Journal of Physiology (1991), **432**, *pp.* 65–80
With 8 figures
Printed in Great Britain

THE CALCIUM ANTAGONIST D600 INHIBITS CALCIUM-INDEPENDENT TRANSIENT OUTWARD CURRENT IN ISOLATED RAT VENTRICULAR MYOCYTES

BY ISABEL A. LEFEVRE*, ALAIN COULOMBE AND EDOUARD CORABOEUF

From the Laboratoire de Physiologie Comparée et Laboratoire de Biomembranes et des Ensembles Neuronaux associé au CNRS (URA 1121), Université Paris-Sud, Bât. 443, 91405 Orsay Cedex, France

(*Received 6 March 1990*)

SUMMARY

1. The whole-cell voltage-clamp technique was applied to isolated rat ventricular myocytes to investigate the effects of D600 (10^{-9}–10^{-3} M) on the intracellular calcium-independent component of transient outward current, I_{to}, recorded in a sodium-free medium containing $0{\cdot}5 \times 10^{-3}$ M-cadmium and 10^{-6} M-ryanodine.

2. Externally applied D600 reduced I_{to} in a dose-dependent, reversible manner, and accelerated the decay of the current.

3. Current–voltage relationships and corresponding activation curves (determined assuming I_{to} to be a pure potassium current) were shifted towards positive potentials in the presence of 10^{-3} M but not 10^{-5} M-D600. Steady-state inactivation curves were not affected by either low or high concentrations of D600.

4. Under control conditions, the inactivation of I_{to} is composed of a fast and a slow component. The amplitude of the slow component was more strongly reduced by D600 than that of the fast one. In the presence of 10^{-3} M-D600, the slow component was entirely suppressed.

5. Both the time to peak I_{to} and the time constant of the fast component of inactivation were markedly reduced at all potentials by D600. The time constant of the slow component was less sensitive to the drug.

6. When the relative quantity of charge carried by each kinetic component of I_{to} was plotted *versus* the concentration of D600, the data could be fitted by two distinctly separate dose–response curves with an almost 100-fold difference between the two apparent dissociation constants, of which the values were $2{\cdot}88 \times 10^{-6}$ M for the slow phase of inactivation and $2{\cdot}07 \times 10^{-4}$ M for the fast one, with Hill coefficients of 0·68 and 0·73 respectively.

7. The inhibition of I_{to} by D600 displayed little or no use dependence, one of the major characteristics of the effects of phenylalkylamines on the cardiac calcium current I_{Ca}.

8. Our results show that at least part of I_{to} is sensitive to D600 in the same range of concentrations as I_{Ca}. Although the effects of D600 on the two currents differ in

* To whom all correspondence and reprint requests should be sent.

several points, this observation raises the possibility that, besides clear differences, certain similarities may exist between the channels responsible for I_{to} and I_{Ca}.

INTRODUCTION

In rat ventricle, one of the major currents involved in the control of the amplitude and duration of the action potential plateau is the transient outward current (Josephson, Sanchez-Chapula & Brown, 1984). This current is generally assumed to be a voltage-dependent potassium current, which activates and inactivates rapidly upon depolarization. It has been shown to be composed of two components in various cardiac preparations (Coraboeuf & Carmeliet, 1982; Escande, Coulombe, Faivre, Deroubaix & Coraboeuf, 1987; Giles & Imaizumi, 1988; Hiraoka & Kawano, 1989). One of these components depends on a transient increase of intracellular calcium and is therefore dependent on the slow inward calcium current (I_{Ca}) and the resulting release of calcium from the sarcoplasmic reticulum. The other component, which is present even in the absence of I_{Ca}, is inhibited by 4-aminopyridine (4-AP), and has been found to be modulated by noradrenaline via the β-adrenergic pathway in Purkinje fibres (Nakayama & Fozzard, 1988), whereas it is depressed by α-adrenergic stimulation both in rat ventricular myocytes (Apkon & Nerbonne, 1988) and rabbit atrial cells (Fedida, Shimoni & Giles, 1989). This calcium-independent component of the transient outward current, previously referred to as I_{to} in Purkinje fibres (Coraboeuf & Carmeliet, 1982), appears similar to the neuronal I_A current (Connor & Stevens, 1971).

Increasing evidence obtained from the structure of channel proteins and the expression of functional channels from cloned cDNA indicates that cation-permeable channels controlled by voltage-dependent activation and inactivation mechanisms exhibit striking primary and secondary structure similarities; for example, the type A potassium channel subunit resembles one of the four sodium or calcium channel pseudo-subunits (for review, see Catterall, 1988). Recently, an electrophysiological and pharmacological approach has shown that functionally equivalent dihydropyridine receptors are present in both calcium and sodium channels of cardiac muscle and this has been taken as evidence of the homology between them (Yatani, Kunze & Brown, 1988). It has also been observed that sodium current inhibitors such as quinidine (Imaizumi & Giles, 1987) and disopyramide, but not lidocaine (Coraboeuf, Deroubaix, Escande & Coulombe, 1988), strongly depress the cardiac transient outward current. Furthermore, in rat ventricular cells, the voltage-dependent inactivation of I_{to} is enhanced by tedisamil, which also inhibits the sodium current (Dukes & Morad, 1989), and in Purkinje fibres a similar effect on I_{to} was evoked by bepridil, a drug that also blocks I_{Ca} (Berger, Borchard & Hafner, 1989).

The aim of the present work was to search for a possible inhibitory effect of calcium current antagonists on I_{to}. We chose to study D600, the methoxy-derivative of verapamil, because it has already been shown that this substance also inhibits the cardiac sodium current (Bustamante, 1985) and depresses in various cardiac preparations the delayed outward potassium current (Kass & Tsien, 1975; Nawrath, Ten Eick, McDonald & Trautwein, 1977; Kass, 1982; McDonald, Pelzer & Trautwein, 1984*a*; Hume, 1985) and the muscarinic potassium current (Mubagwa & Carmeliet,

1987). The results of the present study show that D600 inhibits the calcium-independent transient outward current I_{to} in rat ventricular myocytes, with a most marked effect on the inactivation kinetics and a much stronger effect on the slow than on the fast component of the current decay.

METHODS

Preparation and solutions

Single ventricular cells from adult rat heart were isolated by an enzymatic dissociation procedure derived from the techniques of Irisawa & Kokubun (1983) and Mitra & Morad (1985).

The standard extracellular solution in which the myocytes were maintained contained (mM): NaCl, 135; KCl, 5·4; $MgCl_2$, 1·1; $CaCl_2$, 0·18; $CdCl_2$, 0·5; HEPES, 10; ribose, 1; glucose, 10; ryanodine, 0·001; atropine sulphate, 0·01; pH was adjusted to 7·4 with NaOH. The intracellular medium was a nominally calcium-free solution with EGTA and no added calcium, the pipette containing (mM): potassium aspartate, 115; KCl, 5; sodium pyruvate, 5; Na_2ATP, 4; $MgCl_2$, 7; EGTA, 5 (free Mg^{2+} approximately 2·7 mM); HEPES, 10; pH was adjusted to 7·2 with KOH (total K^+ was thus approximately 140 mM). The standard superfusion medium was a sodium-free medium derived from the standard extracellular medium in order to exclude the possible contribution of either sodium-activated potassium currents (Kameyama, Kakei, Sato, Shibasaki, Matsuda & Irisawa, 1984), or of transient currents generated by Na^+ translocation through the Na^+–K^+ pump (Nakao & Gadsby, 1986); atropine was added to eliminate any choline-activated muscarinic potassium currents. The slow inward calcium current was completely blocked by 0·5 mM-Cd^{2+}, and ryanodine was added in order to inhibit the release of calcium from the sarcoplasmic reticulum so that the intracellular calcium-dependent component of transient outward current was also entirely suppressed. Control experiments were performed at a potential close to the reversal potential of I_{Ca}, and the effects of D600 were found to be the same whether or not Cd^{2+} was present, indicating that in spite of the effect of Cd^{2+} itself on I_{to} (Benndorf & Nilius, 1987; Mayer & Sugiyama, 1988), the two compounds did not interfere. Therefore the standard superfusion medium contained (mM): choline chloride, 135; $MgCl_2$, 1·1; $CaCl_2$, 1·8; $CdCl_2$, 0·5; HEPES, 10; ribose, 1; glucose, 10; ryanodine, 0·001; atropine sulphate, 0·01; pH was adjusted to 7·4 with KOH (total K^+ was thus 4–5 mM). Racemic D600 (Knoll AG, Ludwigshafen, FRG) was prepared as stock solution dissolved in distilled water, or directly added to the standard superfusion solution. All experiments were conducted at room temperature (20–27 °C).

Current recordings and analysis

Macroscopic current recordings were obtained with the whole-cell voltage-clamp method as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981) using a patch-clamp amplifier with a 100 megaohm feedback resistor (Model 8900, Dagan Corp., Minneapolis, MN, USA). Patch pipettes were pulled from Pyrex capillaries (Corning 7740) and were not fire-polished before use. The resistance in series with the cell membrane was compensated using dynamic series resistance control compensation to provide the fastest possible capacity transient without ringing. Neither capacitive current nor leakage current was compensated. Currents were elicited by 700 ms voltage steps in 10 mV increments from a holding potential of −80 mV. Under our experimental conditions, the total outward current was composed of a transient component identified as the calcium-independent current I_{to} and a time-independent component I_c. Voltage steps were applied at a frequency of 0·2 Hz which allowed complete recovery of I_{to} between pulses. Indeed, in our conditions (20–27 °C, holding potential −80 mV), the removal of inactivation of I_{to} could be described by a biexponential process, with time constants of $\tau_1 = 22 \pm 3$ ms and $\tau_2 = 931 \pm 29$ ms (mean ± standard deviation of $n = 3$ determinations), so that the current reached 98% of its initial value within less than 3 s (see also Josephson *et al.* 1984).

Whole-cell currents and voltages were recorded and stored with a betamax video cassette recorder (Sony) after 16-bit digitization at 22 kHz with a pulse code modulator (Sony PCM-701-ES). Macroscopic currents were further digitized at 4 kHz with a microcomputer (Compaq, Deskpro 286) using an S200 interface (Cambridge Research Systems, UK), analysed and retrieved

on a HP 7475A plotter. The data for the activation and steady-state inactivation curves were fitted to the theoretical Boltzmann function using the non-linear least-squares gradient–expansion algorithm of Marquardt, which was also used for the dose–response relationships. The kinetic analysis of current inactivation was performed by fitting a sum of exponential decays to experimental data, using the non-linear regression program DISCRETE (Provencher, 1976). The data could be adequately fitted by a sum of two or exceptionally three exponential terms and a constant. Because, when present, the third and slowest time constant was indeed very slow, and its amplitude very small, we did not study it systematically, and considered for our investigation of the effects of D600 on I_{lo} only two exponential terms, i.e. the data were fitted by an equation of the form:

$$I_o(t) = A_f \exp(-t/\tau_f) + A_s \exp(-t/\tau_s) + A_c, \quad (1)$$

where $I_o(t)$ is the total outward current, τ_f and A_f are the time constant and initial amplitude of the fast phase of inactivation, respectively, τ_s and A_s are the corresponding parameters for the slow phase of inactivation and A_c is the amplitude of a time-independent component. When possible, results are given as mean ± standard deviation of n determinations.

RESULTS

D600 reduces I_{lo} even when I_{Ca} is blocked

Figure 1*A* shows the families of current traces obtained before, during and after exposure to 1 μM-D600. Under control conditions (Fig. 1*Aa*), depolarizations to membrane potentials more positive than −20 mV elicited large, rapidly activating transient outward currents, which increased in amplitude with increasing depolarization. No inward current was activated by the depolarizing voltage steps; the slow inward calcium current, which would be expected to begin to develop at −30 mV and to be maximum around 0 mV, was clearly completely inhibited under these conditions. The transient outward current decayed quite rapidly over the first 100 ms of the pulse, then more slowly, suggesting the contribution of two kinetic components to the time-dependent fraction of outward current. At the end of the 700 ms voltage step there remained a large sustained component of outward current. The peak of I_{lo} was reached within 20 ms for strong depolarizations and the time to peak decreased with increasing membrane depolarization.

The external application of 1 μM-D600 (Fig. 1*Ab*) induced a decrease of both the peak transient outward current and the current measured at the end of the pulse; both effects occurred within 25 s, reached a maximum after 2 min, and were completely reversible (Fig. 1*Ac*). Figure 1*B* shows the current traces elicited by a depolarization to +60 mV before and during exposure to 10 μM-D600, showing a stronger reduction of both peak I_{lo} and the sustained current than in the presence of 1 μM-D600. In the presence of 1 mM-D600 (Fig. 1*C*), the peak I_{lo} was even more markedly reduced and the sustained current was strongly inhibited; the decay of I_{lo} was greatly accelerated and the time to peak was considerably decreased, whereas the slowly inactivating component seemed to be completely abolished. The onset of the effect of 1 mM-D600 on I_{lo} kinetics appeared within 5 s, and maximum inhibition was reached within less than 1 min. During wash-out of the drug, I_{lo} recovered almost completely within 2 min after returning to the control solution. Figure 1*D* illustrates the inactivation time course of I_{lo} in the absence and in the presence of 10 μM-D600, taken from the current traces shown in Fig. 1*B* and represented using log current *versus* time. In this experiment, the decay of the current was well described by the sum of two exponential terms and a constant, both under control

conditions and during application of the drug; the effects of D600 were to decrease the amplitude of the slow component of inactivation without notable modification of the slow time constant, and to reduce both the amplitude and the time constant of the fast component.

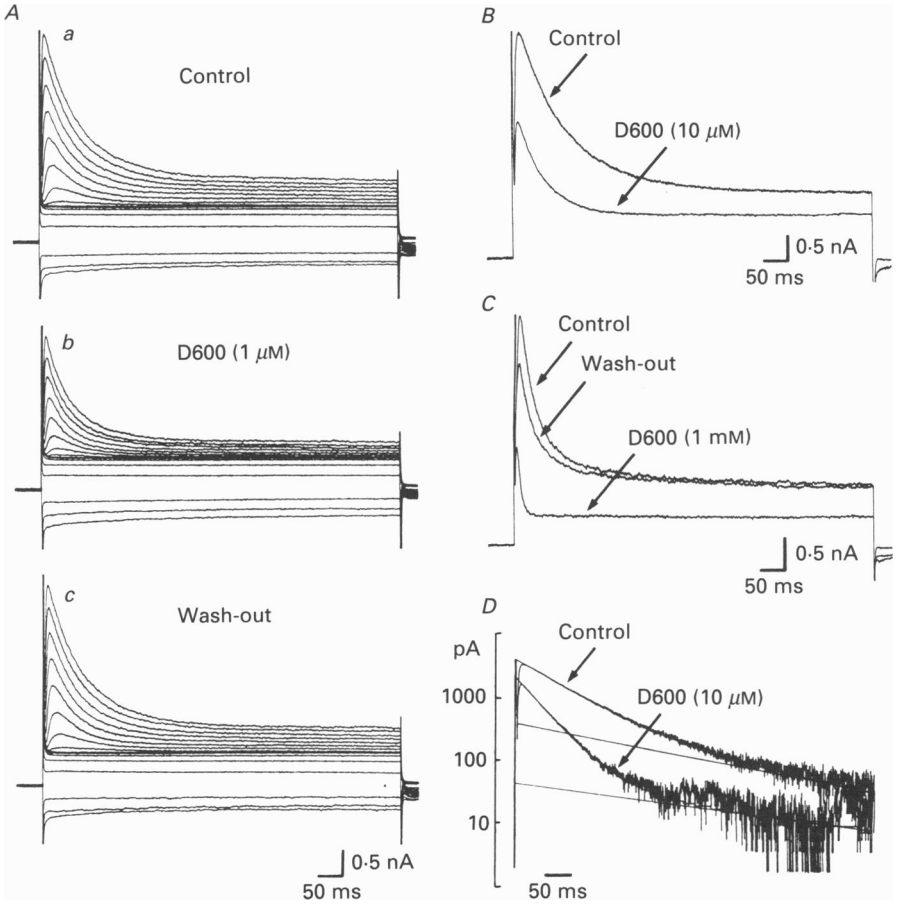

Fig. 1. Effects of D600 at concentrations of 1 μM (*Aa*, *b* and *c*), 10 μM (*B* and *D*) and 1 mM (*C*) on I_{1o}. Currents were elicited by 700 ms depolarizing or hyperpolarizing voltage steps applied at 0·2 Hz from a holding potential of −80 mV, in 10 mV increments between −110 and +60 mV (*A*), to +60 mV (*B* and *D*) and to +50 mV (*C*). In *D*, the current traces shown in *B* are represented using log current *versus* time; the continuous lines are the computer-calculated fits for the slow component of inactivation of I_{1o} and for the total I_{1o}. The currents shown in *A*, *B* and *C* were recorded from three different cells.

The current–voltage (*I–V*) relationship of the total time-dependent outward current (I_{1o}) was determined using the kinetic analysis indicated in the Methods section. The effects of 1 μM- and 1 mM-D600 on the *I–V* relationship of I_{1o} are shown in Fig. 2. I_{1o} began to activate between −10 and 0 mV and continued to increase with increasing depolarization. In the presence of 1 μM-D600, the amplitude of I_{1o} at +60 mV was reduced to 72·1 ± 4·3 % ($n = 4$) of its control value. The voltage at

which I_{1o} activated did not seem to be affected by this concentration of D600. When 1 mM-D600 was applied, I_{1o} at +60 mV was reduced to 48·1 ± 5·9 % ($n = 4$) of its control value, and its threshold was shifted to potentials more positive than +5 mV.

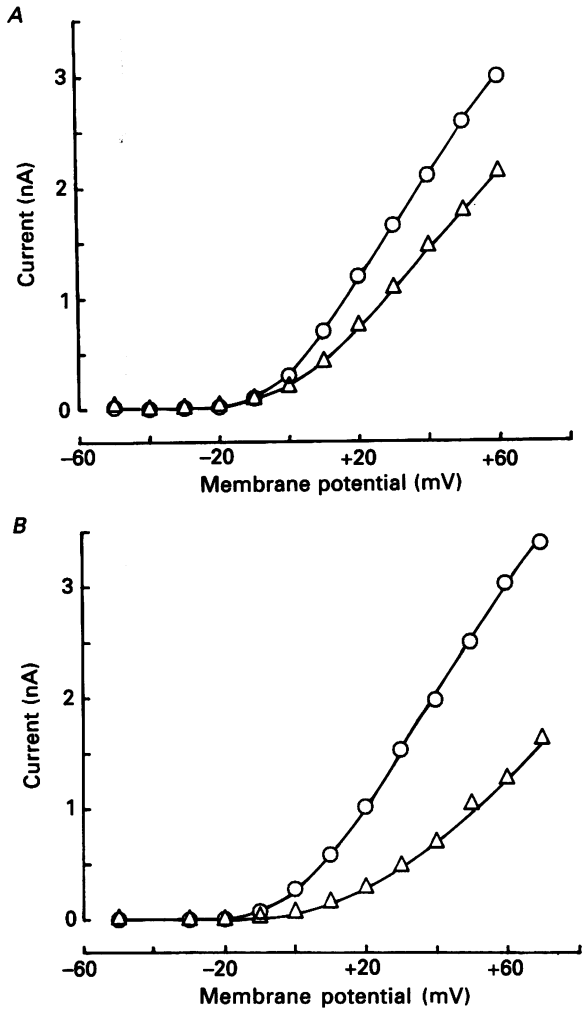

Fig. 2. Effects of a low (1 μM, *A*) and a high (1 mM, *B*) concentration of D600 on the current–voltage relationship of I_{1o} (same cells as in Fig. 1*A* and *C* respectively). Peak current amplitudes were measured with respect to the time-independent component of outward current determined at each potential by kinetic analysis, and plotted *versus* membrane potential under control conditions (○) and in the presence of D600 (△). The lines were drawn by eye.

Thus, in the absence of I_{Ca}, and under conditions in which intracellular calcium movements have been minimized, the addition of D600 reduces the transient outward current. It is therefore clear that D600 inhibits I_{1o} by a mechanism that does not depend on inhibition of calcium influx by this drug. The results also suggest that

D600 affects several properties of I_{lo}. Therefore, we first investigated the effects of D600 on the voltage dependence of activation and steady-state inactivation, then analysed the kinetics of I_{lo}.

Voltage dependence of activation of I_{lo}

The result shown in Fig. 2*B* indicates that a high concentration of D600 shifted the *I–V* relationship towards more positive potentials, thus causing an additional

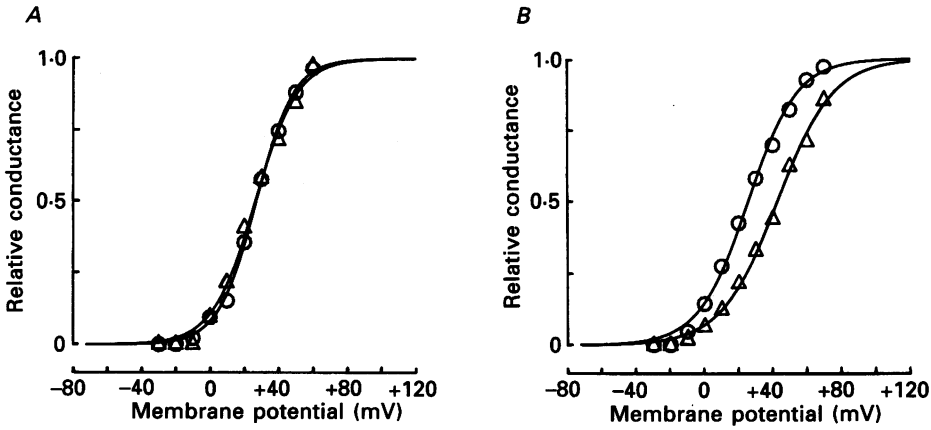

Fig. 3. Activation–voltage relationship: normalized chord conductances of I_{lo} plotted *versus* membrane potential before (○) and during (△) exposure to 10 μM-D600 (*A*) and 1 mM-D600 (*B*). The curves were fitted to experimental data according to the procedures described in the text. In panel *A* the values of $V_{0.5}$ and k (see text) were respectively +27 and +11 mV under control conditions *versus* +26·4 and +12·4 mV in the presence of D600, whereas in panel *B* they were +25·7 and +14·5 mV respectively under control conditions *versus* +42·7 and +16·5 mV respectively in the presence of D600.

reduction in the amplitude of I_{lo}. In order to assess this effect more accurately, the chord conductance was determined as a function of membrane potential, assuming that I_{lo} was a pure potassium current, i.e. that it reversed at the K^+ equilibrium potential E_K. In our different experiments E_K varied between −85 and −90 mV. As the maximum experimental values of I_{lo} did not reflect the maximum chord conductances, we determined the latter using a computer-calculated Boltzmann fit, according to the equation:

$$G = G_{max}/(1+\exp((V_{0.5}-V_m)/k)), \quad (2)$$

where G_{max} is the maximum chord conductance, G the chord conductance calculated at the membrane potential V_m, $V_{0.5}$ the potential at which the conductance is half-maximally activated, and k is the slope factor describing the steepness of the activation curve. Using the values of G_{max} obtained with this procedure, we then plotted the normalized whole-cell conductance (G/G_{max}) against membrane potential, under control conditions and for two different concentrations of D600 as shown in Fig. 3.

For low concentrations of D600, no significant shift of the conductance–voltage curve was observed (Fig. 3*A*). On the other hand, 1 mM-D600 shifted the

conductance–voltage curve in a depolarizing direction by 17 mV, as estimated from the difference between the half-activation voltage of the control and D600 curves (Fig. 3*B*). In the presence of 1 mM-D600, the slope factor of the activation curve was increased by 2 mV, and it cannot be excluded that this increase of slope factor,

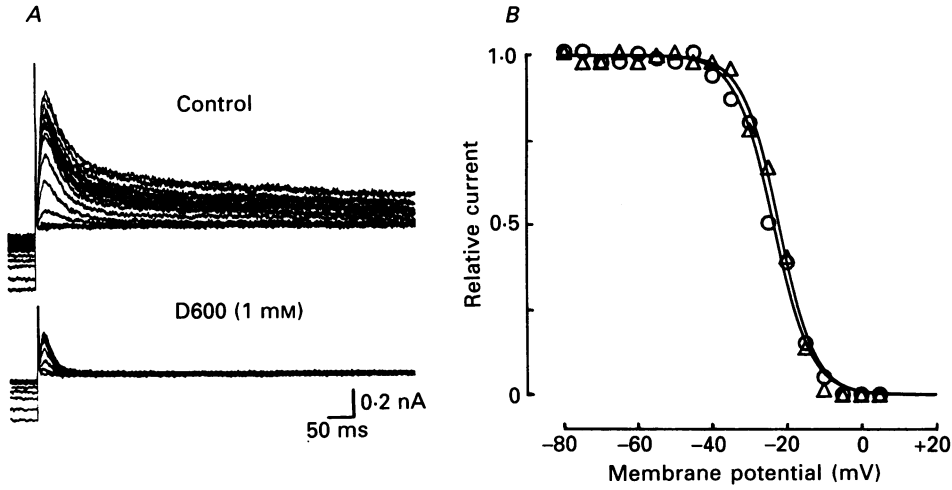

Fig. 4. *A*, current records obtained by holding the cell membrane for 2 s at different potentials (between −80 and +10 mV in 5 mV increments) before applying the 700 ms test pulse (to +20 mV), under control conditions and in the presence of 1 mM-D600. *B*, steady-state inactivation–voltage relationship of I_{lo} under control conditions (○) and in the presence of 1 mM-D600 (△). The curves were fitted to experimental data according to the equation: $I/I_{max} = 1/(1+\exp((V_{0.5}-V_m)/k))$, where $V_{0.5} = -23{\cdot}6$ mV, $k = -5$ mV under control conditions, *versus* $V_{0.5} = -22{\cdot}2$ mV and $k = -4{\cdot}8$ mV in the presence of the drug.

associated with the strong reduction of current amplitude, may have artificially accentuated the voltage shift by a few millivolts.

Steady-state inactivation–voltage relationship

To determine the voltage dependence of inactivation of I_{lo}, the membrane was held at different potentials in the range −80 to +10 mV for 2 s before applying the test pulse to a fixed voltage of +20 mV. Figure 4*A* gives an example of the current records obtained following this procedure. Figure 4*B* shows the steady-state inactivation–voltage relationships of I_{lo}, under control conditions and in the presence of 1 mM-D600. At potentials more negative than −50 mV, inactivation was almost completely absent; complete inactivation was achieved at potentials positive to 0 mV. Whatever the concentration of D600 used (between 1 μM and 1 mM), there was no significant shift in the voltage dependence of the inactivation of I_{lo}.

Kinetic analysis of the effects of D600 on I_{lo}

As indicated in the Methods, the time course of decay of I_{lo} can be divided into a fast and a slow component. Figure 4*A* shows that, under control conditions, the peak of the transient outward current decreased at first slowly with conditioning potential

in the range −80 to −50 mV, and this decrease was accompanied by an almost equal reduction in the amplitude of the current at the end of the test pulse. For conditioning potentials between −45 and −30 mV, both the peak amplitude and the current at the end of the test pulse decreased concomitantly. For conditioning

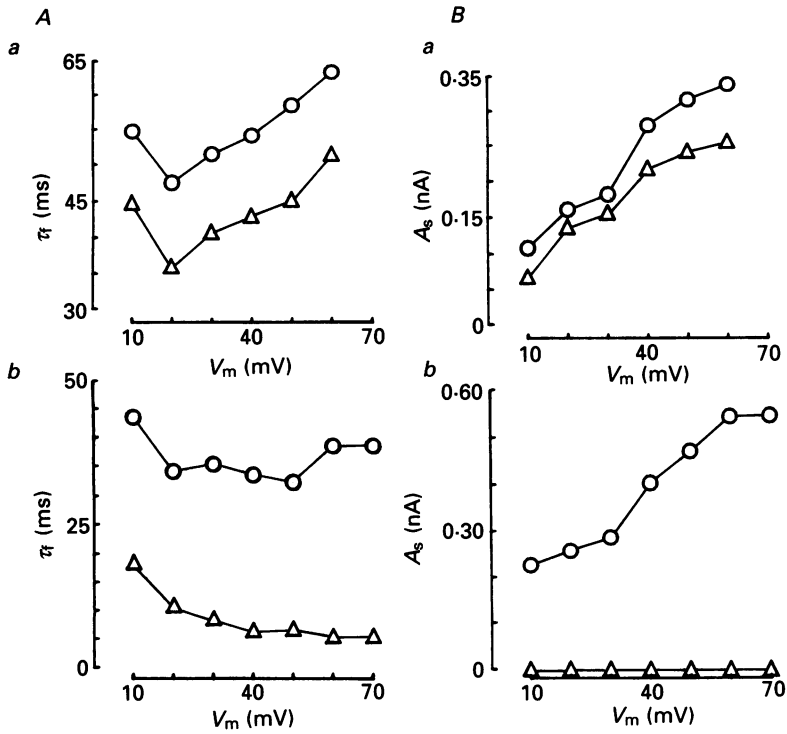

Fig. 5. Effects of D600 on the kinetic parameters of the inactivation of I_{lo}. *A*, time constant of the fast component of inactivation of I_{lo} (τ_f) plotted *versus* membrane potential (V_m) under control conditions (○) and in the presence (△) of 1 μM- (*a*) or 1 mM- (*b*) D600. The room temperature at which the experiments were performed was 20 °C in *a*, and 27 °C in *b*. *B*, amplitude of the slow component of inactivation (A_s) plotted *versus* membrane potential, under control conditions (○) and in the presence (△) of 1 μM- (*a*) or 1 mM- (*b*) D600. Note the different vertical scales for *a* and *b* in both *A* and *B*.

potentials positive to −30 mV, the current at the end of the test pulse remained constant, whereas peak I_{lo} continued to decrease in a voltage-dependent manner. In Figure 4*A*, the inhibition of the slowly inactivating component of I_{lo} in the presence of 1 mM-D600 was so marked that only the inactivation of the peak I_{lo} could be seen on the current traces, the current measured at the end of the pulse being constant for all conditioning potentials tested.

The kinetics of inactivation of I_{lo} were studied in the absence and the presence of D600. In the absence of drug, the time constant of the fast component of inactivation, τ_f, ranged between 40 and 60 ms, whereas the value of the time constant of the slow phase, τ_s, was between 200 and 500 ms; the fast and the slow components of inactivation represented in amplitude approximately 60 and 15% of the total

outward current respectively, the remainder of which was the time-independent component. D600 did not affect both kinetic components of inactivation of I_{1o} in the same manner, the predominant effects being a reduction of the time constant of the fast phase and a reduction of the amplitude of the slow one, without significant

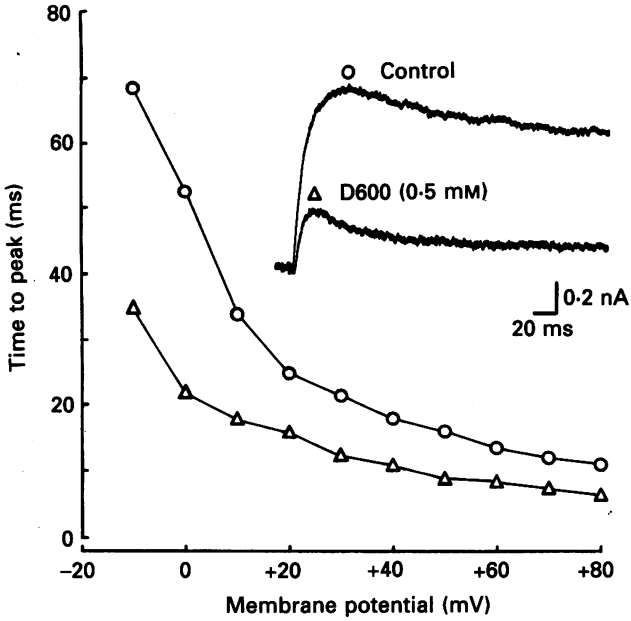

Fig. 6. Time to peak I_{1o} measured from the onset of the command pulse plotted *versus* membrane potential under control conditions (○) and during application of 0·5 mM-D600 (△). The current traces corresponding to a depolarization to 0 mV from a holding potential of −80 mV before (○) and during application of 0·5 mM-D600 (△) are shown in the inset.

modification of τ_s (see for example Fig. 1*D*). The effect of D600 on the fast phase of inactivation is illustrated in Fig. 5*A*, which shows the relationship between τ_f and membrane potential, under control conditions and for a low and a high concentration of D600. Under control conditions, the value of τ_f showed a slightly U-shaped dependence towards potential. The major effect of D600 was to reduce τ_f in a dose-dependent manner, thereby accelerating the initial decay of the current. When high concentrations of the drug were applied, only one exponential term was required to fit the decay of the current, with a very fast time constant for strong depolarizations, e.g. 5·5 ms at +70 mV. The relation between τ_f and voltage may have been slightly modified in the presence of 1 mM-D600. Figure 5*B* gives the amplitude of the slow phase of inactivation as a function of membrane potential, before and after exposure to a low and a high concentration of D600. For 1 mM-D600, the slow component of inactivation was totally suppressed at all potentials. Although in some experiments and for some depolarizations a slow component could also be determined in the presence of high concentrations of D600, its amplitude was never more than 1% of the total outward current.

Figure 6 gives the relationship between the time to peak I_{1o} and membrane potential, with the inset showing the activation phase of I_{1o} for a depolarization to 0 mV from a holding potential of −80 mV, before and during exposure to 0·5 mM-D600. As noted above (see for example Fig. 1), the time to peak I_{1o} shortened with

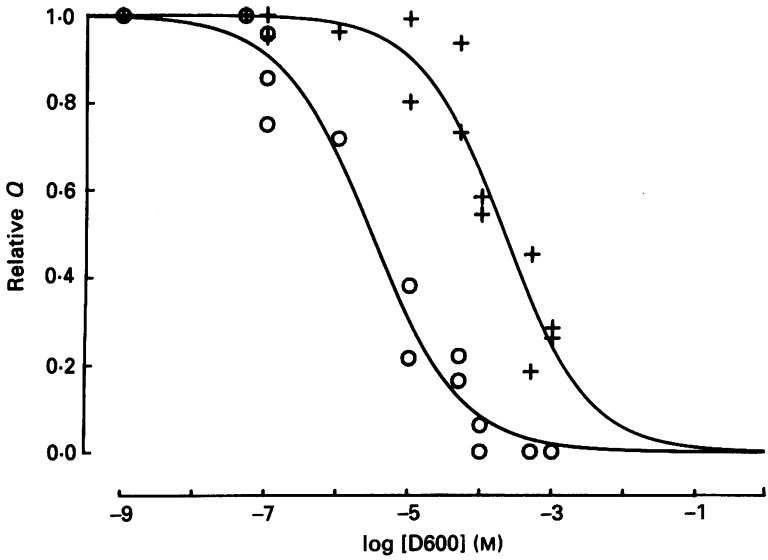

Fig. 7. Log dose–response relationships for inhibition of I_{1o} by D600. The relative quantity of charge Q (normalized with respect to control values) was determined for each component by kinetic analysis and plotted *versus* log concentration of D600. Relative Q was found to be independent of test potential between +30 and +70 mV, the range in which measurements were performed. The data represent the values (slow component, O; fast component, +) obtained from four experiments in which each of four cells was superfused with four different concentrations of D600. The lines are the computer-calculated fits to the weighted combined data according to eqn (3), with apparent dissociation constants of $2{\cdot}88 \times 10^{-6}$ M for the slow component and $2{\cdot}07 \times 10^{-4}$ M for the fast component and Hill coefficients of 0·68 and 0·73 respectively.

increasing membrane depolarization, both in the absence and in the presence of the drug. The apparent effect of D600 was to reduce time to peak at all potentials, without significantly altering the voltage dependence.

Dose–response relationship

Because the effect of D600 on current kinetics appeared to be a major factor in the inhibition of I_{1o}, we chose to use quantity of charge, Q, rather than peak current amplitude or maximum conductance, as the parameter for the dose–response relationship. The quantity of charge Q transferred during a pulse, corresponding to the total I_{1o}, was determined in the presence of different concentrations of D600 and normalized with respect to the control values obtained in the absence of drug. When the values of Q corresponding to the total I_{1o} (fast and slow components) were plotted *versus* the concentration of D600, inhibition of I_{1o} began for concentrations of D600

greater than 10^{-8} M and was complete only for concentrations higher than 10^{-3} M (not illustrated). When the data were fitted with a single function of the form:

$$Q_D/Q_C = 1/(1+(D/K)^{n_H}), \quad (3)$$

where Q_D/Q_C is the relative quantity of charge, D the concentration of D600 (in M), K the apparent dissociation constant and n_H the Hill coefficient, the best fit gave the following values for these parameters: $K = 1{\cdot}32 \times 10^{-5}$ M and $n_H = 0{\cdot}42$, with a minimum χ^2 value of $9{\cdot}2 \times 10^{-4}$. A concentration-dependent effect of a substance over such a large range is an unusual observation. A Hill coefficient so clearly less than one can be explained by considering either the possibility of a negative co-operativity of the binding of the molecules of D600 to their sites, or the participation of at least two current components to the total I_{lo}, which D600 could affect differentially. Because the fast component of I_{lo} was clearly more resistant to D600 than the slow component (see for example Fig. 1), we determined separate dose–response curves for each kinetic component, using as parameters the quantity of charge carried by each component. The data were obtained from four different experiments in which four D600 concentrations had been successfully tested on each of four cells. The result shown in Fig. 7 demonstrates that clearly distinct dose–response curves can indeed be determined for each component, with an almost 100-fold difference between the two apparent dissociation constants: the slow component of I_{lo} is thus considerably more sensitive to D600 than the fast component. When the weighted combined data were fitted for each separate curve to eqn (3), the values of the apparent dissociation constants were $2{\cdot}88 \times 10^{-6}$ M for the slow component *versus* $2{\cdot}07 \times 10^{-4}$ M for the fast component, whereas the corresponding Hill coefficients were 0·68 and 0·73 respectively and the respective minimum χ^2 values were $4{\cdot}9 \times 10^{-3}$ and $7{\cdot}7 \times 10^{-3}$.

Absence of use dependence of the effects of D600 on I_{lo}

A well-established characteristic of the action of phenylalkylamines on cardiac calcium channels is their marked use dependence, i.e. the fact that the inhibition of I_{Ca} is enhanced by repetitive membrane depolarization (use-dependent or frequency-dependent block) with respect to the block that occurs when the membrane is maintained at the resting potential (resting block). A simple way of testing the relative amounts of resting and use-dependent block involves: (i) depolarizing the membrane at a regular rate; (ii) discontinuing the depolarizations and applying the drug for a certain period of time, for example 3 min, without further depolarization; (iii) resuming stimulation at the previous rate (see Lee & Tsien, 1983, their Fig. 5). Figure 8 shows the result of such an experiment in which 100 μM-D600 was added at the beginning of a 3 min quiescent period. It can be seen that the block induced by the drug at this concentration occurred essentially during the period of rest (presence of a large resting block) and was not further increased (or to a very limited extent) during post-rest depolarizations (absence of use-dependent block). Similar results were obtained in three other cells, in which no use-dependent component of block was detected.

DISCUSSION

The results of the present work show that the calcium channel antagonist D600 inhibits the intracellular calcium-independent transient outward current, I_{to}, of rat ventricular myocytes in a reversible, dose-dependent manner. The D600-induced

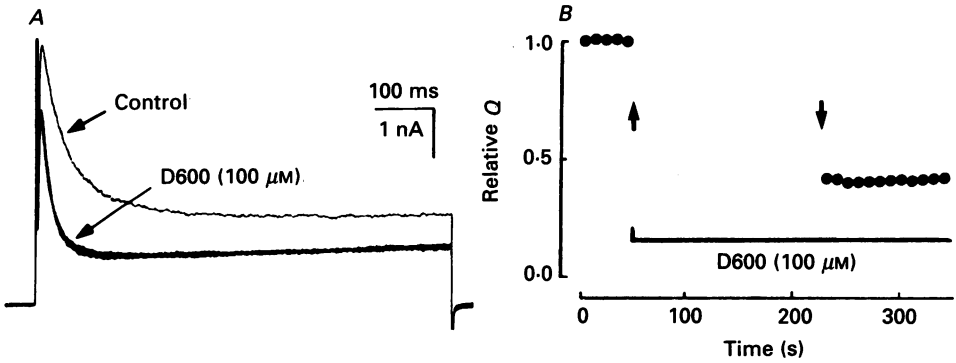

Fig. 8. Absence of use dependence of the effects of D600 on I_{to}. *A*, I_{to} was elicited by depolarizations to +60 mV from a holding potential of −80 mV. The control trace was recorded just before stopping stimulation and applying D600; after a 3 min quiescent period, stimulation was resumed in the presence of the drug, as illustrated by the twelve superimposed current traces (D600 (100 μM)). *B*, total quantity of charge carried by I_{to} was normalized with respect to the control value and plotted *versus* time: the arrows indicate the times at which stimulation was stopped (upward arrow) and resumed (downward arrow), and the horizontal bar indicates the time during which D600 was applied.

inhibition of I_{to} is due to (i) a decrease of the total conductance, (ii) a shift of the activation towards more positive potentials in the presence of high concentrations of the drug, and (iii) an acceleration of the inactivation kinetics. Under control conditions, the decay of I_{to} can be described by a biexponential process, involving a fast and a slow component, which represent approximately 80 and 20% of the amplitude of the total time-dependent component respectively. In the presence of D600, the amplitude of the fast component was reduced and its time constant was greatly accelerated, whereas the effect of D600 on the slow component was essentially a strong reduction of amplitude with little effect on the slow time constant. At high concentrations of D600, the slow component of inactivation of I_{to} was completely suppressed, whereas an accelerated fast component remained, suggesting that the slow component was more sensitive to the inhibitory effect of the drug than the fast phase. Our results show that the time to peak I_{to} decreased in the presence of D600. This effect could result either from a direct effect of the drug on the kinetics of activation or from the acceleration of inactivation, or from a combination of both effects. We have not at present investigated whether or not activation kinetics are directly modified by D600 but obviously a large part of the decrease of time to peak is due to the acceleration of the current decay.

The separate dose–response curves (Fig. 7) calculated for the fast and slow components of inactivation of I_{to}, using the quantity of charge carried by each

component, clearly show that the slow component is by far the more sensitive to D600. Because the Hill coefficients of the two theoretical curves (0·68 and 0·73) are relatively different from unity it cannot be excluded that drug effects on different parameters, such as conductance and gating properties, are mixed up in our measurements using relative quantity of charge as a parameter. Nevertheless, in spite of this possible limitation, this parameter appears more appropriate than, for example, maximal initial amplitudes (i.e. the current amplitudes extrapolated to the time of onset of the pulse). Indeed we often observed that such an extrapolation can lead to a marked apparent increase of initial amplitude of the fast component in the presence of D600 in spite of a dramatic reduction of quantity of charge. Such a discrepancy might result from a possible undetected delay preceding the beginning of inactivation as has been described for the sodium current (Goldman & Kenyon, 1982).

The question should be raised as to whether both components of the inactivation of I_{lo} involve the same channel. The contribution of two kinetic components to the inactivation process can be taken as the sign of the existence of either one channel population having two inactivation states, or of two different channel populations, one of which undergoes inactivation more slowly than the other. In the absence of single-channel data it is difficult to resolve this issue. The observation that, in the presence of high concentrations of D600, a rapidly inactivating peak of current persists whereas the slow component of inactivation of I_{lo} is suppressed, can be interpreted as an argument in favour of the hypothesis that two channel populations with different pharmacological sensitivities (or of two different pathways) underlie the I_{lo} current, as recently suggested for the I_A current (Greene, Haas & Reiner, 1990). However, this result can also be explained by a preferential and relatively fast binding of the drug to open I_{lo} channels leading to an apparent acceleration of current inactivation (Carmeliet, 1987). In the case of preferential drug binding on activated channels, the drug unbinds when the channel inactivates (i.e. when the channel becomes blocked by the closing of its inactivation gate rather than being blocked by the drug). Depending upon the unblocking rate and the interval between successive depolarizations, some block may persist thus inducing some use-dependent current inhibition (Hondeghem & Katzung, 1984). Under our experimental conditions most of the block occurs during rest whereas use-dependent block is very weak. This is at variance with the effect of D600 on cardiac calcium current where in similar experimental conditions resting block is absent and use-dependent block accounts for more than 95% of the drug effect (Lee & Tsien, 1983).

The inhibitory effects exerted by D600 on I_{lo} and I_{Ca} differ in at least three points, namely: (i) the inactivation kinetics of I_{Ca} are not accelerated by D600 (McDonald *et al.* 1984*a*; Cohen & Lederer, 1987; McDonald, Pelzer & Trautwein, 1989) in contrast with the present findings for I_{lo}; (ii) the absence of resting block and the presence of a prominent use-dependent block of I_{Ca} (Lee & Tsien, 1983; McDonald, Pelzer & Trautwein, 1984*b*) whereas the reverse is observed for I_{lo}; (iii) the concentration ranges in which the inhibition develops, i.e. approximately between 3×10^{-7} and 3×10^{-4} M for I_{Ca} in rat ventricular trabeculae (Payet, Schanne, Ruiz-Ceretti & Demers, 1980) in comparison with, for I_{lo}, between 10^{-7} and 10^{-4} M (slow component) and 2×10^{-6} and 2×10^{-3} M (fast component). It should be noted that, if

the sensitivity of the fast component is particularly low and could correspond to some non-specific effects of D600 (in spite of a very rapid reversibility), in contrast, the sensitivity of the slow phase of inactivation of I_{1o} is quite similar to that of I_{Ca} and therefore of true pharmacological significance. It is impossible at the present time to draw valid conclusions concerning possible relationships between structure and activity (or pharmacological sensitivity) of I_{1o} and I_{Ca}. Nevertheless, our results underline a clearly surprising point of analogy between a potassium and a calcium current, in that both of these currents are sensitive to the calcium channel blocker D600 at concentrations as low as 10^{-7}–10^{-6} M. The analysis of macroscopic current recordings does not enable us at present to discriminate further between the various hypotheses which could account for our observations. However, in this respect, D600 could provide a useful tool for further investigation of the I_{1o} current at the single-channel level.

We thank Paulette Richer for preparing the isolated cells, and Gerard Sadoc for computer programming. We also wish to thank Dr Edith Deroubaix for fruitful discussions and for help in preparing the manuscript, Dr Ian Findlay for critical reading of the manuscript and judicious suggestions, Dr Jean-Marc Dubois for helpful comments and Knoll AG (Ludwigshafen, FRG) for the generous supply of D600. This work was supported by grants from Association Française contre les Myopathies and from Fondation pour la Recherche Medicale.

REFERENCES

Apkon, M. & Nerbonne, J. M. (1988). Alpha$_1$-adrenergic agonists selectively suppress voltage-dependent K^+ currents in rat ventricular myocytes. *Proceedings of the National Academy of Sciences of the USA* **85**, 8756–8760.

Benndorf, K. & Nilius, B. (1988). Different blocking effects of Cd^{++} and Hg^{++} on the early outward current in myocardial mouse cells. *General Physiology and Biophysics* **7**, 345–352.

Berger, F., Borchard, U. & Hafner, D. (1989). Effects of the calcium entry blocker bepridil on repolarizing and pacemaker currents in sheep cardiac Purkinje fibres. *Naunyn-Schmiedeberg's Archives of Pharmacology* **339**, 638–646.

Bustamante, J. O. (1985). Block of sodium currents by the calcium antagonist D600 in human heart cell segments. *Pflügers Archiv* **403**, 225–227.

Carmeliet, E. (1987). Voltage-dependent block by tetrodotoxin of the sodium channel in rabbit cardiac Purkinje fibers. *Biophysical Journal* **51**, 109–114.

Catterall, W. A. (1988). Structure and function of voltage-sensitive ion channels. *Science* **242**, 50–61.

Cohen, N. M. & Lederer, W. J. (1987). Calcium current in isolated neonatal rat ventricular myocytes. *Journal of Physiology* **391**, 169–191.

Connor, J. A. & Stevens, C. F. (1971). Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *Journal of Physiology* **213**, 21–30.

Coraboeuf, E. & Carmeliet, E. (1982). Existence of two transient outward currents in sheep cardiac Purkinje fibers. *Pflügers Archiv* **392**, 352–359.

Coraboeuf, E., Deroubaix, E., Escande, D. & Coulombe, A. (1988). Comparative effects of three class I antiarrhythmic drugs on plateau and pacemaker currents of sheep cardiac Purkinje fibres. *Cardiovascular Research* **22**, 375–384.

Dukes, I. D. & Morad, M. (1989). Tedisamil inactivates transient outward K^+ current in rat ventricular myocytes. *American Journal of Physiology* **257**, H1746–1749.

Escande, D., Coulombe, A., Faivre, J. F., Deroubaix, E. & Coraboeuf, E. (1987). Two types of transient outward currents in adult human atrial cells. *American Journal of Physiology* **252**, H142–148.

Fedida, D., Shimoni, Y. & Giles, W. R. (1989). A novel effect of norepinephrine on cardiac cells is mediated by alpha$_1$-adrenoceptors. *American Journal of Physiology* **256**, H1500–1504.

Giles, W. R. & Imaizumi, Y. (1988). Comparison of potassium currents in rabbit atrial and ventricular cells. *Journal of Physiology* **405**, 123–145.

GOLDMAN, L. & KENYON, J. L. (1982). Delays in inactivation development and activation kinetics in Myxicola giant axons. *Journal of General Physiology* **80**, 83–102.

GREENE, R. W., HAAS, H. L. & REINER, P. B. (1990). Two transient outward currents in histamine neurones of the rat hypothalamus *in vitro*. *Journal of Physiology* **420**, 149–163.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HIRAOKA, M. & KAWANO, S. (1989). Calcium-sensitive and insensitive transient outward current in rabbit ventricular myocytes. *Journal of Physiology* **410**, 187–212.

HONDEGHEM, L. M. & KATZUNG, B. G. (1984). Mechanism of action of antiarrhythmic drugs. In *Physiology and Pathophysiology of the Heart*, ed. SPERELAKIS, N., pp. 459–476. Martinus Nijhoff Publishing, Boston.

HUME, J. R. (1985). Comparative interactions of organic Ca^{++} channel antagonists with myocardial Ca^{++} and K^+ channels. *Journal of Pharmacology and Experimental Therapeutics* **234**, 134–140.

IMAIZUMI, Y. & GILES, W. R. (1987). Quinidine-induced inhibition of transient outward current in cardiac muscle. *American Journal of Physiology* **253**, H704–708.

IRISAWA, H. & KOKUBUN, S. (1983). Modulation by intracellular ATP and cyclic AMP of the slow inward current in isolated single ventricular cells of guinea-pig. *Journal of Physiology* **338**, 321–337.

JOSEPHSON, I. R., SANCHEZ-CHAPULA, J. & BROWN, A. M. (1984). Early outward current in rat single ventricular cells. *Circulation Research* **54**, 157–162.

KAMEYAMA, M., KAKEI, M., SATO, R., SHIBASAKI, T., MATSUDA, H. & IRISAWA, H. (1984). Intracellular Na^+ activates a K^+ channel in mammalian cardiac cells. *Nature* **309**, 354–356.

KASS, R. S. (1982). Delayed rectification in the cardiac Purkinje fiber is not activated by intracellular calcium. *Biophysical Journal* **45**, 837–839.

KASS, R. S. & TSIEN, R. W. (1975). Multiple effects of calcium antagonists on plateau currents in cardiac Purkinje fibers. *Journal of General Physiology* **66**, 169–192.

LEE, K. S. & TSIEN, R. W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* **302**, 790–794.

MCDONALD, T. F., PELZER, D. & TRAUTWEIN, W. (1984*a*). Cat ventricular muscle treated with D600: effects on calcium and potassium currents. *Journal of Physiology* **352**, 203–216.

MCDONALD, T. F., PELZER, D. & TRAUTWEIN, W. (1984*b*). Cat ventricular muscle treated with D600: characteristics of calcium channel block and unblock. *Journal of Physiology* **352**, 217–241.

MCDONALD, T. F., PELZER, D. & TRAUTWEIN, W. (1989). Dual action (stimulation, inhibition) of D600 on contractility and calcium channels in guinea-pig and cat heart cells. *Journal of Physiology* **414**, 569–586.

MAYER, M. L. & SUGIYAMA, K. (1988). A modulatory action of divalent cations on transient outward current in cultured rat sensory neurones. *Journal of Physiology* **396**, 417–433.

MITRA, R. & MORAD, M. (1985). A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. *American Journal of Physiology* **249**, H1056–1060.

MUBAGWA, K. & CARMELIET, E. (1987). Interaction of AQA 39, D600, verapamil and diltiazem with cardiac cholinergic effects. *Archives Internationles de Pharmacodynamie et de Thérapie* **286**, 71–84.

NAKAO, M. & GADSBY, D. C. (1986). Voltage dependence of Na translocation by the Na/K pump. *Nature* **323**, 628–630.

NAKAYAMA, T. & FOZZARD, H. A. (1988). Adrenergic modulation of the transient outward current in isolated canine Purkinje cells. *Circulation Research* **62**, 162–172.

NAWRATH, H., TEN EICK, R. E., MCDONALD, T. F. & TRAUTWEIN, W. (1977). On the mechanism underlying the action of D-600 on slow inward current and tension in mammalian myocardium. *Circulation Research* **40**, 408–414.

PAYET, M. D., SCHANNE, O. F., RUIZ-CERETTI, E. & DEMERS, J. M. (1980). Inhibitory activity of blockers of the slow inward current in rat myocardium, a study in steady state and of rate of action. *Journal of Molecular and Cellular Cardiology* **12**, 187–200.

PROVENCHER, S. W. (1976). A fourier method for the analysis of exponential decay curves. *Biophysical Journal* **16**, 27–41.

YATANI, A., KUNZE, D. L. & BROWN, A. M. (1988). Effects of dihydropyridine calcium channel modulators on cardiac sodium channels. *American Journal of Physiology* **254**, H140–147.