: membrane potential, membrane current and tension. Depolarizations (300 ms to 0 mV at 0·33 Hz) elicited inward current transients that are mainly due to I_{Ca} (McDonald & Trautwein, 1978). Compared to the pre-drug amplitudes, I_{Ca} and developed tension were enhanced after 2 min D600, and depressed after 4 min D600.

The block of cardiac I_{Ca} by D600 and DHPs is enhanced by rapid stimulation from depolarized potentials, and alleviated by short rests at negative potentials (McDonald, Peltzer & Trautwein, 1980, 1984*b*; Sanguinetti & Kass, 1984). Conversely, stimulation by Ca^{2+} channel blockers appears to be blunted by rapid pulsing from depolarized potentials, and enhanced by slow pulsing from negative holding potentials (Brown *et al.* 1986; Kokubun *et al.* 1986; Scott & Dolphin, 1987). In agreement with the foregoing, there was no transient stimulation of I_{Ca} when guinea-pig myocytes ($n = 4$) and cat ventricular muscles ($n = 2$) were stimulated at 0·33–1 Hz from a −35 mV holding potential. Unfortunately, we did not obtain complementary results with protocols designed to tilt the balance towards stimulation. A transient stimulation of I_{Ca} was not detected when 2 μM-D600 was applied to cat ventricular muscles pulsed at 0·01 Hz from a −85 mV holding potential ($n = 4$), nor was stimulation unmasked by 2 min hyperpolarizations to −140 mV after 30–90 min D600 ($n = 3$). In addition, transient stimulation of force was not observed when guinea-pig papillary muscles hyperpolarized with 1·5 mM-K^+ superfusates (resting potential, *circa* −105 mV) were stimulated at 0·1 Hz and treated with either 0·2 or 2 μM-D600 ($n = 6$ each). It may be that the modulatory effect of holding potential on D600 stimulation will be easier to detect in single Ca^{2+}

channel studies. Until these have been performed, it is an open question whether stimulation by D600 in heart cells is actually transient or just appears so because it is quickly overcome by powerful inhibition (see Discussion).

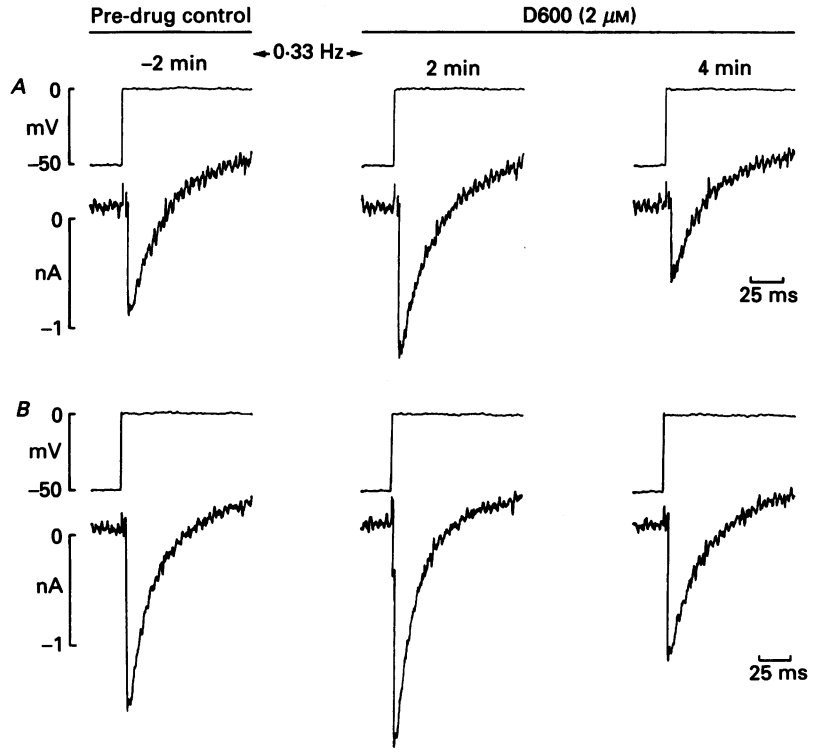

Fig 3. Voltage-clamp records from ventricular myocytes isolated from (*A*) cat and (*B*) guinea-pig hearts. The records (top, voltage; bottom, current) were obtained during 300 ms depolarizations from −50 to 0 mV before and during superfusion with 2 μM-D600 solution. Inhibition (right-hand traces) was preceded by a transient increase in inward-going I_{Ca} (middle traces). Stimulation rate, 0·33 Hz.

Stimulation and inhibition of single cardiac Ca^{2+} channel currents

In three of eleven patch clamp experiments on guinea-pig myocytes superfused with 2 μM-D600 solution, a transient stimulation preceded inhibition of single Ca^{2+} channel current carried by Ba^{2+}. An example of the dual action is presented and analysed in Figs 4–6. In this experiment, the membrane patch was depolarized from the cell resting potential (estimated to be −50 mV) to +25 mV for 300 ms at 0·5 Hz. The data were divided into a control 3 min phase and three successive 3 min drug phases (stimulatory, early inhibitory and intermediate inhibitory). Sets of twenty consecutive records (*a′–d′*, see time bars, Fig. 5*A*) selected from each of these four phases are reproduced in Fig. 4. During the pre-drug phase, the channel sometimes failed to open during a depolarizing pulse and the current record was 'blank' (e.g. bottom left record). There were fewer blanks during the 0–3 min stimulatory phase but, as stimulation gave way to inhibition, the number of blanks increased and eventually surpassed pre-drug values (*c′* and *d′*, Fig. 4).

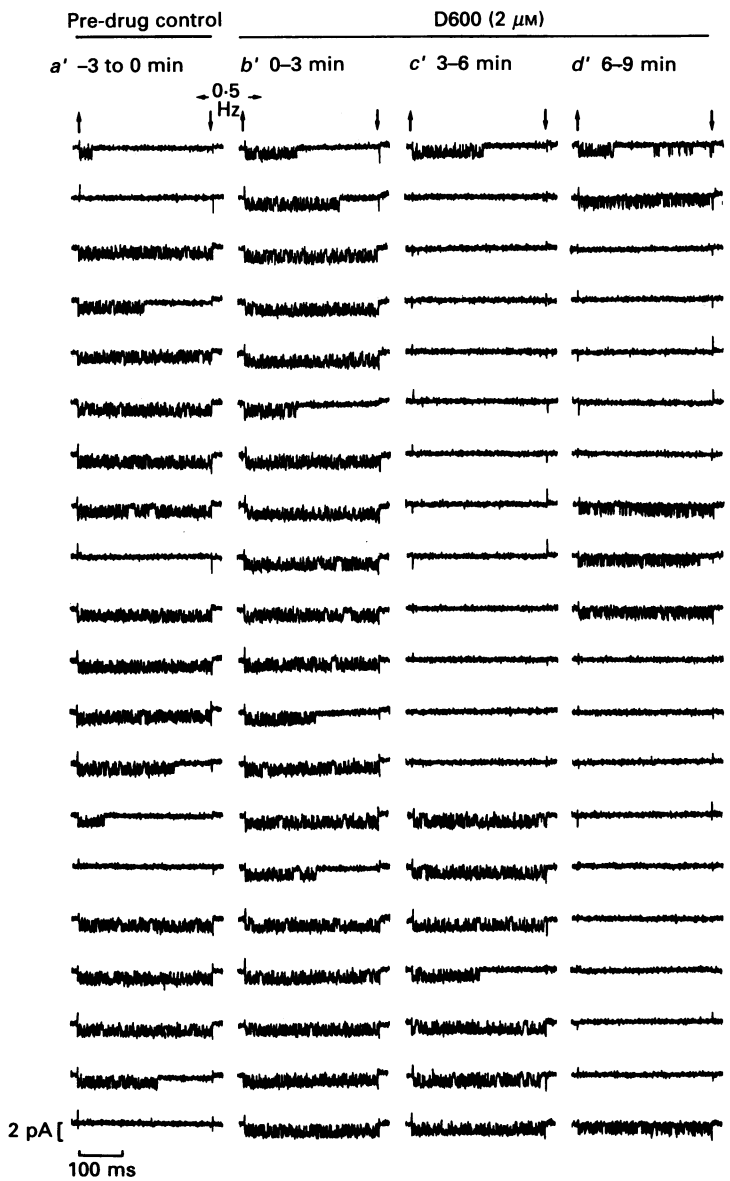

Fig. 4. The dual action of D600 on single Ca^{2+} channel currents in a cell-attached patch of a guinea-pig ventricular myocyte. The twenty consecutive records shown in each of the four columns are from 40 s intervals (*a'–d'*: see Fig. 5) within four designated phases of the experiment: pre-drug, and 0–3, 3–6, and 6–9 min after 2 μM-D600 solution. A 90 s break between pre-drug and 0 min drug was incorporated to allow for solution change in the bath. There were fewer blanks in the 0–3 min D600 phase than in the pre-drug interval; this stimulation gave way to a pronounced increase in blanks (inhibition) shortly thereafter (*c'* and *d'*). Inhibition was also marked by the appearance of records with long closings interspersed with short openings (compare top and subsequent records in *d'* with records in other columns). The patch was pulsed from the resting potential (about −50 mV) to +25 mV for 300 ms at 0·5 Hz.

In addition to an increment in blanks, the onset of inhibition was marked by a change in channel bursting activity. During intervals a'–c', visual inspection suggests that the channel was open more often than it was closed during bursting, i.e. tracings are denser along the lower (open) level (e.g. top two and bottom two records of b' and c'). Although this is also evident in the early bursting of the top record in d', inhibition then set in: the early burst was followed by a long closing, a very short burst, another long closing, and so on. Thereafter, non-blank records give a visual impression (upper-level density) opposite to that given by earlier records.

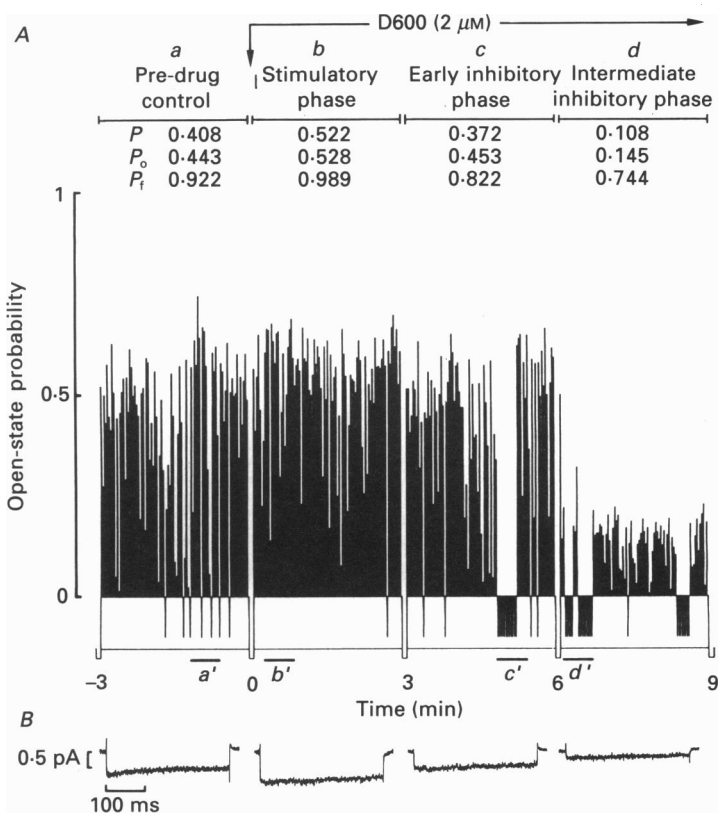

Fig. 5. Open-state probabilities and average single Ca^{2+} channel currents from a cell-attached patch of a guinea-pig ventricular myocyte before and during superfusion with 2 μM-D600 solution. *A*, each upward-going bar reflects the average time in the open state during each 300 ms depolarization; a downward deflection indicates a blank. The ensemble probabilities above the bar graph are as follows: P, ensemble average open-state probability; P_o, average open-state probability in non-blank records; P_f, probability that the channel opened once or more during a depolarization (channel availability). *B*, the computed average currents for the four 3 min phases. Same experiment as that in Fig. 4.

The open-state probabilities calculated for this experiment are shown in Fig. 5*A*. The bar graph indicates that, aside from blanks (downward-going bars), the open-state probability ranged from 0·02 to 0·7 during the first three phases. The average (P_o) in non-blank records was 0·44 during the pre-drug phase, 0·53 during the stimulatory phase, and 0·45 during the early inhibitory phase. Channel availability,

or the probability of channel opening in response to a depolarization (P_f), also followed an up and down pattern during D600 treatment. Pre-drug P_f was 0·92, the stimulatory phase P_f was 0·99, and the early inhibitory phase P_f was 0·82.

The trend to more blanks per ensemble continued with increasing time of D600 treatment (Fig. 5*A*). Although there were only four blank episodes in the intermediate inhibitory phase, three of these four episodes comprised five to nine successive depolarizations, and this resulted in a further lowering of P_f from 0·82 to 0·74. Aside from two early sweeps, the open-state probability during intermediate inhibition ranged from about 0·01 to 0·25, compared to the 0·02–0·7 range during early inhibition. Thus, the average P_o for non-blank records declined from 0·45 to 0·15 as inhibition gathered force.

The average open-state probability for each entire ensemble (non-blanks plus blanks) is listed under P at the top of Fig. 5*A*. The P values for the control and three drug phases were 0·41, 0·52, 0·37 and 0·11; normalization to pre-drug P gives 1:1·27:0·90:0·25. The average single Ca^{2+} channel currents for the four phases are shown in Fig. 5*B*. The normalized peak amplitudes are 1:1·37:0·81:0·27, in good agreement with the relative P values. This correspondence is expected if D600 has little effect on the time course of Ca^{2+} channel activation and inactivation.

An analysis of the fast gating behaviour of the single Ca^{2+} channel was performed on digitized records from the four experimental phases. At the top of each quadrant (*A–D*) of Fig. 6 we show example 32 ms segments from 300 ms records that were digitized at 12·5 kHz, and spline-interpolated for visual representation. In the pre-drug phase, open-channel activity was punctuated by channel closings in the submillisecond to millisecond range. The net effect was that the channel spent slightly more than half the time in the open state. In the presence of D600, the dwell times in the open and closed states depended on the length of exposure to drug. The channel was open about 70% of the time during the stimulatory phase, 55% during the early inhibitory phase, and 15% during the intermediate inhibitory phase.

Frequency histograms of open times and closed times were compiled from records digitized at 6 kHz. The distributions of open times were fitted with single exponentials, and the distributions of closed times by sums of two exponentials (Fig. 6). During the stimulatory phase (Fig. 6*B*), the longer openings are reflected by the increase in time constant τ from 1·35 ms (pre-drug) to 1·92 ms, and the shorter closings by decreases in τ_{fast} and τ_{slow} from 0·3 and 1·5 ms (pre-drug) to 0·25 and 1 ms, respectively. Fast gating during the early inhibitory phase (Fig. 6*C*) was practically the same as that prior to drug, but during the next 3 min (Fig. 6*D*) there was a marked shortening of open times (τ = 0·34 ms) and lengthening of closed times (τ_{fast} = 0·9 ms, τ_{slow} = 4 ms).

Reduced inhibition by D600 after pre-treatment with adrenaline

Inhibition by D600 is characterized by an increase in the fraction of depolarizations that elicit blanks (Figs 4–6). Since the up-regulation of Ca^{2+} channel activity by β-adrenergic agonists has the opposite effect (Trautwein & Pelzer, 1985; Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986), we examined whether pre-treatment of myocytes with adrenaline modifies D600 inhibition.

Under control conditions (not shown), the low P_o channel investigated had a mean

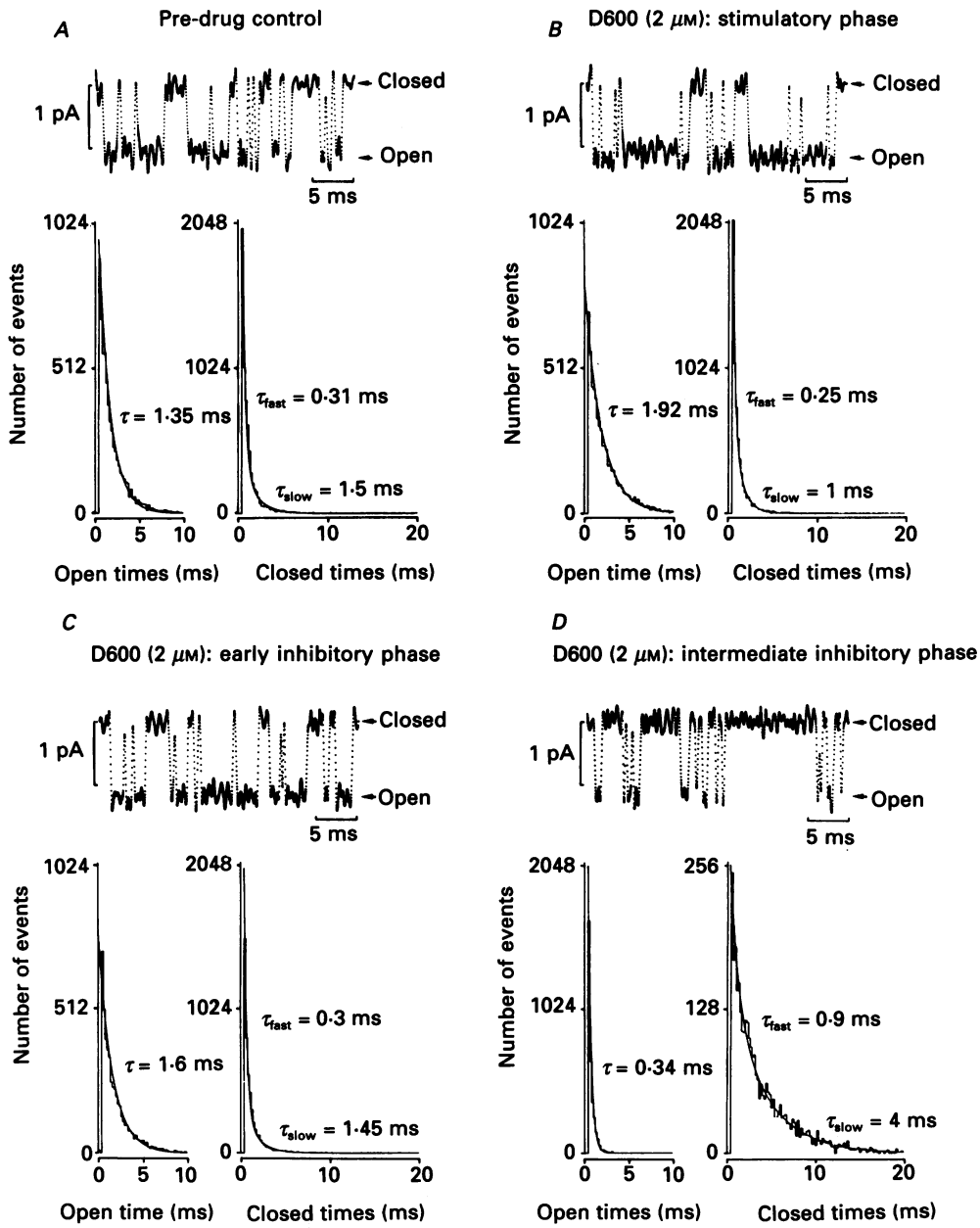

Fig 6. The fast gating behaviour of a single Ca^{2+} channel before and during stimulatory and inhibitory action by 2 μM-D600. *A–D*, the four phases of the experiment. In each phase, a 32 ms segment of a record digitized at 12 kHz has been selected and spline-interpolated (see Colquhoun & Sigworth, 1983) for improved visual representation. The histograms of open times are fitted with single exponentials, and those of closed times with two exponentials. The latter do not include terminal closings (closed times truncated by end of depolarization). Compared to pre-drug control, there were longer average openings and shorter average closings during the stimulatory phase. Thereafter, the average open time became progressively shorter and the average closed time progressively longer. Infrequent, unusually long, non-terminal closings during intermediate phase inhibition (*d'*, Fig. 4) are not included here. Same experiment as that in Figs 4 and 5.

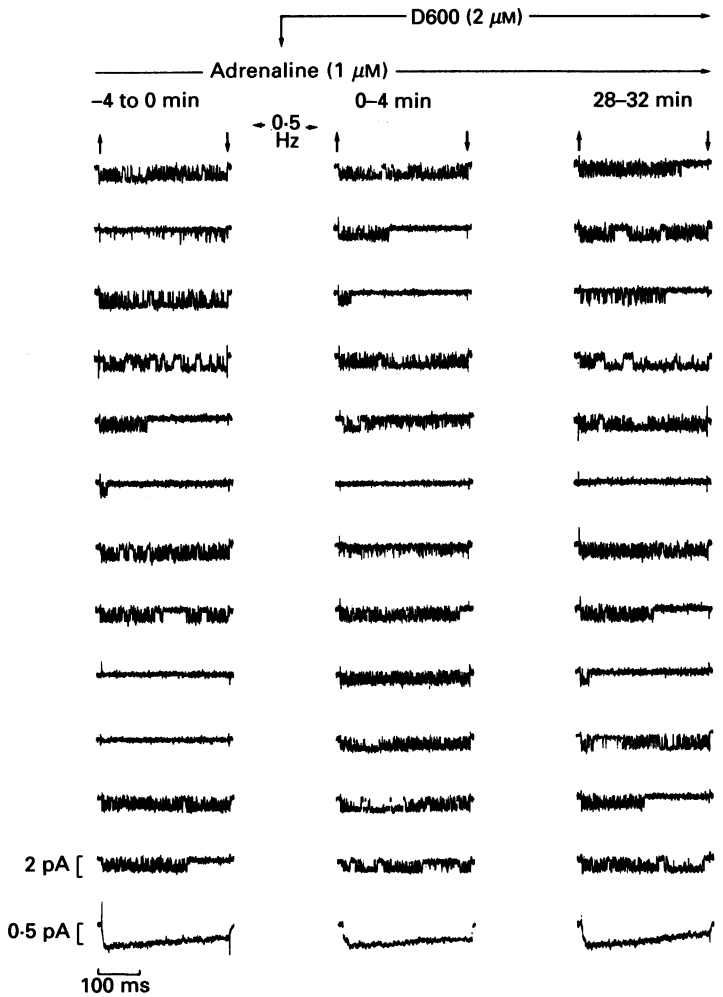

Fig. 7. Lack of effect of D600 after adrenaline-induced up-regulation of a single Ca^{2+} channel in a cell-attached patch of a guinea-pig ventricular myocyte. Left-hand column: selected records from an ensemble of 120 records after cell superfusion with 1 μM-adrenaline for 15 min. Bottom record is the average current, which was about 3-fold larger than control average current (not shown). Middle column: selected records from an ensemble of 120 records gathered during the first 4 min after the addition of 2 μM-D600 to the adrenaline solution. Bottom record is the average current. Right-hand column: selected records from an ensemble of 120 records gathered 28–32 min after addition of D600. Bottom record is the average current, whose amplitude was about 85% of that before D600. The ensemble numbers of the selected records (top to bottom) are: left, 3, 29, 30, 35, 43, 52, 53, 63, 66, 73, 80 and 91; middle, 15, 26, 29, 30, 53, 55, 67, 73, 76, 87, 109 and 118; right, 6, 7, 28, 30, 41, 50, 73, 78, 95, 109, 113 and 117.

open time of 0·59 ms, mean closed times of 0·35 and 1·8 ms, P_f of 0·77, and average current amplitude of 0·16 pA. After 15 min adrenaline (Fig. 7, left-hand column) longer openings (τ = 1·24 ms), shorter closings (τ values of 0·17 and 0·9 ms), and larger P_f (0·90) produced an average current amplitude of 0·51 pA. These changes,

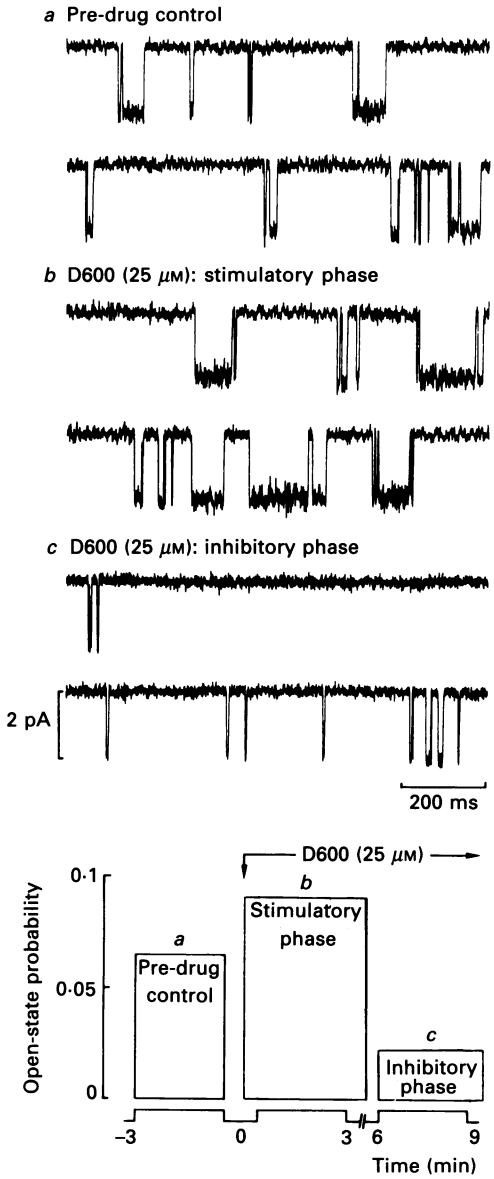

Fig. 8. The dual action of D600 on a purified DHP-binding site reconstituted to a functional Ca^{2+} channel in a lipid bilayer. The bilayer was bathed in symmetrical solutions (90 mM-$BaCl_2$, 5 mM-HEPES, 0·02 mM-TTX), held at a constant intrapipette of −100 mV, and exposed to solution containing 25 μM-D600 at 0 min. *A*: segments of continuous 3 min records immediately before drug (*a*), just after drug addition (*b*), and 6–9 min after drug addition (*c*). The stimulation (*b*: longer openings, shorter closings) observed shortly after the addition of drug was transitory and followed by marked inhibition (*c*: fewer, shorter openings, longer closings). *B*: channel open-state probability during the experimental phases. The open-state probability in each phase was obtained by dividing the average current by the average open-channel current amplitude.

and the increase in time to half-inactivation from about 110 to 270 ms, are typical of the action of β-adrenergic agonists (Tsien *et al.* 1986). After the addition of 2 μM-D600, there were no signs of early stimulation (Fig. 7, middle column). More surprisingly, there were few indications of inhibition in the ensemble gathered 30 min later (Fig. 7, right-hand column). Multiblank episodes were conspicuously absent ($P_f = 0{\cdot}93$), and while non-blanks exhibiting sprays of short openings separated by long closings were present (e.g. third from top, right), they were infrequent. The difficulty in attributing record-long or short-lasting infrequent spraying to drug inhibition is that spraying can also be observed under control (not shown) and adrenaline conditions (e.g. second and fourth top sweeps, left). This quandary in dealing with individual records does not extend to ensemble analysis, and the latter indicated that D600 had caused a 40% reduction in mean open time, with little change in mean closed times. The net effect was a 15% reduction in average current amplitude, a slight delay in time to peak, and an unchanged rate of decay (Fig. 7, bottom). This inhibition is quite modest compared to that caused by much shorter exposures to D600 in the absence of adrenaline (e.g. Figs 5 and 6).

Dual action on unitary currents through reconstituted DHP-binding sites

Purified DHP-binding sites from the transverse tubules of rabbit fast skeletal muscle were incorporated into lipid bilayers and bathed with symmetrical solutions containing 90 mM-$BaCl_2$, 5 mM-HEPES, and 0·02 mM-TTX. At a constant intra-pipette potential of -100 mV, pre-drug current had a unitary amplitude of 2 pA as illustrated by the two records in Fig. 8*A a*. The channel opened sporadically for variable times, many in the 10–100 ms range. D600 was then added to the bath solution to give a final concentration of 25 μM (roughly double that reported by Palade & Almers (1985) as the EC_{50} (holding potential, -70 mV) for inhibition of Ca^{2+} channel currents in rabbit skeletal muscle fibres). Channel activity was stimulated (longer openings, shorter closings) in the first 3 min after drug equilibration (Fig. 8*A b*). As in the cardiac preparations, stimulation was short-lived, and subsequent inhibition (6–9 min) was characterized by a marked attenuation of open times and prolongation of closed times (Fig. 8*A c*).

The open-state probability was calculated by dividing the average current during each 3 min phase by the average elementary current amplitude. The probability increased from 0·065 (pre-drug) to 0·092 (0–3 min) before declining to 0·016 (6–9 min) (Fig. 8*B*). Normalization relative to the control open-state probability gives 1 : 1·42 : 0·24, which is not unlike the results obtained from cardiac single Ca^{2+} channels.

DISCUSSION

Inhibition of Ca^{2+} channel activity by D600 was sometimes preceded by transient stimulation. The dual action was expressed in tissues, cells and reconstituted channels, and was independent of charge carrier and temperature. It resembled that described for primary DHP inhibitors of Ca^{2+} channels, and this raises the question of common mechanism(s). The Discussion begins with this theme and then focuses more closely on the dual action of D600.

Dual action: comparison of DHP inhibitors and D600

The dual action of primary DHP inhibitors resembles that of D600 in the following ways. (1) Stimulation by DHP has not been a regular finding (e.g. Lee & Tsien, 1983; Brown *et al.* 1986). (2) Stimulation is short lived, and enhanced by slow pulsing from negative holding potentials (Brown *et al.* 1986; Kokubun *et al.* 1986; Kass, 1987; Scott & Dolphin, 1987). (3) Maximum stimulation of macroscopic current amplitude appears to be around 30–40% (Brown *et al.* 1984, 1986; Kass, 1987; Scott & Dolphin, 1987). (4) Stimulation of single Ca^{2+} channel current is due to longer openings and fewer blanks; inhibition is due to an increased number of blanks (Hess *et al.* 1984; Reuter *et al.* 1985; Brown *et al.* 1986). (5) Inhibition is relieved by membrane hyperpolarization (Sanguinetti & Kass, 1984; Brown *et al.* 1986).

A few differences can be pinpointed. DHP-stimulated and DHP-inhibited currents nearly always have an enhanced rate of inactivation (Hess *et al.* 1984; Reuter *et al.* 1985; Brown *et al.* 1986; Kokubun *et al.* 1986). By contrast, neither an enhanced decay of macroscopic current (McDonald *et al.* 1984*a*, *b*; Uehara & Hume, 1985), nor an enhanced late silence of single channels (Fig. 4), is apparent during D600 inhibition. A second difference is that DHP inhibition does not seem to include large changes in open and closed times (Hess *et al.* 1984; Reuter *et al.* 1985; Brown *et al.* 1986).

Regulatory G-protein and the stimulatory actions of DHP inhibitors and D600

Scott & Dolphin (1987) have proposed that G_i/G_o is involved in the stimulatory effects of DHP and D600 on Ca^{2+} channel current in cultured DRG neurones. Both 5 μM-nifedipine and 10 μM-D600 caused a transient 10–30% stimulation of Ba^{2+}-carried current that was absent after pertussis toxin pre-treatment. Intracellular application of the guanosine triphosphate (GTP) analogue, GTP-γ-S, left a small residual L-type current which was increased up to 5-fold by nifedipine or D600. The authors suggested that activated G_i/G_o stabilizes the channel resting state and enhances ligand binding to stimulatory sites. The concept of distinct stimulatory sites was invoked earlier to explain DHP stimulation of cardiac Ca^{2+} channel activity. One group (Kokubun *et al.* 1986) proposed that binding to these sites switches the channel into mode 2, the high P_o mode of intrinsic tri-modal channel gating (cf. Hess *et al.* 1984); the other group (Brown *et al.* 1986) felt that stimulatory binding caused multiple changes in channel kinetics.

The dual action of D600

In cell-attached patch experiments, D600 was not present in the solution bathing the external face of patch membranes. Drug in the cell superfusate reached the channel via intramembrane diffusion from extrapatch membrane or, more likely, via intracellular diffusion after transmembrane passage. This rules out stimulation of the patch channel by extracellular charged drug but provides no direction on whether non-external neutral drug, charged drug or a particular isomer is the active form. The results also do not allow us to reach definite conclusions on two important aspects of D600 action: (1) the concentration dependence of the stimulation, and (2) the issue of one or more active binding sites.

Stimulation

The equilibration of D600 in cardiac preparations is a relatively slow process (McDonald *et al.* 1984*a*). Therefore, it is possible that stimulation was recorded as an early transient event because at later times concentration-dependent inhibitory action overwhelmed the stimulatory action. Alternatively, stimulation was short-lived due to a run-down of some nature. Since inhibitory action can be relieved by long hyperpolarizations (McDonald *et al.* 1984*b*), it would seem that long-lasting stimulation masked by inhibition could easily be revealed by suitable voltage manipulations. In an extensive series of experiments on cat ventricular muscle treated with 2 μM-D600 for 60 min or longer, I_{Ca} larger than control was not detected after long hyperpolarizations to potentials as negative as -140 mV (McDonald *et al.* 1984*b*). Similarly, Uehara & Hume (1985) did not unmask stimulation after maximal unblock of Ca^{2+} channels in frog atrial cells treated for 30 min with D600, diltiazem or DHP. Despite these results, it is possible that a stimulatory drug action was present at these later times but masked by 'tonic' inhibition resistant to unblocking procedures.

Scott & Dolphin (1987) have implicated G_i/G_o in D600 and DHP stimulation of Ca^{2+} channel current in DRG cells, and a similar situation may exist in heart cells. On the other hand, Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown (1987) found that activated G_s regulated protein (but not an activated G_i/G_o ('G_K')) has a direct stimulatory action on cardiac Ca^{2+} channels, and proposed that G_s is an endogenous channel regulator. An interplay between D600 binding and a regulatory G_s system is an attractive proposition. At low drug concentrations, infrequent occupancy of an inhibitory binding site by drug causes fleeting inhibition. However, drug binding enhances activated G_s association, and longer dwell time in the up-regulated mode more than offsets the depression produced by short dwells in the drug-bound mode. Unfortunately, a display of records showing apparent fleeting inhibition during the stimulatory phase does not provide much support for the hypothesis because inhibitory-like sequences can also be found in the absence of D600 (Fig. 7). On the other hand, the shorter, less frequent periods of low P_o observed during the stimulatory phase are consistent with enhanced dwells in the up-regulated mode. Factors causing a tilt towards inhibition could include enhanced drug concentration and inhibitory site occupancy with time, down-regulation with time, positive holding potentials, and exhaustion of local GTP supply.

Adrenaline seems to produce an environment that hinders drug inhibition. The resistance might be related to Ca^{2+} channel phosphorylation or to activation of G_s since both are consequences of β-adrenergic stimulation (see Trautwein, Kameyama, Hescheler & Hofmann, 1986; Yatani *et al.* 1987). Channel phosphorylation may not be very protective since 2 μM-D600 fully suppressed cardiac Ca^{2+} channel current stimulated by injection of C subunit (Osterrieder, Brum, Hescheler, Trautwein, Hofmann & Flockerzi, 1982). On the other hand, GTP greatly accelerated the dissociation of [^{3}H]verapamil from muscle membrane binding sites (Galizzi, Fosset & Lazdunski, 1984) raising the possibility that an activated G_s–channel complex may present a very low-affinity target for D600 (a consequence which would also favour early stimulation by drug as described above).

A major problem with the G_s scheme (or with a G_i/G_o scheme) is that D600 also stimulated current flow through reconstituted DHP-binding sites. This seems to rule out an associated co-factor that requires the presence of a high-energy activating compound, but further experiments with G-protein inhibitors are necessary for a definitive answer.

Inhibition

As speculated for the dual action of DHPs, there may be separate stimulatory and inhibitory sites for D600 rather than the single inhibitory site discussed above. The issue does not affect consideration of the basis of the inhibition. Inhibition by D600 is characterized by (a) an increase in the occurrence of blanks, and (b) shorter openings and longer closings in non-blank records. The increase in blanks seems to be due to a stabilization of the inactivated state. Whether the marked changes in fast gating kinetics can be attributed to sporadic stabilization is very questionable because exit from the 'non-stabilized' inactivated state during a depolarization is infrequent (Cavalié *et al.* 1986; McDonald *et al.* 1986). One would have to postulate a drug-induced pseudo-inactivation state from which escape is imminent upon drug unbinding. It is also difficult to attribute this aspect of D600 action to an alteration in the kinetics between closed and open states. Suitable alterations (e.g. slower forward rate constants, slower backward rate constants) would necessarily result in greatly delayed times to first openings and macroscopic peak currents, neither of which have been observed. Physical occlusion of the open channel is a possibility that is not easily discounted.

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