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CALCIUM-SENSITIVE AND INSENSITIVE TRANSIENT OUTWARD CURRENT IN RABBIT VENTRICULAR MYOCYTES

BY MASAYASU HIRAOKA AND SEIKO KAWANO

From the Department of Cardiovascular Diseases, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Yushima, Tokyo 113, Japan

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SUMMARY

1. A suction pipette whole-cell voltage-clamp technique was used to record membrane currents and potentials of isolated ventricular myocytes from rabbit hearts.

2. Transient outward current (I_{to}) was activated by voltage steps positive to −20 mV, increasing in amplitude with further depolarization to reach a maximum around +70 mV. The current attained its peak within 10 ms and then it inactivated for 100–200 ms.

3. A large portion of I_{to} still remained after the calcium current (I_{Ca}) was blocked when depolarizing pulses were applied at a frequency of 0·1 Hz or less. Therefore, this current component is referred to as calcium-insensitive I_{to} or I_t.

4. I_t showed voltage- and time-dependent inactivation similar to that observed in Purkinje fibres and other cardiac preparations.

5. The reversal potential of I_t depended on external K^+ concentration, $[K^+]_o$, with a slope of 32 mV per 10-fold change in the presence of a normal $[Na^+]_o$ (143 mM), while the slope was 48 mV per 10-fold change in low $[Na^+]_o$ (1·0 mM).

6. I_t was completely inhibited by 2–4 mM-4-aminopyridine. I_{to} in the presence of I_{Ca} was also partially blocked by 4-aminopyridine and the remainder was abolished by 5 mM-caffeine.

7. The calcium-insensitive and caffeine-sensitive I_{to} differed in their decay rates as well as in their recovery time courses. The former was predominantly available at a slow pulsing rate, while the latter increased its amplitude with high-frequency depolarization.

8. The caffeine-sensitive I_{to} was inhibited by a blockade of I_{Ca}, by replacing Ca^{2+} with Sr^{2+}, by external application of ryanodine and by internal application of EGTA. This indicates that the current is calcium-sensitive and is dependent on increased myoplasmic Ca^{2+} through Ca^{2+} influx via the sarcolemma and Ca^{2+} release from the sarcoplasmic reticulum. The current is therefore designated as $I_{K,Ca}$.

9. The physiological functions of $I_{K,Ca}$ and I_t are indicated by their contribution to ventricular repolarization at fast and slow heart rates, respectively.

INTRODUCTION

The presence of a transient outward current in cardiac Purkinje fibres in response to depolarizing clamp pulses positive to -20 mV was recognized soon after introduction of the voltage-clamp technique to cardiac electrophysiology (Deck & Trautwein, 1964). Since then, various studies have been carried out to clarify the characteristics of this current using Purkinje fibres (Dudel, Peper, Rudel & Trautwein, 1967; Fozzard & Hiraoka, 1973; Kenyon & Gibbons, 1977; 1979*a*, *b*). The current is thought to form the initial phase of repolarization of the Purkinje action potential. The presence of the transient outward current (I_{to}) has recently been described in other cardiac preparations as well (Kukushkin, Gainullin & Sosunov, 1983; Maylie & Morad, 1984; Josephson, Sanchez-Chapula & Brown, 1984; Mitchell, Powell, Terrar & Twist, 1984; Watanabe, Delbridge, Bustamente & McDonald, 1984; Nakayama & Irisawa, 1985; Giles & Van Ginneken, 1985). Since the current displays voltage- and time-dependent inactivation (Fozzard & Hiraoka, 1973), it contributes significantly to frequency-dependent changes in action potential repolarization and duration (Hiraoka & Hiraoka, 1975; Kenyon & Gibbons, 1977; Boyett, 1981*a*, *b*).

There remains some uncertainty as to the selectivity of this current. Most recent reports have indicated that potassium ions are the major charge carrier rather than Cl^-, as originally suggested (Dudel *et al.* 1967; Fozzard & Hiraoka, 1973; Kenyon & Gibbons, 1977; 1979*a*, *b*; Siegelbaum & Tsien, 1980; Nakayama & Irisawa, 1985; Giles & Van Ginneken, 1985). It is not clear, however, whether the current is a pure K^+ current or whether other ions such as Na^+ and Cl^- may contribute to the current.

Another important question concerning I_{to} is related to its control mechanism. Siegelbaum & Tsien (1980) and Maylie & Morad (1984) have indicated that I_{to}, which is assumed to be carried by K^+, is triggered and controlled by an increase in intracellular Ca^{2+} levels, $[Ca^{2+}]_i$, most probably due to Ca^{2+} influx via the slow channel and release of Ca^{2+} from the sarcoplasmic reticulum. A Ca^{2+}-dependent K^+ current and a Ca^{2+}-dependent Cl^- current have been described in nervous tissues as well (Meech & Standen, 1975; Mayer, 1985). A second type of I_{to}, which was described by Kenyon & Gibbons (1979*b*), represents a K^+ current and does not strongly depend on Ca^{2+} influx or increase in $[Ca^{2+}]_i$, but is blocked by 4-aminopyridine (4-AP). This idea of a Ca^{2+}-insensitive I_{to} was supported by Boyett (1981*a*, *b*) in sheep Purkinjne fibres, and partly by Kukushkin *et al.* (1983) in rabbit ventricular muscles. This type of I_{to} displays voltage- and time-dependent inactivation. A similar type of transient outward current was originally demonstrated in isolated snail neurones (Conner & Stevens, 1971; Neher, 1971) and has been named I_A. Recently, adrenergic modulation of the Ca^{2+}-insensitive I_{to} in isolated canine Purkinje cells has been described (Nakayama & Fozzard, 1988). Coraboeuf & Carmeliet (1982), on the other hand, concluded that sheep Purkinje fibres displayed both types of I_{to}, judging from different sensitivities to caffeine and 4-AP, and from the different kinetics of the two current components.

Several recent studies using individual cells isolated from adult mammalian hearts have pointed out the presence of I_{to} from cell types other than Purkinje cells. Most

of them have shown that the current is insensitive to increases in $[Ca^{2+}]_i$ since it remains even after inhibition of the calcium current (I_{Ca}). The current is blocked by 4-AP, and it shows voltage- and time-dependent inactivation (Mitchell *et al.* 1984; Josephson *et al.* 1984; Nakayama & Irisawa, 1985; Giles & Van Ginneken, 1985). More recently, Escande, Coulombe, Faivre, Deroubaix, & Coraboeuf (1987) have reported both types of I_{to} from human atrial myocytes similar to sheep Purkinje fibres (Coraboeuf & Carmeliet, 1982). These results raise questions concerning what determines and controls the expression of the Ca^{2+}-sensitive and -insensitive I_{to} in different cell types and under different experimental conditions.

In previous reports (Hiraoka & Kawano, 1986, 1987), we described the existence of I_{to} in rabbit ventricular myocytes and its contribution to the modulation of interval-dependent changes in action potential repolarization. This study also indicated the presence of two types of I_{to} in this preparation, similar to sheep Purkinje fibres and human atrial myocytes. Therefore, we examined the nature and control mechanism of the two components of I_{to} to clarify their physiological function and their role in the rabbit ventricular action potential.

METHODS

Preparation. All the experiments were done using individual cells isolated from rabbit ventricles. Single ventricular myocytes were obtained by an enzymatic dissociation procedure similar to that described previously by Hiraoka & Kawano (1987). Briefly, rabbits weighing 2–3 kg were anaesthetized with sodium pentobarbitone (30 mg/kg body weight, I.V.) after heparinization (300 iu/kg, I.V.). The chest was opened under artificial respiration and the aorta was cannulated *in situ* for perfusion with Tyrode solution before the heart was dissected out. After the perfusate was changed to nominally Ca^{2+}-free Tyrode solution for about 5 min, low-Ca^{2+} (30 μM) Tyrode solution with collagenase (0·4 mg/ml, type I, Sigma Chemical Co., St Louis, MO, USA) was recirculated for 30 min using a Langendorff apparatus. The heats were then stored in high-K^+ and low-Cl^- solution (KB medium; Isenberg & Klockner, 1982*a*) at room temperature for 60 min, after the enzyme was washed out. The isolated cells were obtained by gentle agitation of small pieces of ventricles and were stored in the KB medium for 1–2 h. They were then kept in the Tyrode solution at room temperature before use. The cells were then placed in the recording chamber, where pre-warmed Tyrode solution was superfused at a rate of 2–3 ml/min. The temperature of the superfusate in the recording chamber was kept at 33–35 °C, not exceeding 1 °C of variation in any experiment. The recording chamber was set on the stage of an inverted phase-contrast microscope (Diaphot TMD, Nikon Co., Tokyo). Isolated cells were either round or rod-shaped; usually 40–60 % of cells were rod-shaped in Tyrode solution containing 1·8 mM-Ca^{2+}. Single rod-shaped cells having smooth surfaces with clear striations were selected for study and the results given below were obtained from these single cells.

Solutions. The Tyrode solution contained (mM): NaCl, 143·0; KCl, 4·0; $MgCl_2$, 0·5; $CaCl_2$, 1·8; NaH_2PO_4, 0·33; HEPES, 5. The low-Ca^{2+} solution contained 0·03 mM-$CaCl_2$, with other components the same as in the Tyrode solution. The pH of both solutions was adjusted to 7·3–7·4 by adding NaOH. The Co^{2+} solution had 2·0 or 4·0 mM-$CoCl_2$ in the Tyrode solution and the Sr^{2+} solution was made up by replacing $CaCl_2$ with 1·8 mM-$SrCl_2$. For measurement of the reversal potential in different K^+ solutions, appropriate amounts of KCl were added to the Tyrode solution. These different K^+ solutions were adjusted to a constant osmolarity by adding choline chloride. In the low-Na^{2+} solution, 143 mM-choline chloride or 300 mM-sucrose replaced NaCl in the Tyrode solution, and an appropriate amount of KCl was added. A total content of Na^+ in these low-Na^{2+} solutions was about 1·0 mM. These low-Na^+ solutions were adjusted to equal osmolarity by adding extra amounts of choline chloride or sucrose. Tetrodotoxin (TTX; Sankyo Pharm. Co., Tokyo), caffeine (Wako Pure Chemical Co., Tokyo), 4-aminopyridine (4-AP; Sigma Chemical Co., St Louis, MO, USA) and ryanodine (LVOG Inc., Wind-Gap, PA, USA) were added to the Tyrode solution to the final concentrations described in the text. The KB (Kraftbrühe) medium contained

(mM): potassium glutamate, 70·0; taurine, 15·0; KCl, 30·0; KH_2PO_4, 10·0; $MgCl_2$, 0·5; glucose, 11·0; and EGTA, 0·5. The pH was adjusted to 7·4 by adding KOH. The internal solution in the patch electrode contained: KCl, 130·0; K_2ATP, 5·0; creatine phosphate (Sigma Chemical Co., St Louis, MO, USA), 5·0; and HEPES–KOH buffer, 5·0 (pH = 7·3). For the EGTA-containing solution, 20·0 mM-EGTA was added to the above internal solution.

Electrical measurements. Suction pipettes with inner tip diameters of less than 3 μm were fabricated from capillaries of glass with a high melting temperature (Propper Manufact. Co., NY, USA) by the method originally described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). A typical electrode had a tip diameter of 2–3 μm and a resistance of 1·5–3·0 MΩ when the pipette was filled with the internal solution by capillarity and via a syringe. The cells were allowed to settle on the bottom of the recording chamber and were superfused with the Tyrode solution for at least 20–30 min before they were approached by the pipettes. The electrode potential was adjusted to zero immediately before the pipette was attached. After a seal resistance of 5–100 GΩ was established by gentle suction, the cell membrane under the electrode tip was broken by further application of negative pressure (−30 to −60 mmH_2O). A resting potential of −80 to −90 mV was usually recorded. The whole-cell current recordings and the measurement of membrane potentials were done using a patch-clamp amplifier (Model 8900, Dagan Corp., Minneapolis, MN, USA). In the whole-cell-clamp mode, the electrode resistance in-series with the cell membrane was compensated by minimizing the duration of the capacitive surge on the current trace. Action potentials were elicited under the current-clamp condition by applying a square pulse of 2 ms duration and suprathreshold intensity.

The general criteria and cautions used to select the electrically sound and stable cells for the present experiment were as follows. The resting membrane potential exceeded −80 mV and its value did not vary more than 1 mV for the 5 min observation period. The maximum upstroke velocity ($\dot{V}_{max}$) of action potentials exceeded 100 V/s. The configuration of action potential exhibited a distinct plateau like those recorded from multicellular ventricular preparations and the total duration was longer than 150 ms at a constant stimulation frequency of 0·1 Hz. Cells that did not meet all of the above conditions were discarded. Further, the configuration of either action potentials or membrane currents during depolarization to 0 mV from the holding potential of −30 mV was carefully observed with pulse application at 0·1 Hz for 5–10 min before starting electrical measurements. If the action potential duration at the 0 mV level or the amplitude of the peak inward current, mostly representing the calcium current, did not differ by more than 5% of their values in successive measurements, a preparation was judged stable and experiments proceeded. This requirement was based on our observation that the repolarization phase of the action potential and the calcium current seemed to be the most sensitive indicators of electrical conditions of the cells, since changes in these parameters always appears to precede cellular damage and death. Once a stable condition was achieved, it usually lasted for 30–40 min in our preparations. Our experiments were usually completed within 40 min. One exception to the above rule was for studies in the Co^{2+} solution, where the preparations were stable for 60–80 min. Pulse application under the voltage clamp was done at a frequency of 0·1 or 0·067 Hz unless otherwise stated. Following each experiment, the junction potential was measured, and if the value exceeded ±2 mV, correction for the potential measurement was made.

Membrane potential and current signals were monitored by a storage oscilloscope (VC10, Nihon Kohden Co., Tokyo) and recorded simultaneously on an FM tape-recorder (A-45, Sony Co., Tokyo). The voltage and current signals were inscribed with a recorder (Omnicorder 8M 14, NEC San-ei Co., Tokyo) that had a linear frequency response up to 2·5 kHz. In the later half of the study, signals were digitized on-line by a 12-bit A–D converter (ADX-98, Canopus Co., Kobe) at a sampling frequency of 1 kHz and stored in a computer (PC 9801, NEC, Tokyo) for later analysis.

The amplitude of I_{to} in these experiments was measured as the difference between the peak of the transient outward current and the minimum current level during the depolarizing pulse after the peak. This measurement may have included a small portion of the delayed outward K^+ current even in the absence of I_{Ca}. In the presence of I_{Ca} the measurement errors of other currents may be larger. We did not use a subtraction method by application of a blocker for I_{to}, since there was no selective inhibitor of I_{to} available that did not affect other currents.

Curve-fitting procedures. Generally, we fitted our experimental data to the sum of three or less exponential functions plus a baseline. As the first step, we manually chose a length of data to be fitted and set the baseline. Parameters of exponential functions were determined originally by

fitting the data in chosen lengths, using a non-linear optimum seeking fitting method. The optimum objective function was achieved according to a least-squares principle. Variance analysis of the optimized fitting parameters was, then, examined by the F test. If the test approves that the fitting function has a statistical significance between regression sum of square and residual sum, together with a lack of statistical significance between residual sum of square and sum of square of random noise error, a correlation index of the fitting function is calculated. Thus, taking account of the correlation indices, we mainly chose the function of the least-exponential components among those of the three which could pass the F test. In the case of failure to pass the second test item for any of the chosen sum of exponential functions, the one having the largest correlation index was chosen. In the case of failure to pass the first test, we concluded that there was no obvious time-dependent component in the length of data.

Statistical analysis was done using the paired t test, and a P value of less than 0·05 was considered significant.

RESULTS

Transient outward current in the absence of calcium current in rabbit ventricular myocytes

When depolarizing clamp pulses were applied to rabbit ventricular myocytes from the holding potential of −60 mV in normal Tyrode solution, I_{to} was not seen upon depolarizations to around +20 mV, but it appeared at voltages more positive than +40 mV (Fig. 1). However, when 2 mM-Co^{2+} was applied to the preparation to block the calcium current (I_{Ca}), I_{to} was apparent at potentials positive to −20 mV and it increased in amplitude with increasing depolarization (Fig. 1). I_{to} reached its peak rapidly (within 10 ms) from the beginning of the pulse. Current decay was largely over 100–200 ms, depending on the voltage. Current changes similar to those shown in Fig. 1 were confirmed in six preparations after blocking I_{Ca} by 2–4 mM-Co^{2+} and in four other cells treated with 6·8 μM-TTX and Co^{2+}. The threshold voltage for I_{to} activation was found to be −30 mV in two, −20 mV in five and −10 mV in three cells with an average value of −19 mV, when it was examined by applying depolarizing pulses in 10 mV steps from the holding potential of −60 mV. In three other preparations, external Ca^{2+} was removed from the Co^{2+} Tyrode solution (2 mM-Co^{2+}) without change in I_{to}. These data indicate that rabbit ventricular myocytes exhibit I_{to} in response to strong depolarization and that the current overlaps with I_{Ca} at most activating voltages. The current is also activated in the absence of I_{Ca} or external Ca^{2+}. We denote this Ca^{2+}-insensitive I_{to} component as I_t, and the rest of I_{to}, which is described below, as $I_{K,Ca}$, for reasons stated in the following sections. We will use the term I_{to} in the following sections when we deal with the total current or the current including both components.

We first analysed the characteristics of I_t in the absence of I_{Ca} after applying Co^{2+}. Since strong depolarization was necessary to activate I_t and the capacitive current did not subside completely within the first 2–4 ms at potentials that caused I_t to appear in our recording system, a detailed analysis of the activating time course could not be made. Therefore, our analysis focused on the peak value and the inactivation properties of I_t. Figure 2 illustrates the voltage-dependent development of I_t and the analysis of its inactivation time course. Both the peak values of I_t and the time course of inactivation varied with voltage. Semilogarithmic plots of the inactivation phase revealed two decaying phases of I_t in the absence of I_{Ca}, a fast and a slow one. The time constants of inactivation of I_t at different voltages were

examined in six preparations exposed to TTX and Co^{2+}. The time constants (approximately 15 ms) of the faster decaying component remained nearly constant at different voltages, whereas the time constants of the slower component varied with voltage. They were longer at around 0 mV, became shorter with membrane depolarization, reached a minimum at +50 to +60 mV and remained that long or

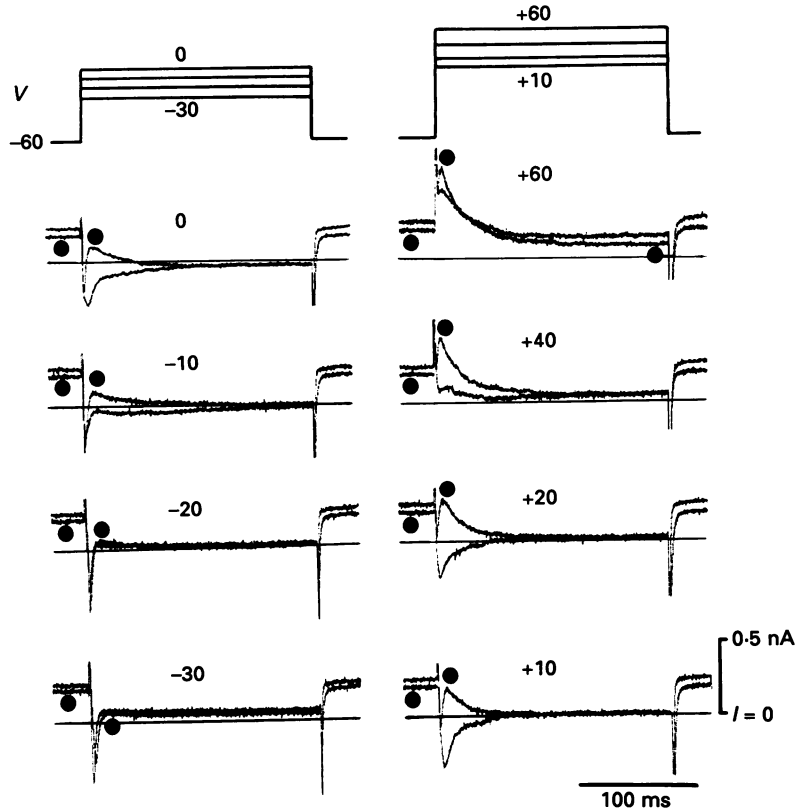

Fig. 1. Transient outward current (I_{to}) in the presence and absence of calcium current (I_{Ca}). The voltage protocol (mV) is shown at the top. Lower four traces in each column are current responses to the depolarizing voltage (mV) steps, indicated above each pair of traces. Each panel shows two superimposed current responses before and after (●) blocking I_{Ca} by applying 2 mM-Co^{2+}. Before application of Co^{2+}, I_{to} is hard to see in depolarizing steps from negative to +20 mV but appears at +40 mV or more positive potentials. After blocking I_{Ca} (●), I_{to} is visible at potentials positive to −20 mV. Throughout the experiment 6·8 μM-TTX was applied, but I_{Na} was not completely blocked in this case, since a small residual inward current is still activated at −30, −20, −10 and 0 mV. Holding potential, −60 mV.

tended to become longer in some preparations upon further depolarization (Fig. 2*C*). The variation in time constants at strong depolarizing voltages may be partly due to overlapping with the delayed outward K^+ current, since it is activated at these potentials (McDonald & Trautwein, 1978).

The activation (q) and inactivation (r) parameters of I_t were determined in the TTX- and Co^{2+}-containing solution. To obtain the parameter for activation, the

amplitude of I_t at each activation voltage was measured for the experiment shown in Fig. 1. The I_t at each potential was obtained from every experiment and its value relative to maximal I_t was calculated; the values are plotted in Fig. 3 (●). This gives the steady-state voltage dependence of activation. Activation of I_t began at −20 mV, increasing with membrane depolarization and becoming maximal at voltages

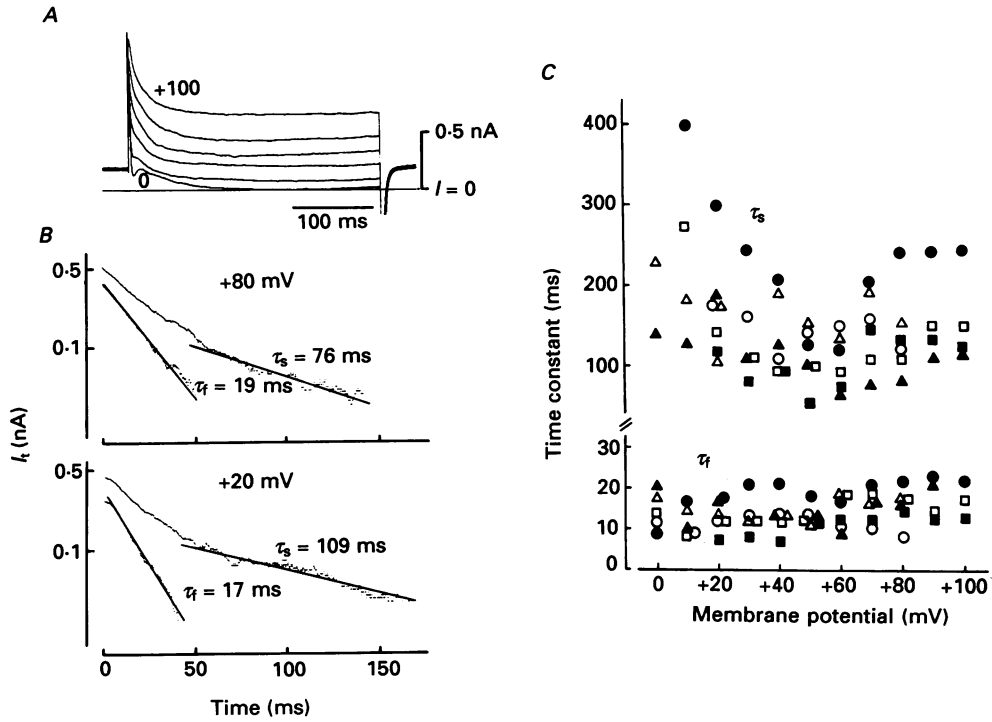

Fig. 2. Time course of inactivation of I_t. *A*, superimposed traces of I_t in response to depolarizing voltage steps to various levels from the holding potential of −60 mV. I_t was elicited by voltage steps between 0 and +100 mV in 20 mV increments. *B*, semi-logarithmic plots of inactivation phase of I_t at voltages of +80 mV (upper) and +20 mV (lower). Two phases of inactivation are present at each voltage. While the time constants of the faster decaying component (τ_f) are similar at both voltages, those of the slower component (τ_s) become shorter with larger depolarizations. *C*, plots of time constants (τ_f, τ_s) obtained from six preparations. Different symbols correspond to data obtained from each preparation.

positive to +70 mV, revealing an S-shaped dependence on membrane potential. The data were reasonably well described by a Boltzmann distribution with voltage at half-maximal activation at approximately +12 mV and a slope factor of −18. The continuous line for the parameter q in Fig. 4*A* was calculated using the following equation:

$$I_t/I_{max} = \frac{1}{1+\exp(V+12{\cdot}3)/-18{\cdot}2}.$$

To determine the inactivation parameter, r, the membrane was held at various levels for 10 s before applying the test pulse to a fixed voltage. The steady-state

inactivation determined by this method is shown in Fig. 3, panel *A*, with examples of experimental records in panel *B*. At potentials more negative than −70 mV, inactivation was almost completely absent; complete inactivation was achieved at potentials positive to 0 mV. Between these membrane potentials, there was an S-shaped relation between voltage and steady-state inactivation. The continuous line

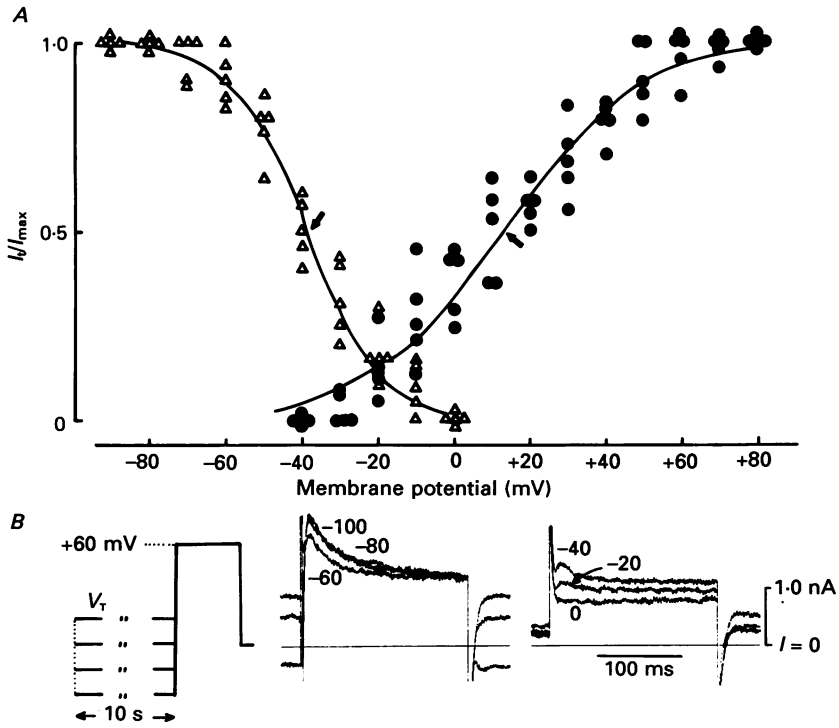

Fig. 3. Steady-state voltage dependence of activation and inactivation of I_t. *A*: ●, activation parameters (q); △, inactivation parameters (r). Data for both parameters obtained from six preparations. Continuous lines drawn according to equations described in text. Arrows, half-maximal activation and inactivation (V_h). *B*, current records demonstrating voltage dependence of steady-state inactivation. Left, the voltage protocol. Middle and right columns, current records during depolarization to a fixed voltage of +60 mV from various membrane potentials (V_T) indicated in mV. V_T was maintained for 10 s before each depolarization. Depolarizations from −80 and −100 mV elicited I_t of equal amplitude, while depolarization from 0 mV caused no I_t.

for the inactivation parameter, r, in Fig. 3*A* was calculated using the following equation:

$$I_t/I_{max} = \frac{1}{1+\exp(V+-38{\cdot}2)/9{\cdot}8},$$

where the voltage at half-maximal inactivation was about −38 mV and the slope factor was 9·8. Again, the experimental data were fitted well by a Boltzmann distribution.

Recovery from inactivation of I_t was examined by applying double pulses of a constant test voltage with various interpulse intervals. Figure 4 shows two examples

of semilogarithmic plots of the recovery of I_t examined by this method. Measurement was done with the preparations in the presence of TTX and Co^{2+} at an interpulse potential of −60 mV. Among six preparations, I_t of two cells recovered on a single-exponential time course, and recovery of four others followed double exponentials with faster and slower time constants. The time constants of the slower components

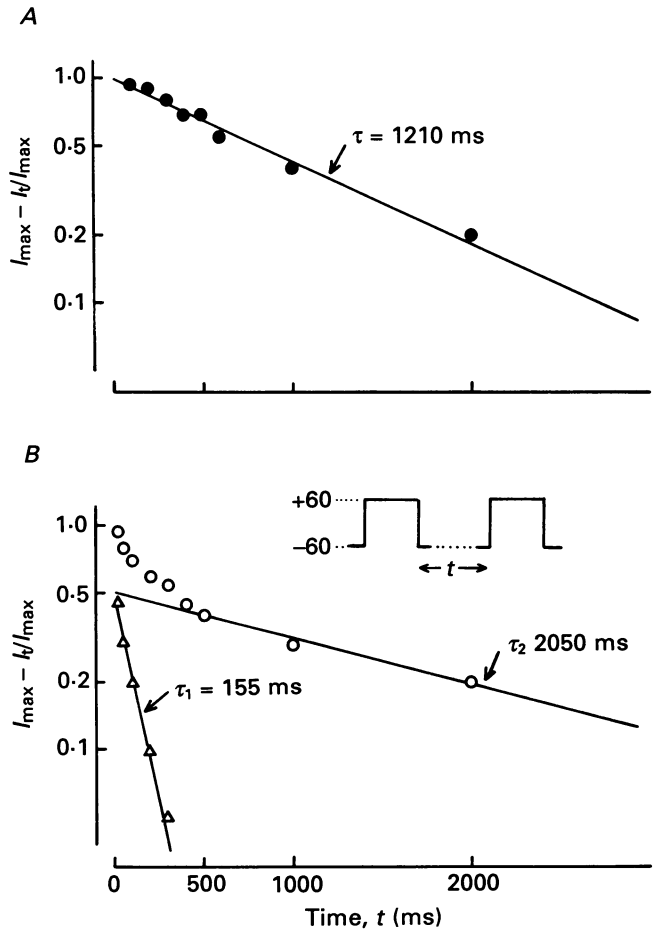

Fig. 4. Semilogarithmic plots of I_t recovery from inactivation. *B*, inset: the experimental protocol. Abscissa, intervals (t) between two pulses. Ordinates, relative I_t calculated as $I_{max} - I_t/I_{max}$. Recovery of I_t was of two types, one composed of a single exponential (*A*) and the other of double exponentials (*B*).

were almost equal to those showing single-exponential recovery; they ranged between 1210 and 2200 ms, with a mean of 1570 ms. When present, the faster time constants gave a mean value of 105 ms ($n = 4$). When the recovery from inactivation of I_t was examined at different holding potentials, its time course varied and became shorter with membrane hyperpolarization. In two preparations, the recovery was examined at three different holding potentials: −40, −60 and −70 mV. The values were 2350 and 2900 ms at −40 mV, 1400 and 1950 ms at −60 mV, and 780 and 950 ms at −70 mV, respectively.

To examine the charge carrier, the reversal potential (E_{rev}) of I_t was determined at different ionic concentrations by applying a short (10–15 ms) depolarizing pulse to activate I_t at a fixed level, and following this with repolarizing pulses to various voltages for 1 s. E_{rev} was measured at three different external K^+ concentrations, $[K^+]_o$, in the presence and absence of external Na^+. When E_{rev} was determined in the

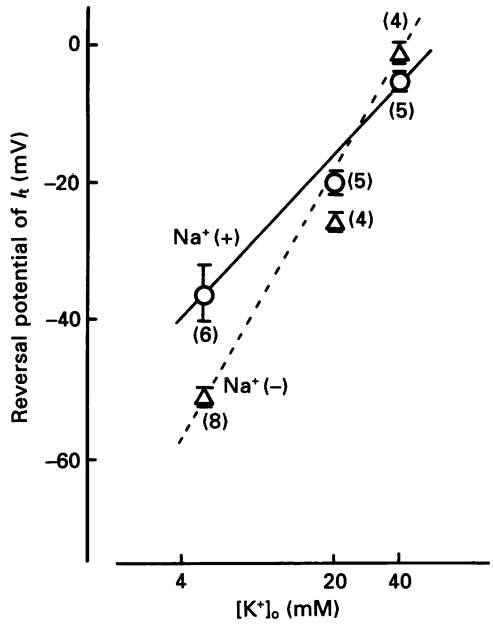

Fig. 5. Reversal potential of I_t at different external K^+ concentrations. Abscissa, $[K^+]_o$ on logarithmic scale. Ordinate, reversal potential of I_t. ○, E_{rev} of I_t in the presence of normal $[Na^+]_o$ (= 143 mM). The slope per 10-fold change in $[K^+]_o$ gives a value of 32 mV. △, E_{rev} of I_t in low $[Na^+]_o$ (= 1·0 mM). The slope gives a value of 48 mV per 10-fold change in $[K^+]_o$.

presence of external Na^+, 40 mM-choline chloride (three preparations) or 80 mM-sucrose (two cells) was added to the Tyrode solution with $[K^+]_o$ = 4 mM. At $[K^+]_o$ = 20 or 40 mM, KCl was exchanged for choline chloride or sucrose to maintain constant osmolarity. In the presence of external Na^+, E_{rev} at $[K^+]_o$ = 4 mM averaged −38 mV (n = 5) and −6 mV at $[K^+]_o$ = 40 mM, yielding a slope for a 10-fold change in $[K^+]_o$ of 32 mV (Fig. 5). At very low $[Na^+]_o$ (1·0 mM), with NaCl replaced by equimolar choline chloride or 300 mM-sucrose, E_{rev} at $[K^+]_o$ = 4 mM was −51 mV and the slope was 48 mV per 10-fold change in $[K^+]_o$. To examine the possible contribution of Cl^- to I_t, external Cl^- concentration was reduced to 8·6 mM by exchanging sucrose (two cells) or 143 mM-sodium aspartate (three cells) for NaCl at $[K^+]_o$ = 4 mM. E_{rev} in the low-Cl^- solutions changed little (2–6 mV) compared with the normal-Cl^- solution. Since changes in junction potential of the low-Cl^- solutions were usually as large as 60–80 mV, the measured shift of E_{rev} could not be considered to be a real change. Therefore, it was concluded that the major charge carrier of I_t is K^+ with Na^+ involved to a small extent, with a negligible Cl^- contribution.

Effects of 4-aminopyridine and caffeine on transient outward current

Since 4-AP and caffeine, either singly or combined, were shown to inhibit I_{to} in Purkinje fibres (Kenyon & Gibbons, 1979*b*; Coraboeuf & Carmeliet, 1982), the effects of these agents on I_t were examined in our preparations. Figure 6*A* and *B* illustrates the complete and reversible inhibition of rabbit ventricular I_t by 2 mM-4-AP after I_{Ca} and I_{Na} were blocked with 2 mM-Co^{2+} and 6·8 μM-TTX. In four of six preparations, 2 mM-4-AP completely abolished I_t and inhibited I_t by more than 85% of the control in the other two. In the latter two cases, application of 4 mM-4-AP eliminated the residual I_t.

Application of 5 mM-caffeine had no effect on peak amplitude or inactivation time course of I_t (Fig. 6*C*). This result was confirmed in five preparations in the presence of Co^{2+} and TTX when drug effects were examined with pulses applied every 15 s.

When the effects of 4-AP and caffeine were examined under different conditions from those shown in Fig. 6, different results were obtained. In the presence of I_{Ca}, the application of 2 or 4 mM-4-AP did not completely inhibit I_{to} but reduced its size to 39% (mean of four cells) and 57% (five cells) of the control, when the current was elicited with depolarizing voltage steps to +50 and +60 mV. These test voltages were chosen because both I_{Na} and I_{Ca} closely approached their reversal potentials, while a relatively large I_{to} could be elicited in normal Tyrode solution. Combined application of 2 mM-4-AP and 5 mM-caffeine rapidly and completely abolished I_{to} (Fig. 7). This result was confirmed in five preparations. In addition to their effects on I_{to}, 4-AP and caffeine changed the holding current differently at −60 or −80 mV. The holding current in the control was 261 ± 46 pA (mean ± S.E.M., $n = 6$) and it significantly increased to 320 ± 44 pA ($P < 0{\cdot}02$) in 2–4 mM-4-AP. On the other hand, 5 mM-caffeine decreased the holding current to 191 ± 50 pA ($P < 0{\cdot}05$) from the control. On the other hand, when 5 mM-caffeine alone was applied to ventricular myocytes in the presence of I_{Ca}, the drug caused a small but consistent reduction of I_{to} to 78% of the control (four cells).

The 4-AP-sensitive and caffeine-sensitive components of I_{to} were subtracted from the total current records in each test solution. Figure 8*A* shows superimposed tracings in the control solution (*a*, ○), 4 mM-4-AP (*b*, ●) and the 4-AP and 5 mM-caffeine solution (*c*, ▲). The subtraction $a-b$ gives the 4-AP-sensitive component, while $b-c$ discloses the caffeine-sensitive component. Figure 8*B* illustrates semi-logarithmic plots of both components. The 4-AP-sensitive I_{to} was composed of two exponentials having fast and slow time courses, while the caffeine-sensitive component was predominantly composed of a single exponential with a fast time course. Results similar to those shown in Fig. 8 were confirmed in five preparations. The time constants of the caffeine-sensitive component were always close to those of the faster ones of the 4-AP-sensitive I_{to}.

Regulation of the caffeine-sensitive transient outward current

The above results showed that the caffeine-sensitive I_{to} was much smaller than the 4-AP-sensitive one (I_t) underlying the total I_{to}. A possible reason for this may be related to our experimental conditions, since we used a slow pulse rate of one per 10 or 15 s. A faster pulse rate would increase Ca^{2+} influx, thus increasing the Ca^{2+}

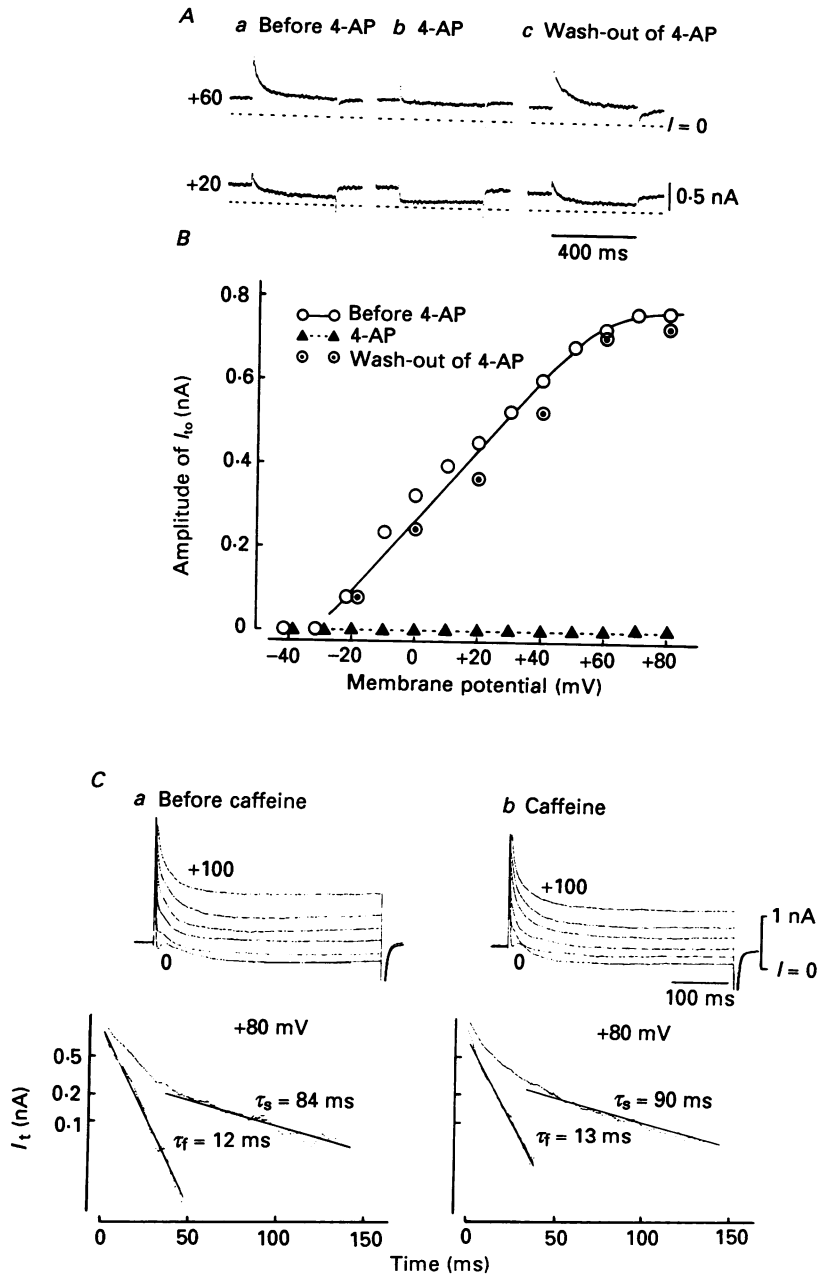

Fig. 6. Effects of 4-AP and caffeine on I_t. *A* and *B*, blocking effect of 2 mM-4-AP on I_t in a cell treated with 2 mM-Co^{2+} and 6·8 μM-TTX. *A*, two representative current traces. *B*, current–voltage relation of I_t and the effect of 2 mM-4-AP. Note that I_t was reversibly abolished by 4-AP. *A* and *B* show results from the same preparation. *C*, effect of 5 mM-caffeine on I_t with I_{Na} and I_{Ca} blocked by TTX and Co^{2+}. Top, superimposed current traces in response to depolarizations to 0 and +100 mV steps. Bottom, semilogarithmic plots of the decay of I_t at +80 mV. Note that caffeine did not affect the amplitude or time course of decay of I_t.

releasable from intracellular stores per unit time. Therefore, I_{to} was examined at a stimulation rate of 2·0 or 2·5 Hz. Figure 9 shows the results of one such experiment where depolarizing clamp pulses of 200 ms were applied at a frequency of 2·5 Hz after a rest period of 15 s. A large I_{to} was elicited at the 1st pulse, but those at the 2nd and 3rd pulses were smaller in amplitude because of the slow recovery from inactivation

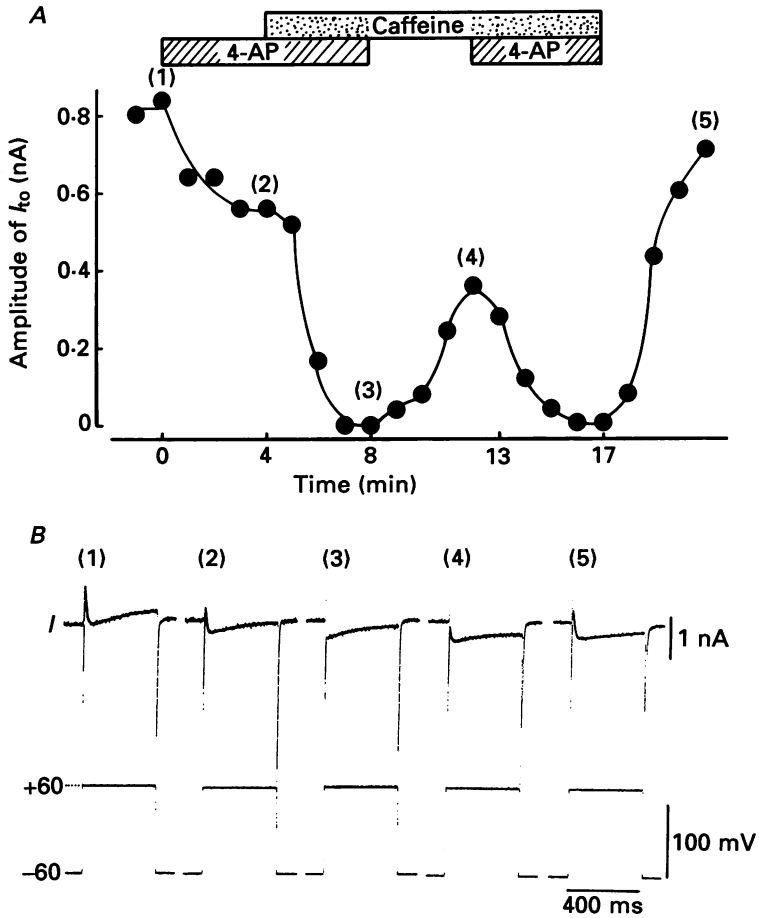

Fig. 7. Effects of 4-AP and caffeine on I_{to} in the presence of I_{Ca}. *A*, time course of changes in I_{to} upon application of 2 mM-4-AP and 5 mM-caffeine. Periods of application of 4-AP and/or caffeine are indicated at top. Holding potential was −60 mV and depolarizing pulse was applied to +60 mV at 0·1 Hz. *B*, representative current (top) and voltage records (bottom). Numbers at top correspond to times indicated in *A*.

(see Fig. 4). However, the amplitude of I_{to} again increased gradually with successive pulses achieving a plateau after about the 30th depolarization (Fig. 9). In this experiment, we assumed that I_{to} reached steady state after the 30th depolarizing pulse at a frequency of 2·0 or 2·5 Hz and considered it to be the steady-state I_{to}. In the case shown in Fig. 9, the relative ratio of I_{to} at the steady state (I_{to} at the steady state divided by that at the 1st pulse) was 0·79. The minimum I_{to} during a train of pulses was usually attained at the 2nd or 3rd pulse. In eleven experiments, the

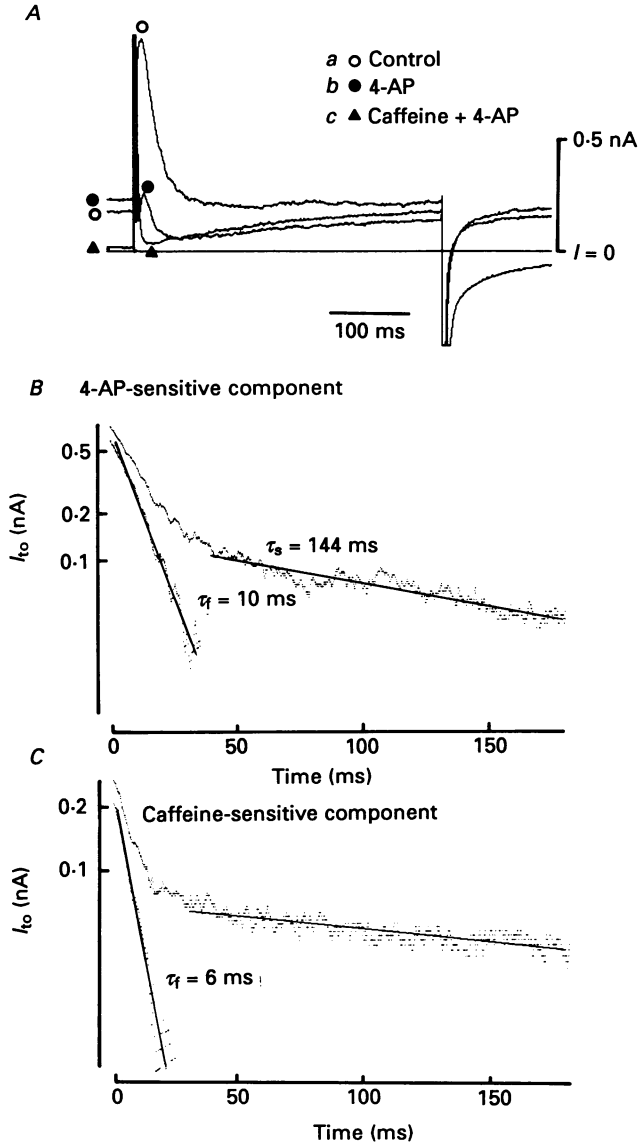

Fig. 8. Separation of the 4-AP-sensitive and caffeine-sensitive I_{to} in the presence of I_{Ca}. *A*, superimposed current traces in control (○), after application of 4 mM-4-AP (●), and after application of 4-AP plus 5 mM-caffeine (▲). Currents were elicited by depolarization to +60 mV from the holding potential of −80 mV. *B*, time course of decay of the 4-AP-sensitive I_{to}. The 4-AP-sensitive component was obtained by the subtraction of I_{to} in the control and in the 4-AP solution. There are two phases of the current decay, with a faster (τ_f) and slower time constant (τ_s). *C*, caffeine-sensitive I_{to}, measured by subtracting I_{to} in the 4-AP and in the caffeine solutions. The decay time course is composed exclusively of the fast component.

relative I_{to} at the 2nd pulse compared with the 1st one was $0{\cdot}33 \pm 0{\cdot}05$ (mean ± S.E.M.), and the one at the steady state was $0{\cdot}71 \pm 0{\cdot}07$. A regrowth of I_{to} always developed during the train of pulses.

While the regrowth of I_{to} was observed during successive depolarizations at a relatively fast rate in the Tyrode solution in the presence (five cells) or absence (six

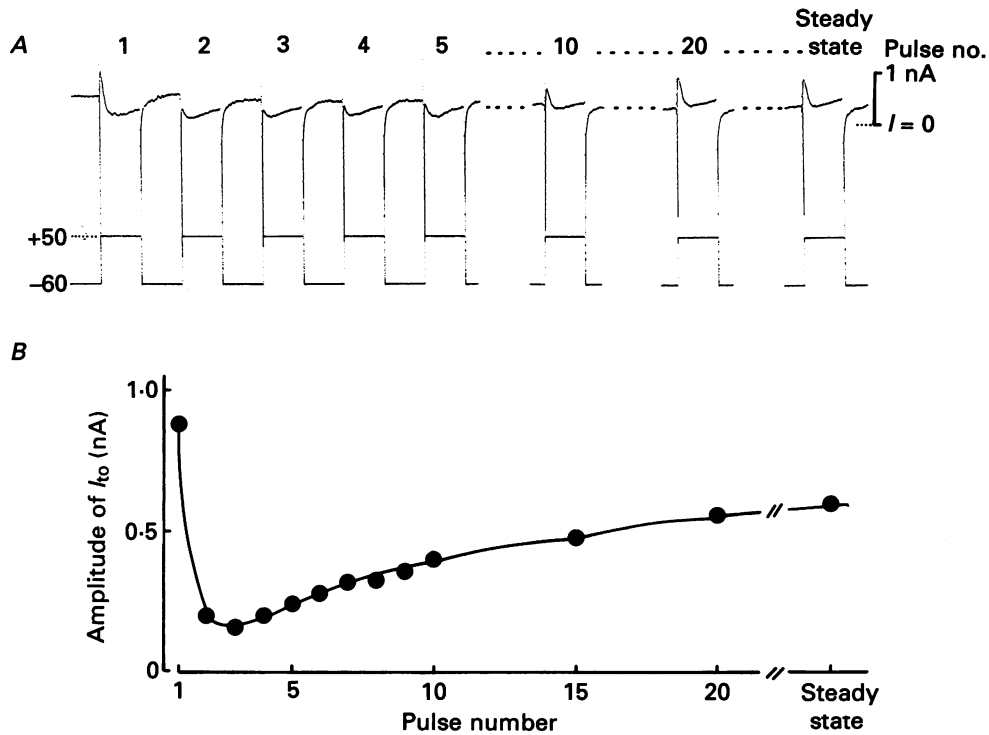

Fig. 9. Successive changes in I_{to} after pulse application at a fast rate. *A*, voltage-clamp records when depolarizing pulses lasting 200 ms were applied at 2·5 Hz from the holding potential of −60 mV to +50 mV. Upper, current records; bottom, voltage. Numbers of pulses are indicated at the top of each record. Steady state: steady-state pulse after the 30th pulse. *B*, change in the amplitude of I_{to} with pulse number. Note that I_{to} greatly decreased in amplitude at the 2nd and 3rd pulse and then increased gradually.

cells) of 15 μM-TTX, the application of 4 mM-Co^{2+} to block I_{Ca} abolished I_{to} regrowth, as shown in Fig. 10*A* and *B*. In six preparations, I_{to} at the 1st pulse was $0{\cdot}56 \pm 0{\cdot}05$ nA in the Tyrode solution and $0{\cdot}58 \pm 0{\cdot}07$ nA in the Co^{2+} solution. The values of the 2nd I_{to} were $0{\cdot}17 \pm 0{\cdot}04$ and $0{\cdot}15 \pm 0{\cdot}04$ nA, respectively. Thus, differences between the two 1st pulses and the two 2nd pulses were not significant. However, the steady-state I_{to} in the Tyrode solution was $0{\cdot}44 \pm 0{\cdot}06$ nA, and this was significantly higher than $0{\cdot}15 \pm 0{\cdot}04$ nA ($P < 0{\cdot}001$) in the Co^{2+} solution.

When 4 mM-4-AP was applied to the myocyte, the 1st I_{to} was markedly reduced in amplitude, but growth in I_{to} during rapid pulsation was similar to that of the control (Fig. 10*C* and *D*). In five experiments, the 1st I_{to} in the 4-AP solution was significantly reduced to $0{\cdot}48 \pm 0{\cdot}08$ nA from $1{\cdot}26 \pm 0{\cdot}07$ nA in the control ($P < 0{\cdot}005$),

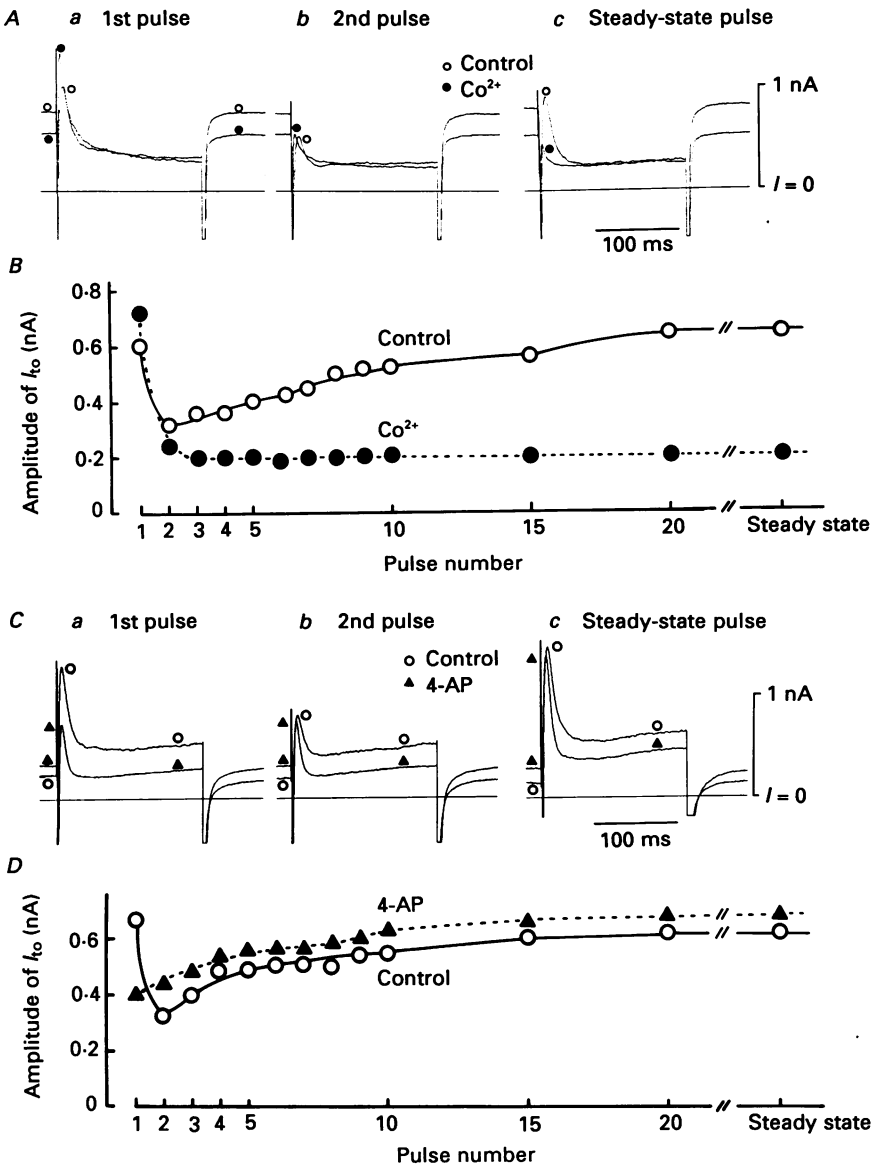

Fig. 10. Effects of Co^{2+} and 4-AP on I_{to} regrowth with pulses. *A* and *B*, effects of 4 mM-Co^{2+}. *A*, superimposed current tracings at the 1st (*a*), 2nd (*b*) and steady-state pulses (*c*) before and after application of Co^{2+}. A 200 ms depolarizing pulse to +50 mV was applied from the holding potential of −60 mV at 2·5 Hz. *B*, analysis of I_{to} with pulses. Although the 1st I_{to} in the presence of Co^{2+} is unchanged, the regrowth of I_{to} with pulses after the 3rd or 4th is completely suppressed. *C* and *D*, effects of 4 mM-4-AP. Arrangement of these panels is similar to *A* and *B*. Holding potential: −80 mV. Depolarizing clamp voltage, +60 mV. Pulses were applied at 2·5 Hz. Although 4-AP suppressed I_{to} at the 1st pulse, it did not block regrowth of I_{to} in the train of pulses.

while I_{to} at the steady state was $0{\cdot}75 \pm 0{\cdot}10$ nA in the latter and $0{\cdot}86 \pm 0{\cdot}16$ nA in the former (not significantly different).

To determine if the I_{to} increase with pulse number represented the caffeine-sensitive I_{to}, 5 mM-caffeine was applied to the myocyte. As shown in Fig. 11*A* and *D*, caffeine completely abolished the I_{to} component and its regrowth with successive pulses. This result was confirmed in five myocytes.

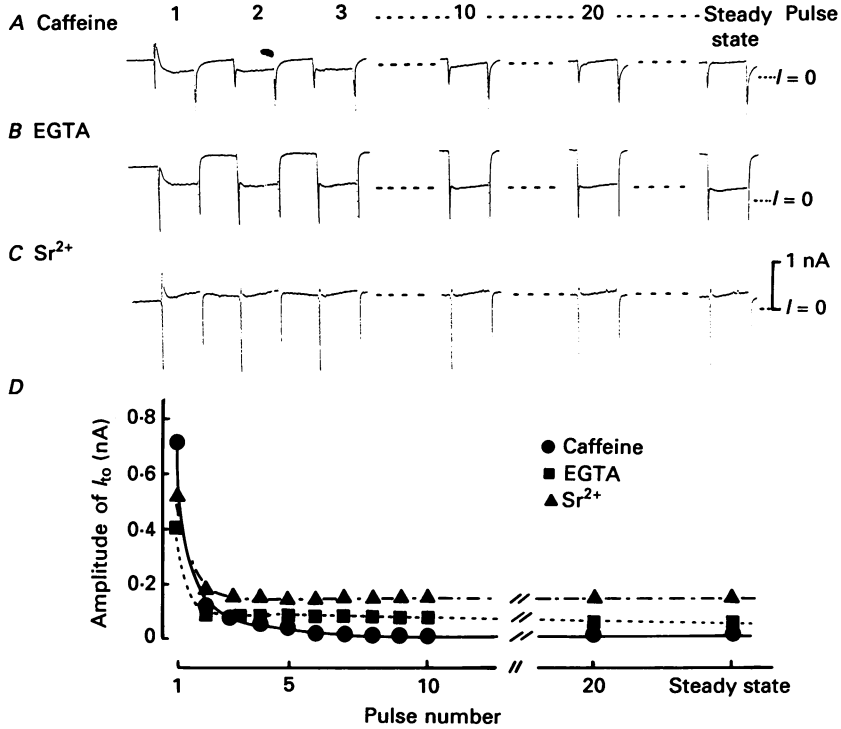

Fig. 11. Effects of caffeine, EGTA and Sr^{2+} on regrowth of I_{to} with pulses. *A–C*, 200 ms current traces elicited at 2·5 Hz. Records *A–C* were obtained from different preparations. *A*, effects of 5 mM-caffeine on I_{to}. Holding potential, −60 mV. Depolarizing clamp voltage, +60 mV. *B*, effects of intracellular application of 20 mM-EGTA on I_{to}. Note absence of regrowth of I_{to} after the 2nd pulse. *C*, effects of Sr^{2+} replacement of external Ca^{2+} on I_{to}. Regrowth of I_{to} after the 2nd pulse is abolished. *D*, summary of I_{to} modulation by trains of depolarization. Regrowth of I_{to} with pulses is absent in three different interventions.

Thus far, our results agreed with the hypothesis that an increase in intracellular Ca^{2+} with rapid pulsation activates the caffeine-sensitive, frequency-dependent I_{to}. To prove this point EGTA was applied intracellularly to determine its effect on I_{to}. This type of experiment has already been useful in defining Ca^{2+}-activated currents in neurones of *Aplysia* (Meech, 1974) and in calf Purkinje fibres (Siegelbaum & Tsien, 1980). Figure 11*B* and *D* illustrates a typical experimental record and a plot of I_{to}. Intracellular application of EGTA suppressed regrowth of I_{to} during successive pulsations (four cells).

Finally, the role of Ca^{2+} released from the sarcoplasmic reticulum in activating the

caffeine-sensitive I_{to} was examined by replacement of external Ca^{2+} with Sr^{2+}. Ca^{2+} and Sr^{2+} have approximately equal permeabilities in the slow channel of cardiac sarcolemma, but release of Sr^{2+} from the sarcoplasmic reticulum (SR) occurs much less effectively than that of Ca^{2+} (Endo, 1977). When 1·8 mM-Sr^{2+} was added to nominally Ca^{2+}-free solution, I_{to} did not increase with successive clamp pulses (Fig. 11*C* and *D*). This was similar to the results obtained with caffeine treatment and

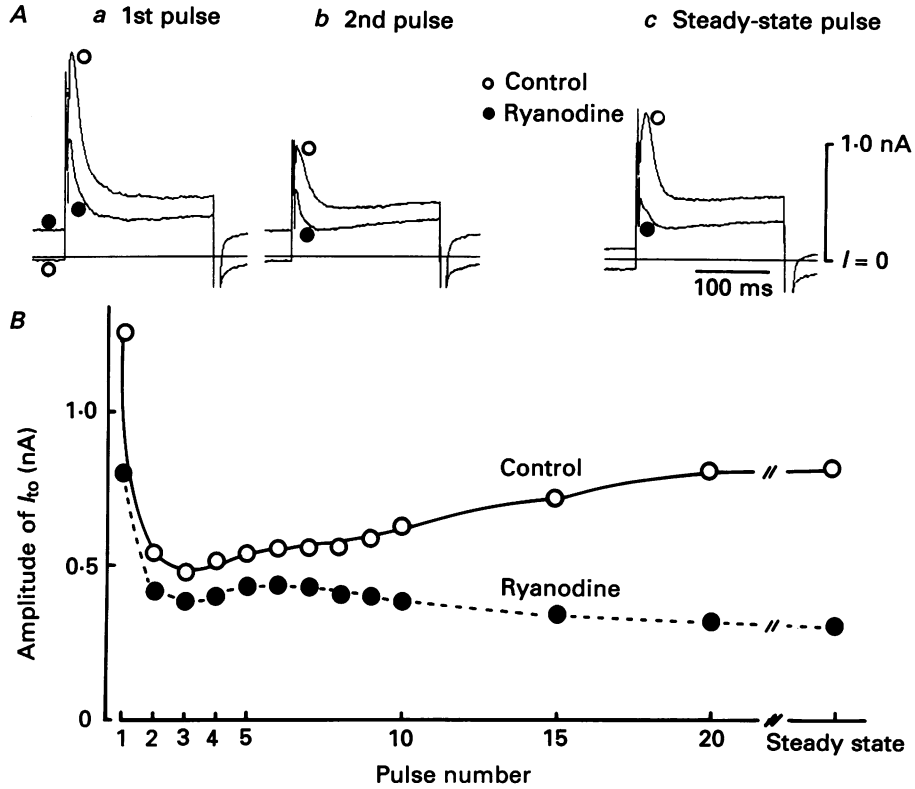

Fig. 12. Effects of ryanodine on frequency-dependent regrowth of I_{to}. Arrangement of the panel is similar to the panels in Fig. 10. Note that 5 μM-ryanodine suppressed I_{to} regrowth as well as the 1st I_{to}. Holding potential, −80 mV. Depolarizing clamp voltage, +60 mV.

with high intracellular EGTA application. Similar results were obtained in four experiments. Another test of the involvement of Ca^{2+} release from SR in the caffeine-sensitive I_{to} is the effect of ryanodine, since this drug has been shown to inhibit Ca^{2+} release from SR with high specificity (Sutko, Ito & Kenyon, 1985). Application of 5 μM-ryanodine abolished the regrowth of I_{to} during a train of pulses (Fig. 12). This result was confirmed in four cells. Ryanodine also depressed the 1st I_{to} to 62 ± 12 % of the control after a rest period longer than 15 s. All of the above results support the hypothesis that the caffeine-sensitive I_{to} is modulated by intracellular Ca^{2+} level controlled through Ca^{2+} influx via the sarcolemma and Ca^{2+} release from the SR. Thus, we use the designation $I_{K,Ca}$ for the caffeine-sensitive I_{to}.

Role of calcium-sensitive and -insensitive transient outward current in ventricular action potential

In a previous report (Hiraoka & Kawano, 1987), we described the contribution of I_t to the modulation of interval-dependent changes in the plateaux of action potentials; namely, the slow recovery of I_t in the face of rapidly recovering I_{Ca} caused

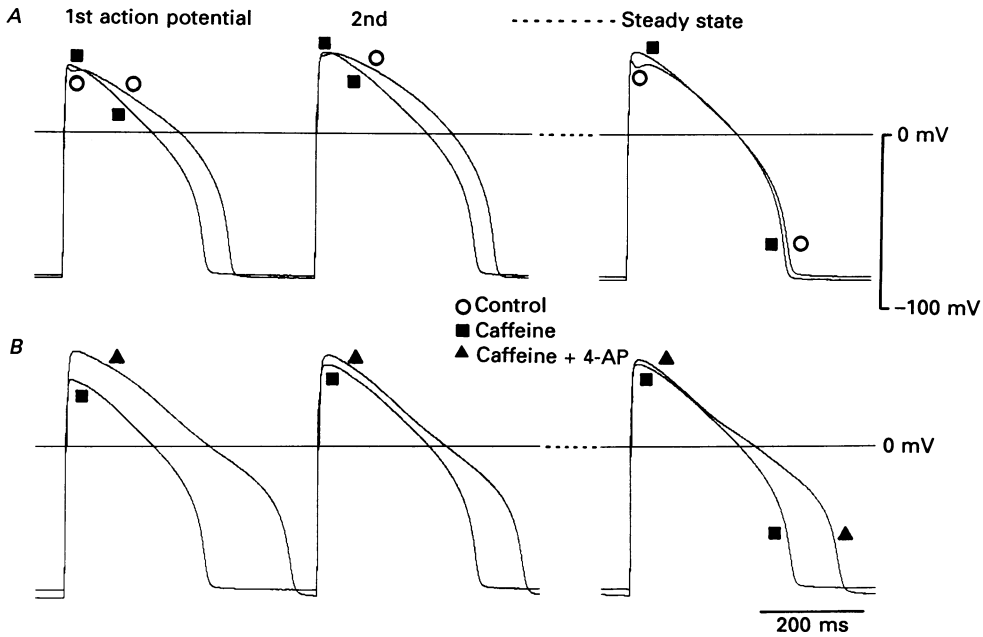

Fig. 13. Effects of 4-AP and caffeine on action potential repolarization following onset of rapid stimulation. Each panel illustrates the 1st, 2nd and steady-state action potentials when rapid stimulation at 2·5 Hz was applied to the ventricular myocyte after a 15 s rest period. Records in *A* and *B* are from the same preparation. The value of $\dot{V}_{max}$ of action potentials was 170 V/s at steady state in this cell. *A*, superimposed tracings before and during application of 5 mM-caffeine. In the control (○), the 1st action potential has a small phase 1 followed by a distinct notch. The 2nd beat shows loss of the notch and attains a higher level and longer duration of the plateau than the 1st one. The steady-state beat again shows a distinct notch after phase 1. The application of caffeine (■) abolished the notch in the 1st and steady-state beats, while the augmented plateau of the 2nd beat was preserved. *B*, superimposed tracings in the presence of caffeine alone (■), and caffeine pulse 4-AP (▲). The presence of 4-AP caused a high and prolonged plateau of the 1st action potential, and the lack of augmentation of the plateau of the 2nd action potential compared with that of the 1st action potential. 4-AP also produced prolongation of each action potential.

paradoxical augmentation and prolongation of the plateau with sudden shortening of the diastolic interval. However, the role of $I_{K,Ca}$ in ventricular repolarization was not clarified in that study. The present study further defines the characteristics of the two components of I_{to} in response to various physiological and pharmacological interventions. To ascertain the different roles of the two I_{to} components in ventricular repolarization, action potential duration during a stimulation train and

the effects of caffeine and 4-AP were examined. When a train of 2·0 or 2·5 Hz stimulation was applied to a myocyte after a rest period of 15 s, the 1st action potential had a small but distinct phase 1 followed by a notch. The 2nd action potential lost phase 1 and the notch, but had augmented plateau and was prolonged as reported previously (Hiraoka & Kawano, 1987). Upon reaching the steady state after successive stimulations, however, phase 1 and the notch reappeared (Fig. 13*A*, ○). Reappearance of the notch on steady-state stimulation was observed in all six preparations examined. The application of 5 mM-caffeine abolished the notch of the 1st action potential and shortened the action potential, but augmentation of the plateau of the 2nd action potential was preserved, similar to the control condition. At steady state, caffeine blocked the reappearance of the notch (*A*, ■). Although shortening of the 1st and 2nd action potentials after caffeine application was not consistent among the six cells, abolition of the notch was consistent in the 1st and steady-state action potentials. Addition of 4 mM-4-AP to the perfusate increased the amplitude and duration of the plateau of the 1st action potential so that they were equal to those of the 2nd one. At steady state, the early phase of the plateau did not change remarkably. The duration of the action potential after caffeine and 4-AP was markedly prolonged throughout the train (Fig. 13*B*, ▲).

The changes in action potential duration were caused not only by I_{to}, but also by both I_{Ca} and the delayed outward K^+ current. The contribution of the latter two currents may be estimated by the alteration of the late current during depolarizing pulses. So, the amplitude of the late current during the train was examined. In normal Tyrode solution, the late current of the 1st pulse was 325 ± 33 pA ($n = 11$). When 200 ms depolarizing pulses were applied from the holding potential of -80 mV to $+60$ mV, the late current of the 2nd pulse significantly decreased to 315 ± 33 pA ($P < 0{\cdot}05$). The late current of the steady-state pulse was 326 ± 45 pA, which was not different from the 1st pulse. Therefore, the late current was mildly decreased with the 2nd depolarization but recovered later during the train. When 4-AP was applied to the myocytes, the late current of the 1st pulse significantly decreased to 181 ± 27 pA ($n = 5$; $P < 0{\cdot}02$) compared to that of the control. However, in the 4-AP solution the late currents of the 1st, 2nd and steady-state pulses did not differ from each other (189 ± 29 and 246 ± 57 pA for the latter two, respectively). In 4-AP plus caffeine, these values were smaller than in the control, but were not different from those in the 4-AP solution. There were, again, no differences among the late currents at the 1st, 2nd and steady-state pulse in 4-AP and caffeine. These results indicate that changes in the action potential duration are also caused by variation of the late current, due mainly to modulation of the delayed outward K^+ current and I_{Ca}, but the latter factors contribute little to the early repolarization.

In summary, the ventricular action potential showed an initial notch in the repolarization phase of the 1st beat after a long rest period and in the steady-state beats during high-frequency stimulation. The 1st beat after a long preceding diastolic interval had a low amplitude, and short plateau, and the 2nd beat after a short diastolic interval had an augmented plateau.

DISCUSSION

The present results demonstrate that rabbit ventricular myocytes exhibit at least two types of transient outward currents with different physiological and pharmacological properties. One is calcium-sensitive and the other is calcium-insensitive. The two components of I_{to} differ in their kinetic properties as well as in their control mechanisms. The charge carrier of the calcium-insensitive I_{to} or I_t is mainly K^+, with Na^+ involved to a much lesser extent.

Transient outward current in ventricular cells

The presence of I_{to} in cardiac preparations was originally described in Purkinje fibres and has long been considered a unique feature of this tissue (see, Carmeliet & Vereecke, 1979). In the past few years evidence has accumulated proving its presence in other cardiac preparations, such as atrial cells (Maylie & Morad, 1984; Giles & Van Ginneken, 1985; Escande *et al.* 1987), A–V node cells (Nakayama & Irisawa, 1985), and ventricular trabeculae and muscle cells (Kukushkin, Gainullin & Sosunov, 1983; Josephson *et al.* 1984; Mitchell *et al.* 1984; Watanabe *et al.* 1984; Tseng, Robinson & Hoffmann, 1987). In our preparations, more than 100 ventricular myocytes were investigated under voltage clamp and every cell examined exhibited prominent I_{to} when depolarizing pulses were applied with the holding potential kept at or more negative than -60 mV. The current was more easily recorded after I_{Ca} was blocked. Therefore, I_{to} seems to exist in most cardiac preparations, although the degree of its development differs. For example, in ventricular preparations, I_{to} is most prominent in rats and much less so in guinea-pig (Josephson *et al.* 1984). I_{to} in rabbit atrial cells seems to be prominent compared to its presence in ventricular preparations (unpublished observations). The factors determining the degree and size of I_{to} development in different cardiac tissues are not known. However, one reason why I_{to} in ventricular muscles is less easily observed than in Purkinje fibres appears to be the large size of the overlapping I_{Ca} in the ventricle (Isenberg & Klockner, 1982*b*; Mitchell, Powell, Terrar & Twist, 1983).

Transient outward current in the absence of calcium current

I_{to} in our preparations was still present when I_{Ca} was blocked by the application of Co^{2+} or the removal of external Ca^{2+}. Therefore, the I_{to} component in the above conditions was relatively calcium-insensitive and is termed I_t. This current was easily abolished by the application of 4-AP. Further, the current exhibited voltage- and time-dependent inactivation. These properties are similar to I_{to} described in isolated atrial cells from the crista terminalis (Giles & Van Ginneken, 1985), A–V nodal cells (Nakayama & Irisawa, 1985) and in rat ventricle (Josephson *et al.* 1984; Mitchell *et al.* 1984). These inactivation properties are similar to those first described in Purkinje fibres (Fozzard & Hiraoka, 1973). This I_t also resembles I_A in neurones (Conner & Stevens, 1971; Neher, 1971; Adams, Smith & Thompson, 1980; Segal, Rogawski & Barker, 1983).

The inactivation time course of I_t was bi-exponential in our preparations (Fig. 2). In contrast Giles & Van Ginneken (1985) reported single-exponential decay in cells from the crista terminals. Nakayama & Fozzard (1988) described two exponential

time constants of inactivation in Ca^{2+}-insensitive I_{to} of isolated canine Purkinje cells. Since our measurements were done only after blocking I_{Ca}, other currents such as the delayed outward K^+ current (I_K) might have been included. However, activation of I_K was small and slow. For example, the amplitude of I_K with depolarizing pulses of 200 ms achieved only about 11% of the maximal level in our preparations ($n = 7$; unpublished observation), where the maximal value was measured by 2–5 s pulses. Since most I_t inactivated after 100–150 ms, the portion of I_K which might have been included in our calculation seemed to be negligible. The application of caffeine did not affect the double-exponential decay in the absence of I_{Ca} (Fig. 6*C*). The time constants of inactivation in two components behaved differently as a function of voltage; the slower component depended on membrane voltage, whereas the faster one did not. These results seem to indicate that double-exponential decay represents a real property of the I_{to} channel. There are also indications that recovery from inactivation of I_{to} in the absence of I_{Ca} had double-exponential time courses, both in previous studies (Nakayama & Irisawa, 1985; Nakayama & Fozzard, 1988) as well as in the present one (Fig. 4).

Determination of the reversal potential of I_t revealed that it depended on external K^+ with a slope of 32 mV for a 10-fold change in $[K^+]_o$. The removal of external Na^+ made the slope steeper for identical $[K^+]_o$ changes. These results indicate that the major charge carrier of I_t is K^+, but a small fraction is also carried by Na^+. This interpretation agrees with the conclusion given by Nakayama & Irisawa (1985), but differs somewhat from the one by Giles & Van Ginneken (1985) in which the E_{rev} of I_t is at a level close to the presumed E_K. It is worth mentioning, however, that the pipette solution in Giles & Van Ginneken's study contained a much higher concentration of K^+ than solutions in the former and the present experiments. This may have possibly given rise to a high K^+ concentration gradient across the cell membrane. The removal of most external Cl^- had little effect on the level of E_{rev}, implying that the contribution of Cl^- to the formation of I_t is negligible.

Transient outward current in the presence of calcium current

In contrast to the condition under which I_{Ca} was absent, I_{to} in the normal Tyrode solution was not abolished completely by the application of 4-AP; however, it was consistently and reversibly inhibited by the co-application of caffeine. These results agree with the observations by Coraboeuf & Carmeliet (1982) in sheep Purkinje fibres, by Escande *et al.* (1987) in human atrial myocytes and by Tseng *et al.* (1987) in canine ventricular myocytes. The caffeine-sensitive I_{to} or $I_{K,Ca}$ increased in amplitude with successive voltage-clamp depolarization at the fast rate of 2·0 or 2·5 Hz, while I_t did not similarly increase because of its slow recovery kinetics. The $I_{K,Ca}$ and I_{to} growth with pulse trains were not seen after the inhibition of I_{Ca} by Co^{2+}. Intracellular application of a high concentration of EGTA also blocked $I_{K,Ca}$. The replacement of Ca^{2+} with Sr^{2+} in the medium, or the application of ryanodine, inhibited this component of I_{to} as well. Caffeine is known to affect Ca^{2+} release and uptake from the sarcoplasmic reticulum and Sr^{2+} can only partially replace Ca^{2+} in bringing about release from the SR (Endo, 1977; Chapman, 1979; Fabiato, 1983). Ryanodine is a potent and highly selective inhibitor of Ca^{2+} release from the SR (Sutko *et al.* 1985). The above results support the idea that increase in myoplasmic

Ca^{2+} through Ca^{2+} influx via the sarcolemma and Ca^{2+} release from the sarcoplasmic reticulum activates $I_{K,Ca}$. The sensitivity of I_{to} to block I_{Ca} was first recognized by Peper & Trautwein (1968), who gave no explanation for their finding. Kenyon & Gibbons (1977) indicated that suppression of I_{to} in the low-Cl^- solution was due to reduced Ca^{2+} activity in the solutions that substituted impermeable anions for Cl^-. Siegelbaum & Tsien (1980) presented evidence supporting the hypothesis that I_{to} in calf Purkinje fibres was activated by intracellular Ca^{2+}. They postulated that the current was carried by K^+. Subsequently, Maylie & Morad (1984) supported this view by showing that I_{to} in atrial cells was closely associated with tension development and I_{Ca}. Although we did not measure the reversal potential of $I_{K,Ca}$ and its dependence on $[K^+]_o$, it might represent K^+ current because of the similarity of its properties to those mentioned in the above reports. Calcium-activated K^+ current has been well described in nervous (Meech, 1978; Hermann & Hartung, 1983) and other tissues (Schwarz & Passow, 1983).

The voltage- and time-dependent characteristics, and the kinetic properties of $I_{K,Ca}$ were not examined in detail in the present study, because of inevitable overlap with I_{Ca}. The subtraction method before and after application of caffeine cannot be verified quantitatively, since caffeine acts in multiple ways on cardiac membrane currents (Eisner, Lederer & Noble, 1979; Yatani, Imoto & Goto, 1984; Hess & Wier, 1984).

Physiological roles of the calcium-sensitive and -insensitive transient outward current

The two types of I_{to} revealed in the present study may have various physiological functions. The calcium-insensitive I_{to}, or I_t, has a slow recovery time course compared with that of $I_{K,Ca}$. Because of this slow recovery, the former contributes predominantly to ventricular repolarization at a slow heart rate and to the modulation of interval-dependent changes in the action potential plateau upon sudden shortening of diastolic intervals (Hiraoka & Kawano, 1987). The present study further demonstrated that $I_{K,Ca}$ grew with successive depolarizations and became almost as large as I_t at a fast pulsation rate. This means that the role of $I_{K,Ca}$ in ventricular repolarization becomes significant at a physiological or faster heart rate. $I_{K,Ca}$ may also stabilize the cell membrane and possibly protect it from damage due to Ca^{2+} overload during tachyarrhythmias, since intracellular Ca^{2+} accumulation is somewhat counteracted by activating the increased outflow of K^+ through the I_{to} channel. This may help to maintain a fully polarized membrane potential. Another implication of $I_{K,Ca}$ in electrical activity is its modulation by changes in I_{Ca}. Interventions affecting I_{Ca} may influence cardiac repolarization by dual mechanisms, one through direct action on I_{Ca} and the other through indirect action on $I_{K,Ca}$.

Determination of steady-state inactivation of I_t revealed nearly complete removal of inactivation at potentials around -70 mV. This may imply that I_t can modify excitability and conduction under certain conditions. If the ventricular cell membrane depolarized to around -70 to -60 mV due to cellular damage or disease processes and therefore the development of I_{Na} became small upon depolarization, the amplitude of I_{Na} would be further reduced by the opposing I_t. When the maximum rate of depolarization ($\dot{V}_{max}$) of the action potential is used as a measure of I_{Na}, the value is distorted by the development of I_t. In such cases, a decrease in $\dot{V}_{max}$ can be

expected depending on the stimulation protocol and the preceding diastolic interval, as can be a decrease in the conduction velocity. In fact, Gilmore, Salata & Davis (1986) reported that the rate-related suppression of excitability and conduction at moderately depolarized states (−70 to −60 mV) was mainly attributable to recovery of 4-AP-sensitive I_{to} in Purkinje and atrial trabeculae. Supernormal conduction observed in the slow response activity (Iinuma & Kato, 1979; Cukierman & Paes De Carvalho, 1982) may also be explained by an interrelation between I_{Ca} and I_t similar to that between I_{Na} and I_t with contribution of the rate-dependent changes in $I_{K,Ca}$.

In neurones, a major role of the calcium-insensitive K^+ current (I_A) is thought to be regulation of the rate of burst activity (Meech, 1978; Hermann & Hartung, 1983). In ventricular cells, deactivation of I_t upon repolarization to the diastolic range of potentials (−80 to −90 mV) appears to be too fast (less than 100 ms) to be significantly involved in diastolic depolarization. It is not certain, however, whether or not deactivation of I_t and $I_{K,Ca}$ becomes slow enough to contribute to the formation of after-potentials in depolarized states. The role of I_{to} in abnormal impulse formation and conduction remains to be clarified.

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REFERENCES

ADAMS, D. J., SMITH, S. J. & THOMPSON, S. H. (1980). Ionic currents in molluscan soma. *Annual Review of Neuroscience* **3**, 141–167.

BOYETT, M. R. (1981*a*). A study of the effect of the rate of stimulation on the transient outward current in sheep cardiac Purkinje fibres. *Journal of Physiology* **319**, 1–22.

BOYETT, M. R. (1981*b*). Effect of rate-dependent changes in the transient outward current on the action potential in sheep Purkinje fibres. *Journal of Physiology* **319**, 23–41.

CARMELIET, E. & VEREECKE, J. (1979). Electrogenesis of the action potential and automaticity. In *Handbook of Physiology, The Cardiovascular System* I. *The Heart*, ed. BERNE, R. M. & SPERELAKIS, N., pp. 269–334. Bethesda, MD, USA: American Physiological Society.

CHAPMAN, R. A. (1979). Excitation–contraction coupling in cardiac muscle. *Progress in Biophysics and Molecular Biology* **35**, 1–52.

CONNER, J. & 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 fibres. *Pflügers Archiv* **392**, 352–359.

CUKIERMAN, S. & PAES DE CARVALHO, A. (1982). Frequency-dependent excitability of 'membrane' slow responses of rabbit left atrial trabeculae in the presence of Ba^{2+} and high K^+. *Journal of General Physiology* **79**, 1017–1039.

DECK, K. A. & TRAUTWEIN, W. (1964). Ionic currents in cardiac excitation. *Pflügers Archiv* **392**, 352–359.

DUDEL, J., PEPER, K., RUDEL, R. & TRAUTWEIN, W. (1967). The dynamic chloride component of membrane current in Purkinje fibres. *Pflügers Archiv* **295**, 197–212.

EISNER, D. A. LEDERER, W. J. & NOBLE, D. (1979). Caffeine and tetracaine abolish the slow inward calcium current in sheep cardiac Purkinje fibres. *Journal of Physiology* **293**, 76–77*P*.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiological Review* **57**, 71–108.

ESCANDE, D., COULOMBE, A., FAIVRE, J. R., DEROUBAIX, E. & CORABOEUF, E. (1987). Two types of transient outward currents in adult human atrial cells. *American Journal of Physiology* **252**, H142–148.

FABIATO, A. (1983). Calcium-induced release of Ca^{2+} from cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

FOZZARD, H. A. & HIRAOKA, M. (1973). The positive dynamic current and its inactivation properties in cardiac Purkinje fibres. *Journal of Physiology* **234**, 469–586.

GILES, W. R. & VAN GINNEKEN, A. C. G. (1985). A transient outward current in isolated cells from the crista terminalis of rabbit heart. *Journal of Physiology* **368**, 243–264.

GILMORE JR, R. F., SALATA, J. J. & DAVIS, J. R. (1986). Effects of 4-aminopyridine on rate-related depression of cardiac action potentials. *American Journal of Physiology* **251**, H297–306.

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.

HERMANN, A. & HARTUNG, K. (1983). Ca^{2+} activated K^+ conductance in molluscan neurones. *Cell Calcium* **4**, 387–405.

HESS, P. & WIER, W. G. (1984). Excitation–contraction coupling in cardiac Purkinje fibers. Effects of caffeine on the intracellular $[Ca^{2+}]$ transient, membrane currents, and contraction. *Journal of General Physiology* **83**, 417–433.

HIRAOKA, M. & HIRAOKA, M. (1975). The role of the positive dynamic current on the action potential of cardiac Purkinje fibers. *Japanese Journal of Physiology* **25**, 705–717.

HIRAOKA, M. & KAWANO, S. (1986). Contribution of the transient outward current to the repolarization of rabbit ventricular cells. *Japanese Heart Journal* **27**, suppl. I, 77–82.

HIRAOKA, M. & KAWANO, S. (1987). Mechanism of increased amplitude and duration of the plateau with sudden shortening of diastolic intervals in rabbit ventricular cells. *Circulation Research* **60**, 14–26.

IINUMA, H. & KATO, K. (1979). Mechanism of augmented premature responses in canine ventricular muscle. *Circulation Research* **44**, 624–629.

ISENBERG, G. & KLOCKNER, U. (1982*a*). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Archiv* **395**, 6–18.

ISENBERG, G. & KLOCKNER, U. (1982*b*). Calcium currents of isolated bovine ventricular myocytes are fast and of large amplitude. *Pflügers Archiv* **395**, 30–41.

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

KENYON, J. L. & GIBBONS, W. R. (1977). Effects of low-chloride solution on action potentials of sheep cardiac Purkinje fibers. *Journal of General Physiology* **70**, 635–660.

KENYON, J. L. & GIBBONS, W. R. (1979*a*). Influence of chloride, potassium, and tetraethylammonium on the early outward current of sheep cardiac Purkinje fibers. *Journal of General Physiology* **73**, 117–138.

KENYON, J. & GIBBONS, W. R. (1979*b*). 4-Aminopyridine and the early outward current of sheep Purkinje fibers. *Journal of General Physiology* **73**, 139–157.

KUKUSHKIN, N. I., GUINULLIN, R. S. & SOSUNOV, E. A. (1983). Transient outward current and rate dependence of action potential duration in rabbit cardiac ventricular muscle. *Pflügers Archiv* **399**, 87–92.

MCDONALD, T. F. & TRAUTWEIN, W. (1978). The potassium current underlying delayed rectification in cat ventricular muscle. *Journal of Physiology* **274**, 217–246.

MAYER, M. L. (1985). A calcium-activated chloride current generates the after-depolarization of rate sensory neurones in culture. *Journal of Physiology* **364**, 217–239.

MAYLIE, J. & MORAD, M. (1984). A transient outward current related to calcium release and development of tension in elephant seal atrial fibres. *Journal of Physiology* **357**, 267–292.

MEECH, R. W. (1974). The sensitivity of *Helix aspersa* neurones to injected calcium ions. *Journal of Physiology* **237**, 259–277.

MEECH, R. W. (1978). Calcium dependent potassium activation in nervous tissues. *Annual Review of Biophysics and Bioengineering* **7**, 1–18.

MEECH, R. W. & STANDEN, N. B. (1975). Potassium activation in *Helix aspersa* neurones under voltage clamp: a component mediated by calcium influx. *Journal of Physiology* **249**, 211–239.

MITCHELL, M. R., POWELL, T., TERRAR, D. A. & TWIST, V. W. (1983). Characteristics of the second inward current in cells isolated from rat ventricular muscle. *Proceedings of the Royal Society* B **219**, 447–469.

MITCHELL, M. R., POWELL, T., TERRAR, D. A. & TWIST, V. W. (1984). Strontium, nifedipine and 4-aminopyridine modify the time course of the action potential in cells from rabbit ventricular muscle. *British Journal of Pharmacology* **81**, 551–556.

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

NAKAYAMA, T. & IRISAWA, H. (1985). Transient outward current carried by potassium and sodium in quiescent atrioventricular node cells of rabbit. *Circulation Research* **57**, 65–73.

NEHER, E. (1971). Two fast transient current components during voltage clamp on snail neurons. *Journal of General Physiology* **58**, 36–53.

PEPER, K. & TRAUTWEIN, W. (1968). A membrane current related to the plateau of the action potential of Purkinje fibres. *Pflügers Archiv* **303**, 108–123.

SCHWARZ, W. & PASSOW, H. (1983). Ca^{2+}-activated K^+ channels in erythrocytes and excitable cells. *Annual Review of Physiology* **45**, 359–374.

SEGAL, M., ROGAWSKI, M. A. & BARKER, J. L. (1983). A transient potassium conductance regulates the excitability of cultured hippocampal and spinal neurons. *Journal of Neuroscience* **4**, 604–609.

SIEGELBAUM, S. A. & TSIEN, R. W. (1980). Calcium-activated transient outward current in calf Purkinje fibres. *Journal of Physiology* **299**, 485–506.

SUTKO, J. L., ITO, K. & KENYON, J. L. (1985). Ryanodine: a modifier of sarcoplasmic reticulum calcium release in striated muscle. *Federation Proceedings* **44**, 2984–2988.

TSENG, G., ROBINSON, R. B. & HOFFMAN, B. F. (1987). Passive properties and membrane currents of canine ventricular myocytes. *Journal of General Physiology* **90**, 671–701.

WATANABE, T., DELBRIDGE, L. M., BUSTAMENTE, O. & MCDONALD, T. F. (1983). Heterogeneity of the action potential in isolated rat ventricular myocytes and tissue. *Circulation Research* **52**, 280–290.

YATANI, A., IMOTO, Y. & GOTO, M. (1984). The effects of caffeine on the electrical properties of isolated, single rat ventricular cells. *Japanese Journal of Physiology* **34**, 337–349.