J. Physiol. (1984), **352**, *pp.* 203–216
With 6 *text-figures*
Printed in Great Britain

CAT VENTRICULAR MUSCLE TREATED WITH D600: EFFECTS ON CALCIUM AND POTASSIUM CURRENTS

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(*Received 10 August 1982*)

SUMMARY

1. In single sucrose-gap experiments on cat ventricular muscle strands stimulated with 300 ms pulses at 0·33 Hz, 2 μM-D600 reduced the Ca-dependent slow inward current (I_{Ca}) by 50 % within 5 min and more than 90 % in 90–120 min. The late outward current was reduced by up to 30 %.

2. During the exposure to D600, Ca channels could be unblocked by hyperpolarizing pulses and blocked again by stimulation with depolarizing pulses. Since the degree of unblocking depended on voltage, and the degree of blocking depended on stimulation pattern, I_{Ca} amplitude could be rapidly manipulated to probe the dependence of K conductance on I_{Ca}.

3. Under control conditions, an increase in stimulation rate from 0·02 to 1 Hz reduced I_{Ca} by 15 % and increased the late outward current by a smaller amount. During exposure to D600, a similar intervention provoked a 60 % reduction in I_{Ca}, but a control-like increase in the late outward current.

4. Two other series of experiments failed to disclose a link between I_{Ca} and K conductance: when a block of Ca channels was reimposed following their unblocking, the outward currents were independent of I_{Ca} amplitude.

5. Unblock–block experiments also provided information on the extent of steady-state I_{Ca} at 0 mV. The fraction of Ca channels not undergoing inactivation appears to be very small.

6. During full D600 block, the inward peak of the current wave form is broad and very much delayed in comparison with pre-drug currents or currents on the first pulse following unblocking. A similar wave form was recorded in D600-treated ventricular myocytes from cat but not guinea-pig. The likely explanation is that D600 unmasks a small transient outward current in cat ventricle.

INTRODUCTION

D600 and verapamil block the Ca-dependent slow inward current (I_{Ca}) in cardiac tissue (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Kass & Tsien, 1975; Nawrath, Ten Eick, McDonald & Trautwein, 1977; Noma & Trautwein, 1978;

McDonald, Pelzer & Trautwein, 1980). Consequent effects include depressed excitability in sino-atrial and atrio-ventricular nodal tissue (Wit & Cranefield, 1974; Cranefield, 1975), a depressed action potential plateau in Purkinje fibres (Rosen, Ilvento, Gelbrand & Merker, 1974; Wit & Cranefield, 1974) and negative inotropy in working myocardium (Singh & Vaughan Williams, 1972; Bayer, Hennekes, Kaufmann & Mannhold, 1975*a*; Bayer, Kalusche, Kaufmann & Mannhold, 1975*b*; Nawrath *et al.* 1977).

These actions are modified by the rate of stimulation. For example, an increase in driving rate induces a more pronounced depression of the ventricular action potential (Bayer *et al.* 1975*b*) and force of contraction (McCans, Lindenmayer, Munson, Evans & Schwartz, 1974; Bayer *et al.* 1975*a*; Siegl & McNeill, 1980). The likely explanation is that these drugs exert their effects by blocking Ca channels in a frequency- and voltage-dependent manner (Ehara & Kaufmann, 1978; McDonald *et al.* 1980).

The blocking action of D600 (or verapamil) is not restricted to Ca channels. D600 depresses current through structures as different as the acetylcholine-operated channel in the frog end-plate (Miledi & Parker, 1980), the Na channel in squid axon (Baker, Meves & Ridgway, 1973) and, as judged by the $\dot{V}_{max}$ of action potential upstrokes, the Na channel in heart (Singh & Vaughan Williams, 1972; Bayer *et al.* 1975*b*; Hirata, Kodoma, Iwamura, Shimizu, Toyama & Yamada, 1979). With regard to K channels, the situation in cardiac tissue remains uncertain. Verapamil is reported to have no effect on the late outward current (Kohlhardt *et al.* 1972; Ehara & Kaufmann, 1978), or to induce a major reduction only when the stimulation rate is increased from 0·1 to 1 Hz (Bassingthwaighte, Fry & McGuigan, 1976). D600 may reduce the outward current (Nawrath *et al.* 1977), have no effect (Noma & Trautwein, 1978), or have dose-dependent effects (Kass & Tsien, 1975). Whether or not these drugs have a direct action on K channels, intracellular Ca (Ca_i)-activated K conductance (g_K) (see Isenberg, 1975; Meech, 1978) may be depressed as a result of the block of I_{Ca} (Bassingthwaighte *et al.* 1976; Nawrath *et al.* 1977). However, in relation to other cardiac conductances, the importance of Ca_i-activated g_K in general, and I_{Ca}-linked g_K in particular, is still unclear (see McDonald, 1982).

In this study we have treated cat ventricular muscles with D600 for up to 3 h to obtain information on the following topics: (a) the time courses of the drug-induced changes in the action potential, I_{Ca} and outward currents, (b) the ionic basis of the frequency-dependent changes in the action potential, (c) the dependence of g_K on I_{Ca} amplitude, and (d) the fraction of Ca channels which activate but do not inactivate. It was possible to study the latter two subjects from a new angle because the degree of Ca channel block could be rapidly altered by voltage-clamp sequences which promote or relieve block (see accompanying paper: McDonald, Pelzer & Trautwein, 1984). In the Discussion we explore the possibility that full block of I_{Ca} by D600 unmasks a small transient outward current.

METHODS

The experiments were performed on right ventricular trabeculae or papillary muscles from cat hearts. Cats were killed following ether narcosis. Hearts were quickly excised and placed in oxygenated Tyrode solution. Free-running bundles of suitable diameter (0·15–0·4 mm) and length (3–5 mm) were tied off with fine silk suture and dissected from the right ventricle.

In the action potential/contraction studies, muscles were mounted horizontally in a plastic bath perfused with Tyrode solution of the following composition (mM): NaCl, 140; KCl, 5·4; $MgCl_2$, 1·0; $CaCl_2$, 1·8; $NaHCO_3$, 12·0; NaH_2PO_4, 0·4; glucose, 5·0. One end of the muscle was held by a clamp and the suture on the other end was tied to a fine rod connected to a force transducer (Statham UC2). After establishing a resting tension of 50–100 mg, the muscle was allowed to recover with regular stimulation at 0·33 Hz for 45 min. Muscles were impaled with micro-electrodes (3 mM-KCl; 10–20 MΩ) which were connected by an Ag–AgCl pellet to a high-impedance voltage follower.

In the voltage-clamp experiments muscles were mounted in a bath having three compartments separated from each other by rubber sheets. The muscles fitted snugly through holes burned into the rubber. The portion of the muscle which protruded into the right-hand (test) compartment had a length of 0·3–0·5 mm and was fixed to the pin of the force transducer. The segment in the middle (sucrose) compartment was 1·5–2·0 mm in length and the rest of the muscle was located in the left-hand (KCl) compartment. Each compartment could be perfused from several sources by hydrostatic pressure, the solution being removed by suction. The mounting of the muscle in the bath was followed by a 45 min recovery period during which all three compartments were perfused with Tyrode solution and the muscle driven at 0·33 Hz. Thereafter, the sucrose gap was established by perfusing the middle compartment with solution containing 304 mM-sucrose (enzymic grade), 5 mM-glucose and 0·01 mM-$CaCl_2$ dissolved in de-ionized water. At the same time the Tyrode solution perfusing the left-hand compartment was replaced by KCl-Tyrode solution (NaCl replaced by KCl).

Tyrode solutions were equilibrated with 95 % O_2 and 5 % CO_2. The temperature of the solutions was 36 ± 1 °C and the pH 7·4 ± 0·05. Tyrode solutions containing D600 (Knoll AG, Ludwigshafen am Rhein, F.R.G.) at desired concentrations were prepared by adding appropriate amounts of drug stock solutions to the normal Tyrode. Tetrodotoxin (Sigma, St Louis, U.S.A.) at 5×10^{-5} M was added to the solutions during the voltage-clamp experiments. This had no detectable effect on the amplitude or time course of I_{Ca}.

In the voltage-clamp method used, membrane potentials were recorded with conventional intracellular micro-electrodes and the membrane current was applied across a sucrose gap. A description of the circuits employed, limitations of the method, trans-gap resistance, etc., may be found in New & Trautwein (1972) and McDonald & Trautwein (1978*a*). Membrane currents, voltage and tension were recorded on f.m. tape. Records were edited, amplified, synchronized and superimposed where required by means of a transient recorder (Biomation, model 1015). The output of the transient recorder could be fed to an oscilloscope for photography and to an *X–Y* pen recorder for further analysis.

RESULTS

Time course and steady-state effect of D600 on the action potential and membrane currents

Fig. 1 illustrates the steady-state (> 90 min) effects of 2 μM-D600 on the membrane currents of cat ventricular muscles stimulated at 0·33 Hz. The configuration of the currents elicited by 300 ms pulses from −50 to 0 mV is changed in three ways (Fig. 1*A*): (a) the early inward I_{Ca} transient is nearly completely suppressed such that no net inward current is recorded, (b) the small inward-directed transient which remains reaches a broad peak in 30–100 ms, and (c) the late outward current flowing at 300 ms is reduced by 10–30 %. The last change can be observed as a reduction in both time-independent outward current and time-dependent K current (I_K) (activation time constant ~300 ms). The depression of I_K as measured from semilogarithmic plots of currents elicited by 2 s long depolarizations (see McDonald & Trautwein, 1978*b*) was about 20 % in the example experiment (Fig. 1*B*) and less than 30 % in three other muscles.

In muscles driven at 0·33 Hz, 2 μM-D600 induced a rapid depression of the action potential plateau during the first 10 min, and then a slower one to steady state by 120 min (Fig. 2*A*). Despite a 40 % reduction in the duration of the action potential

plateau (0 mV) at this time, the duration at 90% repolarization was 10% longer. Commensurate changes were observed in membrane currents elicited by 300 ms pulses from −50 to 0 mV at 0·33 Hz (Fig. 2*B*). There was a progressive, large suppression of the inward I_{Ca} peak and smaller suppression of the late outward current. In seven muscles 2 μM-D600 reduced I_{Ca} to 50% of the control value in about 5 min (Fig. 2*C*), to 30% in 30 min, and to less than 10% in 90–120 min. The rate of decline of I_{Ca} amplitude increased with drug concentration (0·5–5 μM).

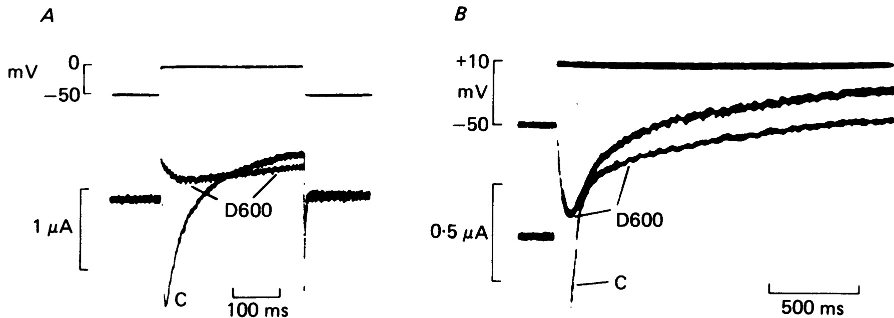

Fig. 1. Steady-state effects of 2 μM-D600 on the membrane currents of cat ventricular muscles. *A*, voltage-clamp pulses from −50 to 0 mV for 300 ms were applied at 0·33 Hz. In comparison with control (C), the records taken after 130 min D600 illustrate the marked suppression of the inward I_{Ca} transient, the emergence of a slow inward-directed transient, and the depression of the late outward current at 300 ms. *B*, membrane currents in response to 2 s depolarizations from −50 to +10 mV before (C) and after 90 min in D600. Regular stimulation with 300 ms pulses at 0·33 Hz was interrupted for 5 s before the 2 s pulses. At this amplification the early phase of the I_{Ca} transient was chopped off in the control record. After D600 treatment there is a delayed time to peak of the inward-directed transient and about a 20% reduction in rising outward I_K. Records in both *A* and *B* are superimposed with reference to zero current.

It is important to note that the D600-induced prolongation of the time to peak I_{Ca} (Figs. 1 and 2) is not an artifact related to a voltage-clamp problem such as high series resistance in the preparation or deterioration of clamp quality with time. First, if series resistance were indeed large enough to produce artifactual prolongation under control conditions (see Attwell & Cohen, 1977; Isenberg & Klöckner, 1982), the depression of membrane conductance during D600 treatment would not further lengthen the time to peak current. Secondly, there could not have been a deterioration of clamp quality with time because inward currents with pre-drug times to peak were recorded following unblocking of Ca channels (e.g. Fig. 4*B*). Finally, the prolongation can also be observed in non-sucrose gap experiments on isolated cat myocytes (Fig. 6). As reasoned in the Discussion, the most likely explanation is that a transient outward current is unmasked by the D600 treatment. Since I_{Ca} amplitude was taken as the difference between the peak inward deflexion and the current level after 250 ms depolarization, the presence of a transient outward current has implications for the accuracy of the measurements. However, the problem is not severe enough to interfere with the general conclusions of the study (see Discussion).

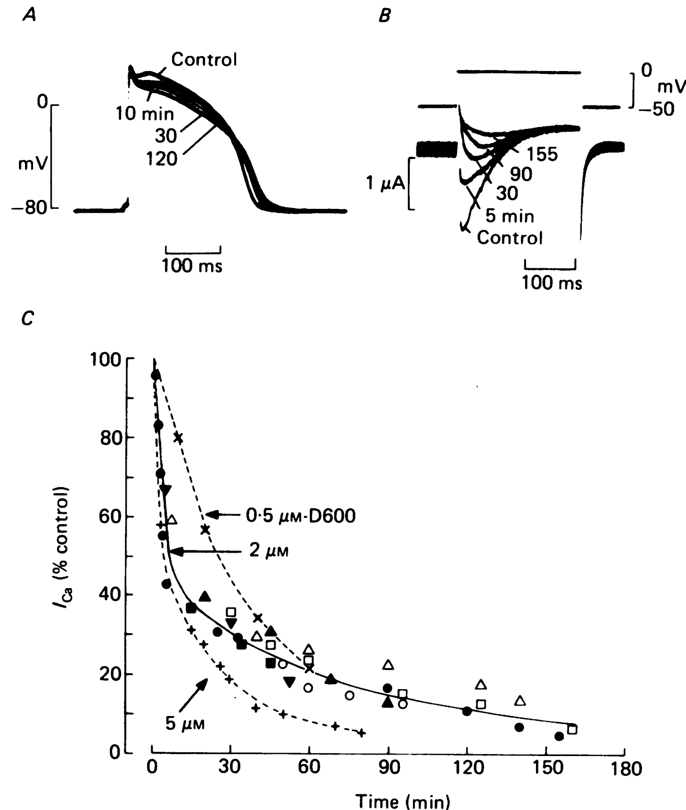

Fig. 2. Time courses of D600 effects on muscles stimulated at 0·33 Hz. *A*, progressive depression of the action potential plateau and prolongation of terminal repolarization during 2 μM-D600 superfusion. *B*, membrane currents accompanying 300 ms depolarizations from −50 to 0 mV before and during 2 μM-D600 treatment. Since the traces were superimposed with reference to the late outward current level at 300 ms, the progressive decline in the late outward current is indicated by the positive shift in holding current. *C*, the time course of I_{Ca} depression in muscles treated with D600 at 0·5 μM ($n = 1$), 2 μM ($n = 7$) or 5 μM ($n = 1$) and stimulated with 300 ms pulses from −50 to 0 mV at a rate of 0·33 Hz.

Outward current and the degree of Ca channel block

In D600-treated cat ventricular muscle, there is a concomitant depression of I_{Ca} and outward current. While this suggests a blocking action on both Ca and K channels, it could also be explained by a block of Ca channels alone with consequent suppression of Ca_i-activated g_K. The frequency- and voltage-dependent features of Ca channel block by D600 provide a means of assessing the impact of Ca channel block on g_K because large changes in the degree of Ca channel block can be achieved within a short time span by (a) changing the frequency of stimulation, or (b) unblocking channels with a hyperpolarizing pulse and then re-blocking them with conditioning depolarizations (conditioned block).

Stimulation frequency. From a holding potential of −80 mV, cat papillary muscle was depolarized with a 200 ms pre-pulse to −50 mV and stimulated with a 300 ms

pulse to 0 mV to activate I_{Ca} and outward current. A train comprising thirty of these two-step commands was applied at 0·33, 1·0 and then 0·02 Hz. The membrane currents recorded on the thirtieth pulse of each train before (control) and after 2 μM-D600 for 60 min are shown in Fig. 3*A*. Under control conditions I_{Ca} declined by about 15 % between 0·02 and 1 Hz, and the outward current increased by a smaller amount (about 5 % of the I_{Ca} amplitude at 0·02 Hz). After D600 treatment the decrease in I_{Ca} with frequency was much more pronounced. At 0·02 Hz, I_{Ca} amplitude was about 75 % of the control value, but at 1 Hz it was down to 35 % of control. Despite the frequency-related differences in I_{Ca} between control and drug trials, the increase in outward current with rate was not affected.

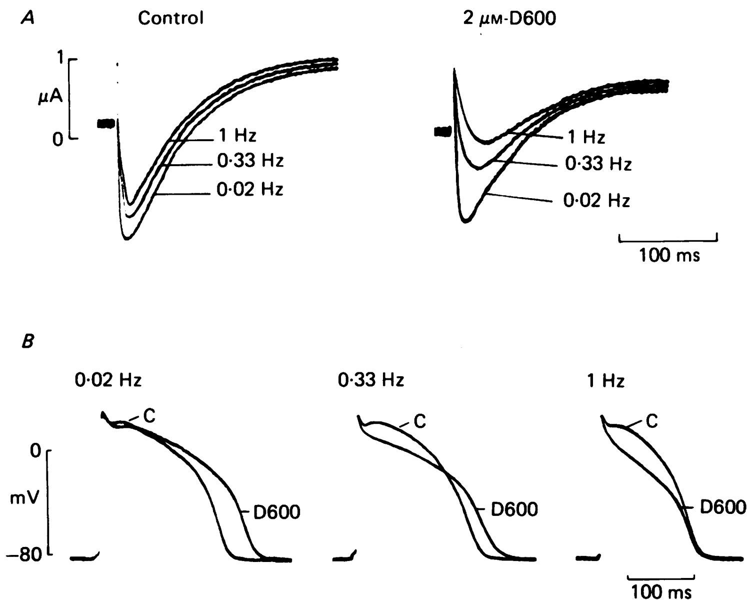

Fig. 3. Rate-dependent changes of membrane currents and action potentials in muscles treated with 2 μM-D600. *A*, membrane currents on the thirtieth pulse of trains applied in the order 0·33, 1 and 0·02 Hz before (control) and after 60 min of treatment with D600. Each 300 ms pulse from −50 to 0 mV was preceded by a 200 ms pre-pulse to −50 mV from the holding potential of −80 mV. The increase in the late outward current with stimulation rate was not affected by the drug despite the large change in I_{Ca}. *B*, action potentials recorded when the driving rate was increased from the basic rate of 0·33 Hz to 1 Hz for 1 min, and then reduced to 0·02 Hz for 10 min before (C) and after 140 min of D600.

Action potentials from another preparation driven at these frequencies are shown in Fig. 3*B*. As expected from the I_{Ca} measurements, the frequency-dependent depression of the action potential plateau was far greater after D600 treatment. The longer action potential duration at 0·02 Hz with the drug compared with the control is explained by the reduction in outward current, whereas the shortening of the duration with rate under both drug and control conditions ensues from the increase in outward current and decrease in I_{Ca}. Similar voltage-clamp and action potential measurements were obtained from nine other preparations.

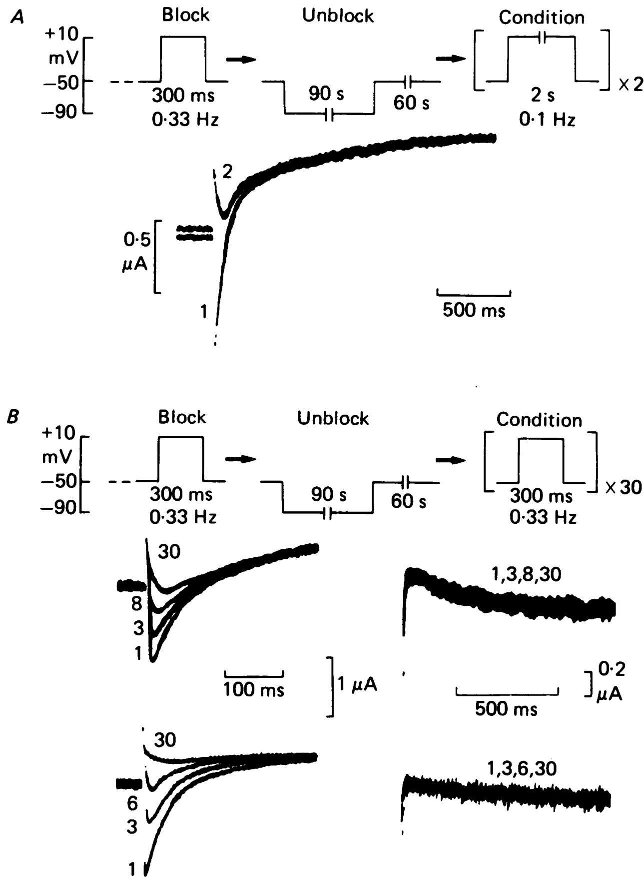

Fig. 4. The influence of I_{Ca} on time-dependent I_K during conditioned blocks. *A*, protocol. In a muscle treated with 2 μM-D600 for 120 min, I_{Ca} was blocked with 300 ms pulses at 0·33 Hz, unblocked (90 s at −90 mV, 60 s at −50 mV), and then conditioned with two 2 s long depolarizations at 0·1 Hz. The currents on the two pulses (1, 2) are superimposed here. Note that the time course and amplitude of I_K were independent of I_{Ca} amplitude (the beginning of the large I_{Ca} transient on pulse 1 was off the oscilloscope screen at this gain). *B*, membrane currents recorded during the conditioned blocks which followed unblocking sequences (see schematic diagram). The muscles were treated with 2 μM-D600 for either 40 min (upper traces) or 90 min (lower traces). Left-hand traces, currents accompanying the conditioning depolarizations; right-hand traces, I_K tail currents on the conditioning repolarizations (these were recorded at a lower sweep speed and higher gain than the currents on depolarization). I_{Ca} restored by the unblocking sequence was blocked again during the conditioning, but there was no detectable influence of I_{Ca} amplitude on I_K activation. The labels refer to the conditioning pulse numbers.

Conditioned block. After full block of Ca channels has been achieved with regular pulsing of D600-treated ventricular muscle, nearly complete unblocking of the channels can be obtained by applying an unblocking voltage-clamp sequence such as 90 s at −90 mV, 60 s at −50 mV. For example, in seven muscles treated with 2 μM-D600 for 2 h or more, this unblocking sequence restored I_{Ca} to 80–85 % of the pre-drug control I_{Ca} (McDonald *et al.* 1984). Channels freed of the drug are blocked

again upon resumption of stimulation, the time course of this conditioned block being dependent on the stimulation pattern. With 300 ms pulses to 0 mV at 0·33 Hz, it develops rapidly over the first eight pulses and reaches steady state within thirty pulses. However, a single depolarization of long duration can also produce effective block (McDonald *et al.* 1984).

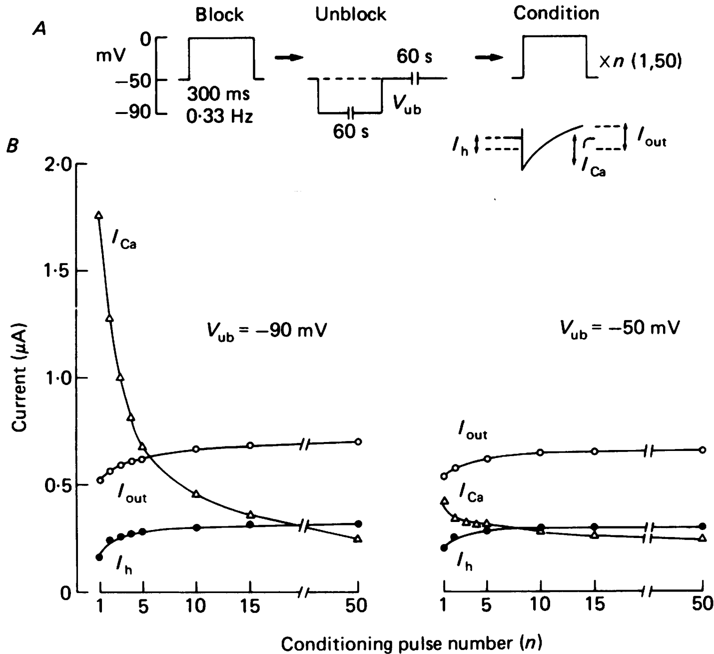

Fig. 5. The amplitudes and time courses of current components during conditioned blocks. *A*, procedure. A muscle exposed to 2 μM-D600 for 100 min was blocked with 300 ms pulses from −50 to 0 mV at 0·33 Hz. Unblocking (60 s at −90 mV, 60 s at −50 mV) was followed by conditioning (50 pulses at 0·33 Hz), a second unblocking (120 s at −50 mV) and similar conditioning. V_{ub} refers to the potential during the first 60 s of the unblocking sequence. The parameters measured during the conditioned block were I_{Ca}, the outward current at the holding potential (I_h), and the late outward current at the end of the 300 ms depolarizations (I_{out}). The latter two amplitudes were taken with reference to zero current (lower dashed line). *B*, the decline of I_{Ca} and increase in outward current during the two conditioned blocks imposed after effective (V_{ub}−90 mV) or ineffective (V_{ub}−50 mV) unblocking. Despite the large difference in I_{Ca} during the two blocks, changes in the outward currents were similar in magnitude and time course.

The activation of I_K is monoexponential with a time constant of 300–400 ms at +10 mV; I_K tail currents deactivate with a similar time constant at −50 mV (McDonald & Trautwein, 1978*b*). We looked for changes in I_K during the conditioned block by recording I_K on depolarization and I_K tails upon repolarization (Fig. 4). Ca channels were unblocked (90 s at −90 mV, 60 s at −50 mV) and then blocked again with conditioning depolarizations. For the experiment on the activation of I_K (Fig. 4*A*) we took advantage of the fact that a single depolarization for 2 s can induce nearly complete block of Ca channels. Thus, the conditioning protocol consisted of a 2 s long pulse to +10 mV followed by a similar pulse 10 s later. The records in Fig. 4*A* show

that I_K flowing at +10 mV after large I_{Ca} (first conditioning depolarization) was not detectably different from I_K after small I_{Ca} (second conditioning depolarization). Outward currents triggered by subsequent pulses in the train (not shown) were superimposable on these two traces. Similar results were obtained on three other muscles.

In muscles with well-developed I_K systems, 300 ms activating pulses elicit measurable outward I_K tail currents when the membrane is repolarized to −50 mV. Fig. 4*B* shows records of conditioned blocks in two muscles treated with 2 μM-D600 for 40 and 90 min, respectively. In each case the superimposed traces on the left-hand side illustrate the progressive block of I_{Ca} while those on the right show the I_K tails at slower sweep speed and higher gain. Although the signal to noise ratio of the I_K tail records is not very favourable at this amplification, the conclusion is the same as that drawn from the experiment in Fig. 4*A*, i.e. large I_{Ca} immediately after unblocking Ca channels provokes no larger an I_K than small I_{Ca} at a later stage of block.

Small changes in the holding current and in the late outward current were consistently observed. We documented their pattern and magnitude during two conditioned blocks, one of which was initiated after a much more efficient unblocking procedure than the other. As depicted in Fig. 5, a muscle exposed to 2 μM-D600 for 100 min was in a blocked state after 0·33 Hz stimulation with 300 ms pulses from −50 to 0 mV. Conditioned blocks were imposed following either an effective unblocking sequence (60 s at −90 mV, 60 s at −50 mV; left-hand plot) or one which provided only token unblocking (120 s at −50 mV; right-hand plot). In each case there was an increase in both the holding current (I_h) and the late outward current (I_{out}) during the conditioning. However, there was only a marginal difference in amplitude and no difference in the pattern of these changes during the two blocks. In experiments on six additional muscles the changes in I_h and I_{out} were expressed as a percentage of I_{Ca} amplitude after the effective unblocking. Mean ± S.D. of the changes were: I_h 8 ± 1% and I_{out} 10 ± 3% following the effective unblocking sequences, and I_h 6 ± 1%, I_{out} 7 ± 2% following the ineffective unblocking sequences.

DISCUSSION

Prolongation of the time to peak I_{Ca}

Nawrath *et al.* (1977) were first to report a prolongation in time to peak I_{Ca} after prolonged exposure of cat ventricular muscle to D600. They proposed that the drug blocks some Ca channels and slows the activation of the remaining non-blocked channels. This explanation cannot be completely dismissed but the following points argue against it. (a) If the prolonged time to peak were related to a drug–Ca channel interaction, there is little reason why it should not have been observed in other cardiac preparations treated with D600. However, it has not been reported in studies on drug-treated rabbit sino-atrial node (Noma & Trautwein, 1978; Noma, Kotake & Irisawa, 1980) or atrio-ventricular node (Kokubun, Nishimura, Noma & Irisawa, 1982). (b) Considering the similarity of Ca channel block and Na channel block by organic agents (see McDonald *et al.* 1984), one might have expected that at least one of the many Na channel blockers examined up to this point would greatly prolong the time to peak I_{Na}; this is not the case (e.g. Strichartz, 1973; Courtney, 1975;

Cahalan, 1978). (c) The final argument concerns the relative outward amplitude of the inward-directed transient during full block. The level of the late outward current (300 ms) at 0 mV reflects outward I_K activated during the step (time constant ~ 300 ms) plus the time-independent background current. However, during the first 10–20 ms of the step (negligible I_K activation) the current is actually outward with respect to the late outward current at 300 ms (Figs. 1*A* and 2*B*). Therefore, the inward-directed transient must be a decaying outward current rather than slowly activating I_{Ca}.

Fig. 6. Membrane currents in ventricular myocytes isolated from guinea-pig (left) and cat heart (right) and perfused with Tyrode solution (36 °C) containing 2 μM-D600 for about 45 min. The currents were recorded during 0·33 Hz stimulation with 300 ms pulses from −50 to 0 mV. A transient outward current is apparent only in the cat myocyte record. Details of the enzymatic dissociation and electrophysiological procedures may be found in Osterrieder *et al.* (1982).

Our conclusion is that in contrast to the situation in some other cardiac preparations, a sharp reduction of I_{Ca} in cat ventricle reveals the presence of a transient outward current (I_{to}). This view is reinforced by data acquired from voltage-clamp experiments on cat and guinea-pig ventricular myocytes exposed to 2 μM-D600: an I_{to} was apparent in the myocytes from cat but not guinea-pig (Fig. 6). It is likely that this current contributes to the early repolarization in cat ventricle (e.g. Figs. 2*A* and 3*B*) and that it is analogous to an I_{to} underlying early repolarization in cardiac Purkinje fibres (see Peper & Trautwein, 1968; Fozzard & Hiraoka, 1973; Siegelbaum, Tsien & Kass, 1977; Kenyon & Gibbons, 1979; Marban, 1981; Boyett, 1981; Coraboeuf & Carmeliet, 1982).

Effect of I_{to} on I_{Ca} measurements. Since D600 may affect I_{to} as well as I_{Ca} (Siegelbaum & Tsien, 1980), it is difficult to gauge the effect of I_{to} on I_{Ca} measurements without having an estimate of their relative amplitudes under pre-drug conditions. We have attempted to find the lower and upper limits of this effect by analysing records such as those in Fig. 1*A*. Calculations of the lower limit depend on the assumption that D600 can completely block I_{Ca} (see Noma *et al.* 1980; Siegelbaum & Tsien, 1980; Isenberg & Klöckner, 1980; Osterrieder, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982), and on the type of graphic analysis used by Siegelbaum

et al. (1977) to measure I_{to} in Purkinje fibres. They estimated I_{to} as the difference between the net current and a linear extrapolation of the late current back to the onset of the step. In the present case I_{to} was taken as the difference current after extrapolation of an exponential (time constant = 300 ms) fitted to rising I_K. Isolated in this manner, I_{to} activates rapidly and inactivates with a time constant of ~ 20 ms. If hidden in the pre-drug net inward current, its presence would result in a 10–15 % underestimate of I_{Ca}. The upper estimate of I_{to} in the control records is not much larger than this lower estimate. Otherwise, there would be a marked deviation from the linearity usually observed in semilogarithmic plots of the net inward currents (McDonald & Trautwein, 1978*a*). We conclude that the amplitude of I_{to} under control conditions is about 10–20 % of I_{Ca} amplitude, and that D600 may partially suppress I_{to}.

Time course of I_{Ca} depression by D600

The depression of I_{Ca} proceeded in two phases (half-times of 3–6 min and 60–90 min), with the initial fast phase accounting for 60 % of the block. Although it is often assumed that steady-state D600 action is reached in 10–30 min, the existence of a slow phase of I_{Ca} block is supported by the action potential measurements, and by the lack of steady-state negative inotropy after 60 min of treatment with D600 (Nawrath *et al.* 1977) or verapamil (Singh & Vaughan Williams, 1972). In addition, there is an interesting resemblance between the time course of I_{Ca} block and that of [^{14}C]D600 uptake observed by Ludwig & Nawrath (1977).

Experiments with D600 and D890 (the permanently charged quaternary derivative of D600) on isolated ventricular myocytes suggest that the uncharged form of the drug crosses the membrane, is protonated, and then acts on the Ca channels from the cell interior (Hescheler, Pelzer, Trube & Trautwein, 1982). Equilibration may be rapid in these guinea-pig myocytes since the maximal effect of external D600 on the action potential plateau appeared to occur within 4–6 min. This suggests that time courses in tissue experiments are distorted by slow diffusion of drug into the tissue bundle. However, it is probably premature to attribute the disparity entirely to intrabundle diffusion because complete time courses of I_{Ca} depression in myocytes have not yet been determined. In addition, these enzymically dissociated cells lack an intact cell coat (Isenberg & Klöckner, 1980) and it is quite possible that the cell coat constitutes a barrier to the penetration of D600 into the cell (cf. Mas-Oliva & Nayler, 1980).

D600 and outward current

A lengthening of the action potential is not observed in all cardiac tissues treated with D600 or verapamil. Important variables include the stimulation rate, drug concentration (Cranefield, Aronson & Wit, 1974; Kass & Tsien, 1975), tissue type (Hirata *et al.* 1979) and species (unpublished observations). Thus, it is hardly surprising that there is a lack of agreement on changes in the outward current with drug (Kohlhardt *et al.* 1972; Kass & Tsien, 1975; Nawrath *et al.* 1977; Noma & Trautwein, 1978). In the present study on cat ventricular muscle, D600 produced a moderate reduction in the outward current. This accounts for the lengthening of the action potential at stimulation rates of 0·33 Hz or less. Higher stimulation rates increased the outward current by about 10 % both before and during drug treatment.

Thus, relative to the shortening of the control action potential, the marked shortening of the D600 action potential at the higher rates is mainly due to the frequency-dependent block of I_{Ca}.

There are two major ways in which D600 could reduce the outward current: (a) by blocking K channels, and (b) by affecting Ca_i-activated g_K. A direct blocking action on K channels is not difficult to accept since it has already been shown that this molecule can block channels other than the Ca channel (Baker *et al.* 1973; Miledi & Parker, 1980). An effect on Ca_i-activated g_K could arise from a general 'tonic' reduction of Ca_i due to prolonged depression of I_{Ca}, and/or from a more dynamic reduction of Ca_i related to each I_{Ca} transient. The present results address the relation between the I_{Ca} transient and g_K: (a) an increase in stimulation rate markedly reduced I_{Ca} but not the outward current, (b) after nearly complete removal of block, the amplitude and time course of I_K were unaffected by the large decrease in I_{Ca} as block was re-established, and (c) the post-rest increase in late outward current was independent of the degree of unblock attained during the rest period. Thus, the results do not support I_{Ca}-linked g_K. This conclusion is consistent with a recent finding by Kass (1982) on Purkinje fibres treated with nisoldipine (10 μM) and D600 (10 μM): D600 reduced the delayed outward current (I_x) despite the fact that I_{Ca} had already been blocked by nisoldipine pre-treatment.

Steady-state I_{Ca}

It is still uncertain whether during a maintained depolarization to plateau potentials, all of the Ca channels activated subsequently undergo inactivation (see McDonald, 1982). If a fraction remain in the conducting state, the late current flowing after the I_{Ca} transient will contain an inward component due to steady-state I_{Ca}. Ca channel block should then abolish the transient and shift the late current in the outward direction.

In calf Purkinje fibres, D600 abolished the I_{Ca} transient and caused an outward shift in the late current (Kass, Siegelbaum & Tsien, 1976; Siegelbaum & Tsien, 1980). From the magnitude of the outward shift, up to 50 % of the Ca channels seemed to be of the non-inactivating type. A similar clear-cut result has not been observed in other studies on D600-treated cardiac preparations (Nawrath *et al.* 1977; Nathan & DeHaan, 1979; Isenberg & Klöckner, 1980; Noma *et al.* 1980). One possibility is that in some preparations treated with the drug a simultaneous depression of outward current and I_{Ca} with time effectively masks the block of the steady-state I_{Ca}. This difficulty is circumvented when the question of non-activating Ca channels is investigated with unblock–block protocols. The result was that the magnitude of the shift in late outward current associated with block was about 10 % of the (unblocked) time-dependent I_{Ca}. However, this would appear to be an over-estimate of the magnitude of steady-state I_{Ca} because (a) there was also an outward current shift of 8 % at the -50 mV holding potential, and (b) the shift in late outward current was nearly as large (7 %) after negligible unblocking as after highly effective unblocking. In summary, the results concur with an earlier conclusion from voltage-clamp studies: the steady-state inactivation of I_{Ca} at 0 mV is on average more than 95 % complete in cat ventricular muscle (McDonald & Trautwein, 1978*a*).

We are grateful to H. Ehrler for skilful electronics support, D. Steimer for technical assistance, and D. Kriesel and E. Nagel for secretarial assistance. This work was supported by the Deutsche Forschungsgemeinschaft, SFB 38, Membranforschung, project G1, and by the Medical Research Council (Canada).

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