Regional differences in the negative inotropic effect of acetylcholine within the canine ventricle

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1. Regional differences in the effects of ACh on sub-epicardial, mid-wall and sub-endocardial cells of the dog left ventricle have been studied.

2. ACh produced a dose-dependent, atropine-sensitive negative inotropic effect that was greatest in sub-epicardial cells and small or absent in sub-endocardial cells.

3. In sub-epicardial (but not sub-endocardial) cells, ACh also resulted in a dose-dependent decrease in action potential duration. The inotropic effect of ACh on sub-epicardial cells was primarily the result of the decrease of action potential duration, because during trains of voltage clamp pulses the inotropic effect of ACh was reduced or abolished. At a holding potential of −80 mV, 10^{-5} M ACh decreased L-type Ca^{2+} current by ~8% and this is thought to be responsible for the small inotropic effect during trains of pulses.

4. Although 4-AP, a blocker of the transient outward current (I_{to}), abolished the 'spike and dome' morphology of the sub-epicardial action potential, it had little or no effect on the actions of ACh on sub-epicardial cells. ACh had no effect on I_{to} in sub-epicardial cells in voltage clamp experiments.

5. ACh activated a Ba^{2+}-sensitive outward current ($I_{K,ACh}$) in sub-epicardial cells, but little or no such current in sub-endocardial cells. In sub-epicardial cells, ACh also inhibited the inward rectifier current, $I_{K,1}$.

6. It is concluded that in left ventricular sub-epicardial cells, ACh activates $I_{K,ACh}$. This results in a shortening of the action potential and, therefore, a negative inotropic effect. In sub-endocardial cells, ACh activates little or no $I_{K,ACh}$ and, therefore, it has little or no negative inotropic effect. This may result from a regional variation in the expression of the muscarinic K^+ channel.

It is well established that acetylcholine (ACh) has a negative inotropic effect on ventricular muscle that has been potentiated by β-adrenergic receptor stimulation (e.g. Levy, 1977). Recent studies have shown that ACh also has a *direct* negative inotropic effect on ventricular cells in the absence of β-agonists in at least two species – ferret and rat (Boyett, Kirby, Orchard & Roberts, 1988; McMorn, Harrison, Zang, Yu & Boyett, 1993).

In the dog ventricle, many investigators have reported major differences in the electrophysiological characteristics and pharmacological responsiveness of cells from different regions within the ventricular wall (for review see Antzelevitch *et al.* 1991). Antzelevitch and his colleagues have drawn attention to differences in the action potential from the sub-epicardial and sub-endocardial regions of the ventricles of the dog – the sub-epicardial action potential has a characteristic 'spike and dome' morphology, whereas the sub-endocardial action potential does not (Antzelevitch *et al.* 1991). Tseng & Hoffman (1989) identified a 4-aminopyridine (4-AP)-sensitive transient outward current (I_{to}) in the dog left ventricle where Liu, Gintant & Antzelevitch (1993) reported that this current is more prominent in the sub-epicardium than in the sub-endocardium and is responsible for the differences in action potential configuration in the two regions. Litovsky & Antzelevitch (1990) have shown that ACh has little or no effect on the sub-endocardial action potential from the right ventricle of the dog, but it either prolongs or markedly abbreviates the sub-epicardial action potential (and effective refractory period). Litovsky & Antzelevitch (1990) suggested that the regional differences in the response to ACh are, in part, the result of the I_{to}-mediated differences in action potential configuration in the two regions. The aim of the present study was to determine whether the ACh-induced changes in the action potential influence the contraction of cells from different regions of the left ventricle of the dog.

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A preliminary account of this study has been presented to the Physiological Society (Yang, Janvier & Boyett, 1995).

METHODS

Isolation of ventricular cells

The isolation method used was a modification of a previously described method (Liu *et al.* 1993). Hearts were taken from dogs at the end of experiments carried out in neighbouring laboratories. Beagle dogs of either sex (~14–18 kg) were anaesthetized by α-chloralose: a dose of 100 mg kg^{-1} was followed by a maintenance dose of 10 mg kg^{-1} every 30 min. Prior to administration of α-chloralose, pentobarbitone sodium or morphine sulphate was sometimes applied to sedate the dog. The heart was quickly removed and placed in ice-cold bicarbonate-buffered Tyrode solution. A piece of ventricular muscle (~50 × 50 mm) centred on the left anterior descending coronary artery was cut from the base region of the left ventricular free wall. The left anterior descending coronary artery was cannulated and the tissue mounted on a Langendorff apparatus. The tissue was perfused at a flow rate of 24–30 ml min^{-1} with various solutions at 37 °C. The tissue was perfused with: (1) isolation solution containing 750 μM $CaCl_2$; (2) Ca^{2+}-free isolation solution containing 1 mM EGTA for 5 min; and (3) enzyme-containing isolation solution for 10 min. (This was recirculated through the tissue.) After perfusion, thin slices of tissue were cut from the sub-epicardium (<2 mm from the epicardial surface), mid-wall (4–7 mm from the epicardial surface) and sub-endocardium (<2 mm from the endocardial surface) using a scalpel and fine scissors. The slices were cut parallel to the surface of the ventricular wall and along the apex–base axis. The tissue from each region was placed in separate flasks and incubated and agitated in enzyme-containing isolation solution supplemented with 1% (w/v) bovine serum albumin for 5 min periods at 37 °C. This protocol was repeated four or five times for each tissue and the cells from each 5 min period were harvested by filtration followed by centrifugation at 400 r.p.m. for 40 s. The cells were washed by resuspending them in isolation solution containing 750 μM $CaCl_2$ and re-centrifuging them. Cells from each region were pooled separately and stored in isolation solution containing 750 μM $CaCl_2$ at 4 °C until use.

Experimental procedures

When required, a drop containing sub-epicardial, mid-wall or sub-endocardial cells was placed in a small chamber (volume, 150 μl) on the stage of a Nikon Diaphot inverted microscope; 5–20 min later, after the cells had been allowed to settle onto the glass bottom of the chamber, the chamber was perfused with Hepes-buffered Tyrode solution at a rate of ~2 ml min^{-1}. Miniature solenoid valves (Lee Products Ltd, Gerrards Cross, UK) were used to direct one of two or four different solutions to the chamber. The temperature of the perfusate was maintained at 37 ± 0·5 °C by feedback control and the solution level in the chamber was controlled using the system described by Cannell & Lederer (1986). The length of a cell was measured optically using a system based on a 1024-element photodiode array (Boyett, Moore, Jewell, Montgomery, Kirby & Orchard, 1988). In addition to cell length, twitch shortening (the shortening of a cell during a contraction) was recorded by means of a sample-and-hold circuit (Boyett & Levi, 1987). Unless specified otherwise, membrane potential and current were recorded with conventional glass microelectrodes (resistance, 15–25 MΩ) filled with 1 M KCl. The duration of action potentials was recorded at −65 mV using an electronic device (Kentish & Boyett, 1983). Membrane current was recorded using the discontinuous switch clamp technique (Model 8800, Dagan Corporation, Minneapolis, MN, USA) with the frequency of switching between voltage measurement and current passing set at ~4·5 kHz. The output of the microelectrode was monitored continuously to ensure that the electrode potential became steady between the current pulses and membrane current was typically filtered at 1 kHz (low pass filter). In some experiments membrane current was recorded with the patch clamp technique in the whole cell configuration (Axopatch-1C, Axon Instruments) with the resistance of the patch pipettes ranging between 1 and 3 MΩ. Pipette capacitance, cell capacitance and series resistance were compensated in the majority of experiments and membrane current was filtered at 1 kHz (low-pass Bessel filter). Cells were stimulated at 1 Hz by stimulus current delivered by platinum electrodes located in the sides of the chamber or stimulus current delivered through the microelectrode or voltage clamp pulses. During an experiment, signals were displayed on an oscilloscope (Tektronix 5000 series) and pen recorder (Gould RS3600 or 2400) and recorded on video tape (using a Neuro-Corder DR-890, Neuro Data Instruments Corp., New York and a JVC video recorder) and computer (using an analog-to-digital converter – CED 1401, Cambridge Electronic Design, Cambridge, UK).

Solutions

Bicarbonate-buffered Tyrode solution contained (mM): NaCl, 93; KCl, 5 ; $NaHCO_3$, 20; Na_2HPO_4, 1; $MgSO_4$, 1; $CaCl_2$, 2; glucose, 10; sodium acetate, 20; and insulin, 5 u l^{-1}. The solution was equilibrated with 95% O_2–5% CO_2. Isolation solution contained (mM): NaCl, 130; KCl, 5·4; Na_2HPO_4, 0·4; $MgSO_4$, 1·4; glucose, 10; Hepes, 5: taurine, 20; creatine, 10; pH 7·3 at room temperature (22 °C). Enzyme-containing isolation solution consisted of isolation solution plus: 1 mg ml^{-1} collagenase (Type 2; Worthington), 0·1 mg ml^{-1} protease (Type XIV; Sigma) and 50 μM $CaCl_2$. Hepes-buffered Tyrode solution contained (mM): NaCl, 137; KCl, 5·4; NaH_2PO_4, 0·33; $MgCl_2$, 0·5; glucose, 5·5; Hepes, 5; $CaCl_2$, 2; pH 7·6 at room temperature (22 °C; ~pH 7·4 at 37 °C). ACh, CsCl, 4-AP, nifedipine and $BaCl_2$ were added to the Hepes-buffered Tyrode solution when required. Stock solutions of 10^{-4} and 10^{-3} M ACh were prepared and stored at −20 °C in small aliquots. One aliquot was thawed and used on the day of an experiment. In whole-cell patch clamp experiments in which the L-type Ca^{2+} current (I_{Ca}) was measured, the patch pipette solution contained (mM): CsCl, 100; tetraethylammonium (TEA) chloride, 20; $MgCl_2$, 4; Hepes, 5; Na_2ATP, 3; EGTA, 10; Na_3GTP, 0·1; pH 7·3 at room temperature (22 °C; ~pH 7·1 at 37 °C). In some experiments GTP was omitted from the solution. In whole cell patch clamp experiments in which I_{to} was measured, the patch pipette solution contained (mM): potassium aspartate, 120; KCl, 20; KH_2PO_4, 1; $MgCl_2$, 5·5; EGTA, 5; Hepes, 5; Na_2ATP, 3; Na_3GTP, 0·1; pH 7·4 at room temperature (22 °C; ~pH 7·2 at 37 °C).

Statistics

Data are given as means ± S.E.M. of n cells. Comparisons between groups were made using an unpaired or paired Student's t test as appropriate. A difference was considered significant if $P < 0·05$.

RESULTS

The negative inotropic effect of ACh

Figure 1*A* shows slow time base recordings of contractions of sub-epicardial cells before, during and after exposure to several concentrations of ACh (from 10^{-9} to 10^{-5} M). The

first four traces were recorded from one cell and the last trace was recorded from a second cell. Fast time base recordings of single contractions are shown on the right of Fig. 1*A*. On application of ACh, there was an immediate decrease in contraction (a negative inotropic effect) within the first 15 s of the exposure to ACh. During the remainder of the 3 min exposure to ACh, the negative inotropic effect faded with time. On wash-off of ACh, there was a 'rebound' increase in contraction above the control level; the contraction then gradually returned to control.

The effects of ACh on contraction were dose dependent. In the examples shown, 10^{-9} M ACh produced little or no effect on contraction, 10^{-8} M ACh produced a 27% decrease in contraction and 10^{-5} M ACh produced a 72% decrease in contraction. The changes in the contraction of thirty-seven sub-epicardial cells on application and wash-off of various concentrations of ACh are summarized in Fig. 1*B*. The filled circles show the mean twitch shortening early during an exposure to ACh at the time of the maximum negative inotropic effect. In this series of experiments 10^{-6} M ACh produced the maximum decrease of twitch shortening of $50{\cdot}0 \pm 4{\cdot}8\%$ ($n = 20$). The filled triangles show the mean twitch shortening at the end of the 3 min exposure to ACh – although the negative inotropic effect faded during the

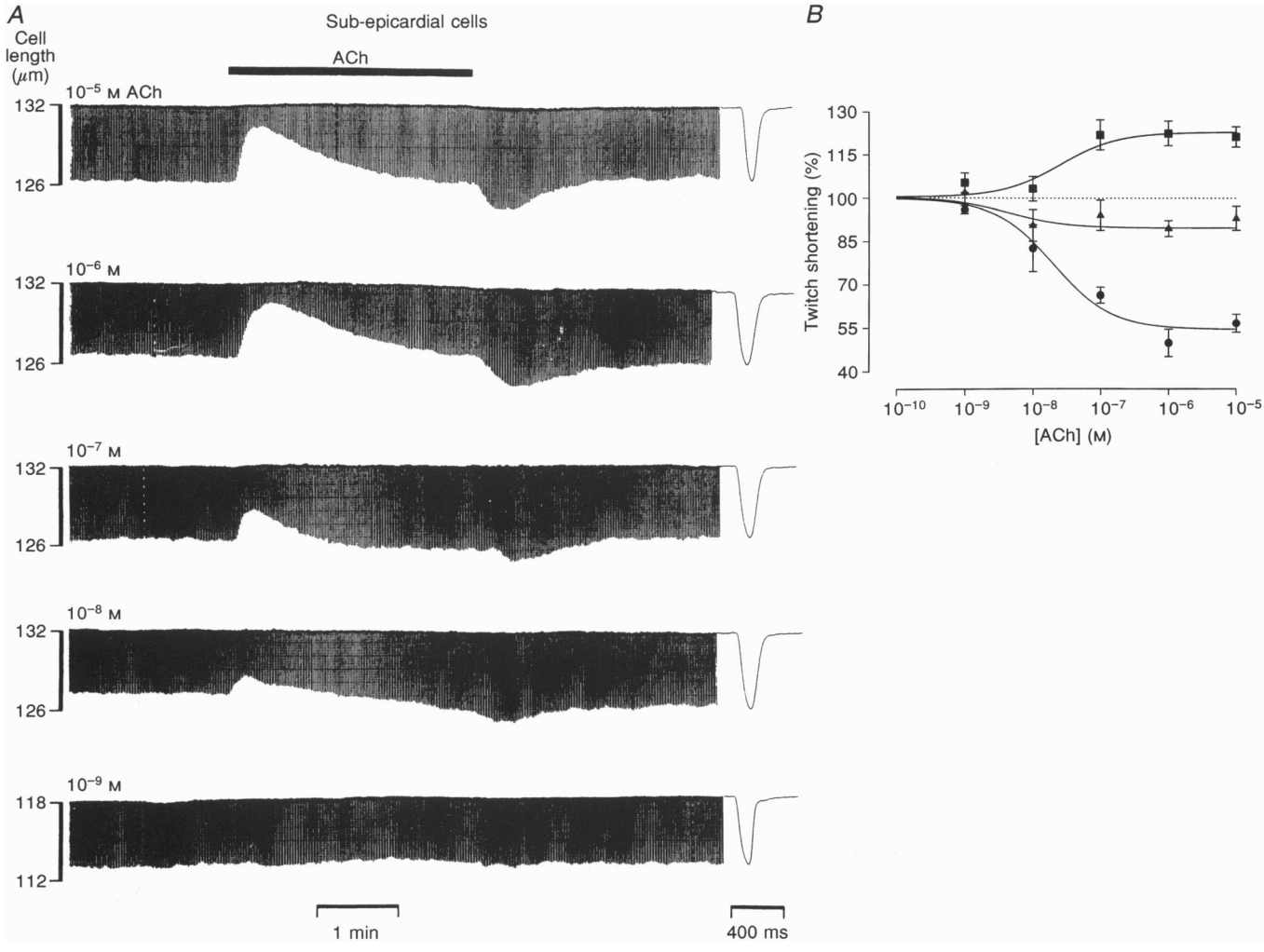

Figure 1. Effect of ACh on the contraction of sub-epicardial cells

A, slow time base chart records of cell length before, during and after the application of various concentrations of ACh. The ACh concentration is noted on the left. The top four records were obtained from one cell and the bottom record from another cell. In this and other figures, the chart speed was increased at the end of each record to illustrate in more detail the signal recorded. In this and other figures, exposure to ACh is indicated by a bar above the top record. *B*, twitch shortening (as a percentage of twitch shortening under control conditions) early (●) and late (▲) during a 3 min exposure to ACh, and early after wash-off of ACh (■) as a function of the ACh concentration. Means ± S.E.M. are shown. Values for n, lowest concentration given first: 5, 5, 27, 20 and 22 (ACh, early) and 3, 6, 22, 23 and 26 (ACh, late; post-control). The data were fitted by eqn (1). $K_{½} = 2{\cdot}0 \times 10^{-8}$, $0{\cdot}7 \times 10^{-8}$ and $2{\cdot}5 \times 10^{-8}$ and $r_{max} = 54{\cdot}6$, 89·4 and 123·0% of the control value early and late during an exposure to ACh and early after wash-off of ACh, respectively.

exposure to ACh, the contraction was still smaller than the control by the end of the exposure (it was 10·5 ± 2·8%, $n = 20$, smaller at the end of the exposure to 10^{-6} M ACh). The filled squares show the mean maximum rebound increase of twitch shortening after wash-off of ACh. After wash-off of 10^{-6} M ACh, there was a maximum rebound increase of twitch shortening of 22·4 ± 4·3% ($n = 20$). In Fig. 1*B*, the data are fitted with typical dose–response curves of the form:

$$r = r_{max}([ACh]/(K_{½} + [ACh])), \quad (1)$$

where r is the response, r_{max} is the maximal response, [ACh] is the molar ACh concentration and $K_{½}$ is the ACh concentration required to produce a half-maximal effect. Based on the fit of eqn (1) to the data, the maximal decreases early and late during the exposure to ACh were 45·4 and 10·6%, respectively, the maximal rebound increase in contraction was 23·0%, and half-maximal effects early and late during the exposure to ACh and on wash-off of ACh were obtained at ACh concentrations of $2·0 \times 10^{-8}$, $0·7 \times 10^{-8}$ and $2·5 \times 10^{-8}$ M, respectively.

The response to ACh varied according to the site of origin of the cell. The effect of 10^{-5} M ACh on a sub-epicardial cell, a mid-wall cell and a sub-endocardial cell is shown in Fig. 2*A*. In the examples shown, 10^{-5} M ACh decreased the contraction by 71% in the sub-epicardial cell and 33% in the mid-wall cell but had little or no effect on the contraction of the sub-endocardial cell. In Fig. 2*B*, the mean initial decrease of twitch shortening in the three cell types is plotted against the ACh concentration. The data are fitted with eqn (1). ACh (10^{-6} M) maximally decreased contraction by 50·0 ± 4·8, 30·5 ± 4·8 and 12·5 ± 4·5% ($n = 37$, 17 and 37, respectively) and half-maximal effects were observed at ACh concentrations of $2·0 \times 10^{-8}$, $3·1 \times 10^{-8}$ and $0·9 \times 10^{-8}$ M ACh in sub-epicardial, mid-wall and sub-endocardial cells, respectively.

The effect of atropine on the negative inotropic effect of ACh in sub-epicardial cells

The effect of 2×10^{-5} M atropine, a blocker of the muscarinic ACh receptor, on the response to 10^{-5} M ACh was investigated in five sub-epicardial cells (not shown). Under control conditions, there was an initial decrease in contraction of 49·3 ± 8·2% on application of ACh. However, in the presence of atropine, the decrease in contraction (2·9 ± 3·6%) was not significant ($P = 0·681$). It is concluded that the negative inotropic effect of ACh on sub-epicardial cells is mediated by the muscarinic ACh receptor.

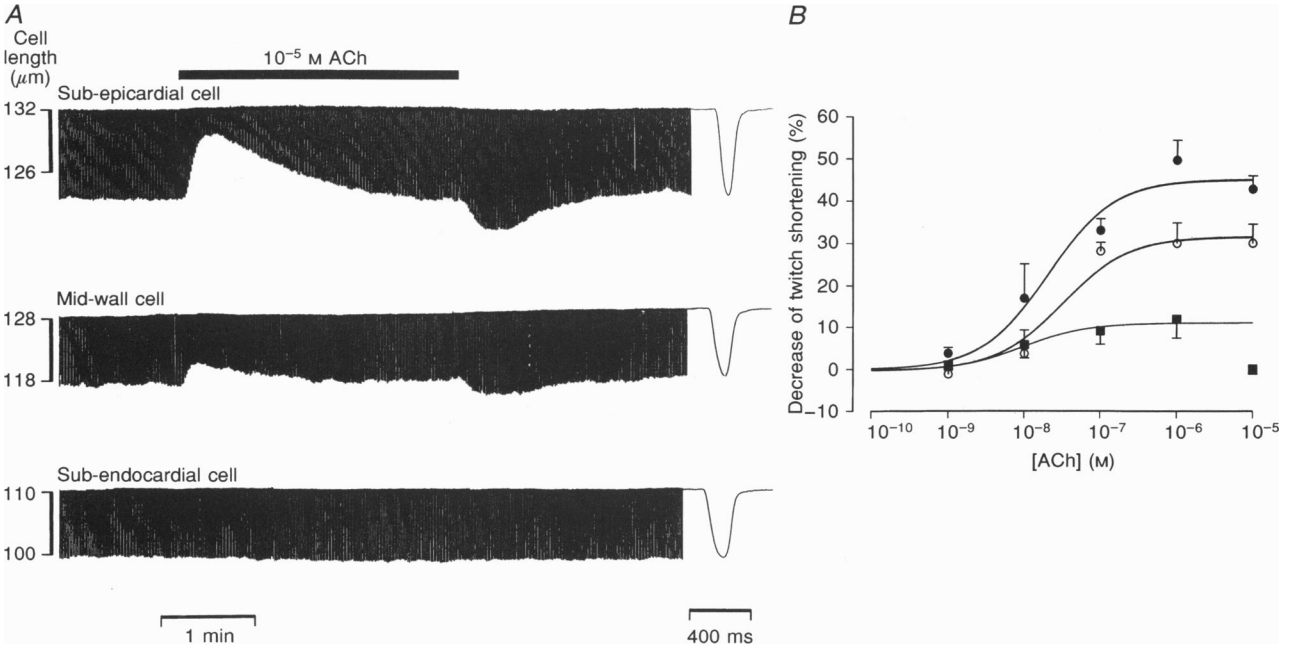

Figure 2. Comparison of the effects of ACh on contraction in different ventricular cell types

A, effect of 10^{-5} M ACh on a sub-epicardial cell (upper record), mid-wall cell (middle record) and sub-endocardial cell (lower record). Slow time base chart records of cell length are shown. *B*, dose–response curves for the action of ACh on contraction in sub-epicardial cells (●), mid-wall cells (○) and sub-endocardial cells (■). The decrease of twitch shortening (as a percentage of twitch shortening under control conditions) early during an exposure to ACh is plotted against the ACh concentration. Means ± S.E.M. are shown. Values for n, lowest concentrations given first: 5, 5, 27, 20 and 22 (sub-epicardial cells), 6, 6, 10, 9 and 11 (mid-wall cells) and 5, 6, 27, 32 and 29 (sub-endocardial cells). The data were fitted by eqn (1). $K_{½} = 2·0 \times 10^{-8}$, $3·1 \times 10^{-8}$ and $0·9 \times 10^{-8}$ and $r_{max} = 45·4$, 32·1 and 12·5 in sub-epicardial, mid-wall and sub-endocardial cells, respectively.

The effect of ACh on the action potential

To investigate the mechanism underlying the negative inotropic effect of ACh and the variation of the negative inotropic effect in the different cell types, the effect of ACh on the action potential was investigated in sub-epicardial and sub-endocardial cells. Figure 3 shows superimposed action potentials recorded from a sub-epicardial cell (Fig. 3*A*) and a sub-endocardial cell (Fig. 3*C*). The sub-epicardial action potential had a spike and dome profile (see Introduction) characterized by a fast phase of repolarization after the action potential peak, giving rise to the 'spike', followed by a secondary depolarization, giving rise to the 'dome'. The sub-endocardial action potential did not have a spike and dome profile, because the initial phase of repolarization after the action potential peak was less prominent. Figure 3*A* and *B* shows the changes in the action potential and contraction of a sub-epicardial cell on application and wash-off of 10^{-5} M ACh. The action potential and contraction under control conditions are identified by filled circles. Immediately after the application of ACh, the plateau of the sub-epicardial action potential was reduced in both height and duration and the accompanying contraction was greatly decreased as shown by the traces identified by the open circles. Later, during the 3 min exposure to ACh, the action potential and contraction had partly recovered as shown by the traces identified by the open triangles. After wash-off of ACh, the action potential and contraction returned to control values as shown by the traces identified by the filled squares. There was little or no change in the action potential and contraction on application of 10^{-5} M ACh in most sub-endocardial cells studied. A typical example is shown in Fig. 3*C* and *D*. Early during an exposure to 10^{-5} M ACh, action potential duration was $96{\cdot}8 \pm 0{\cdot}8\%$ of control in fifteen sub-endocardial cells.

The ACh-induced changes in the sub-epicardial action potential were concentration dependent. Figure 4*A* shows slow time base recordings of action potentials and action potential duration in a sub-epicardial cell. On application of ACh, there was an initial reduction in both the height and duration of the action potential plateau. In the presence of ACh, the effects faded with time and the configuration of

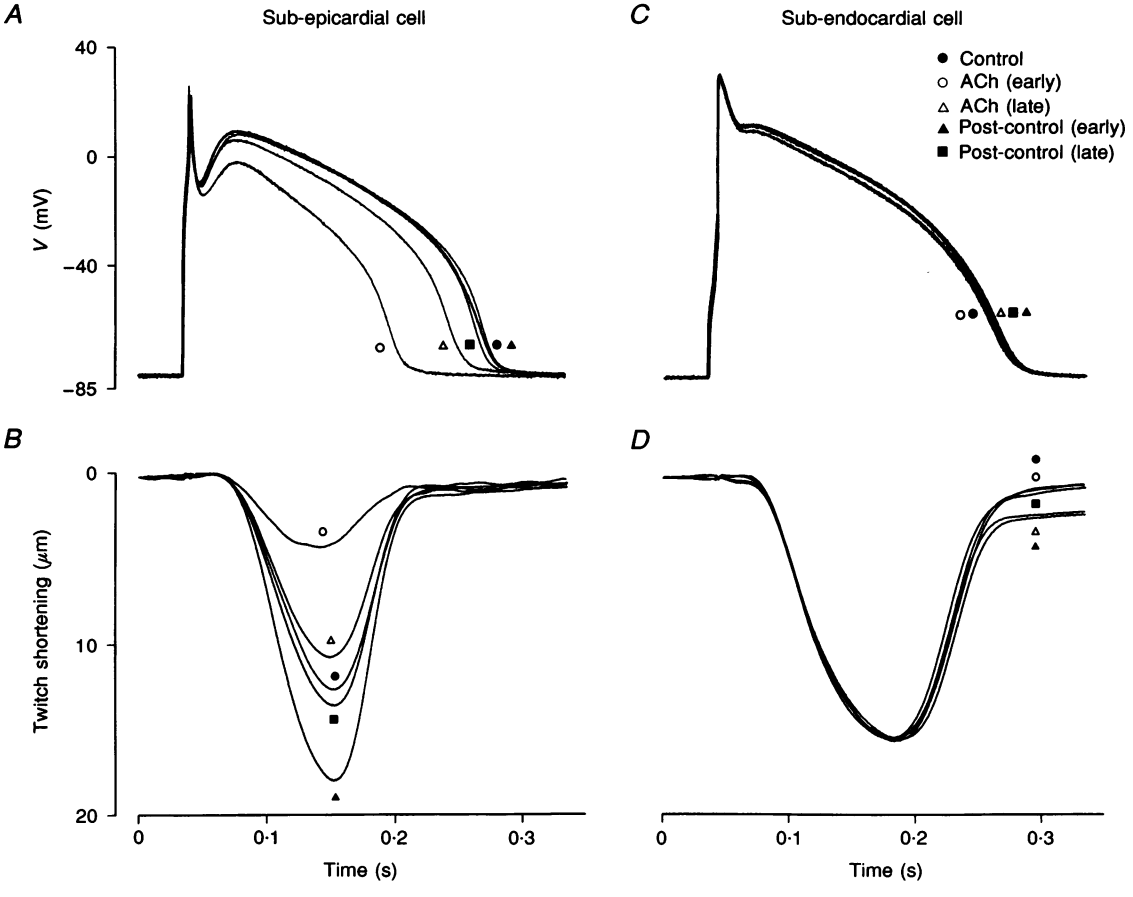

Figure 3. Effect of 10^{-5} M ACh on the action potential in a sub-epicardial cell and a sub-endocardial cell

A–D, fast time base records of superimposed action potentials (*A* and *C*) and accompanying contractions (*B* and *D*) under control conditions (●), early (○) and late (△) during a 3 min exposure to ACh and early (▲) and late (■) after wash-off of ACh in a sub-epicardial cell (*A* and *B*) and a sub-endocardial cell (*B* and *D*).

the action potential partly recovered. The effects on the action potential were greater with the higher concentrations of ACh: for example, in the case shown in Fig. 4*A*, 10^{-7} M ACh decreased action potential duration by 31%, whereas 5×10^{-5} M ACh decreased it by 51%. The effect of a range of ACh concentrations on action potential duration was measured in twenty-two sub-epicardial cells – the mean decrease in action potential duration early during an exposure to ACh is plotted against the ACh concentration in Fig. 4*B*. The data are fitted by eqn (1). ACh (5×10^{-5} M) resulted in a maximum shortening of the action potential of 38·0 ± 5·4%. A half-maximal shortening of the action potential was produced by 1·3 ± 10^{-7} M ACh.

Effect of ACh under voltage clamp control

To investigate whether the changes in contraction of sub-epicardial cells on application of ACh were the result of the parallel changes in action potential duration, the negative

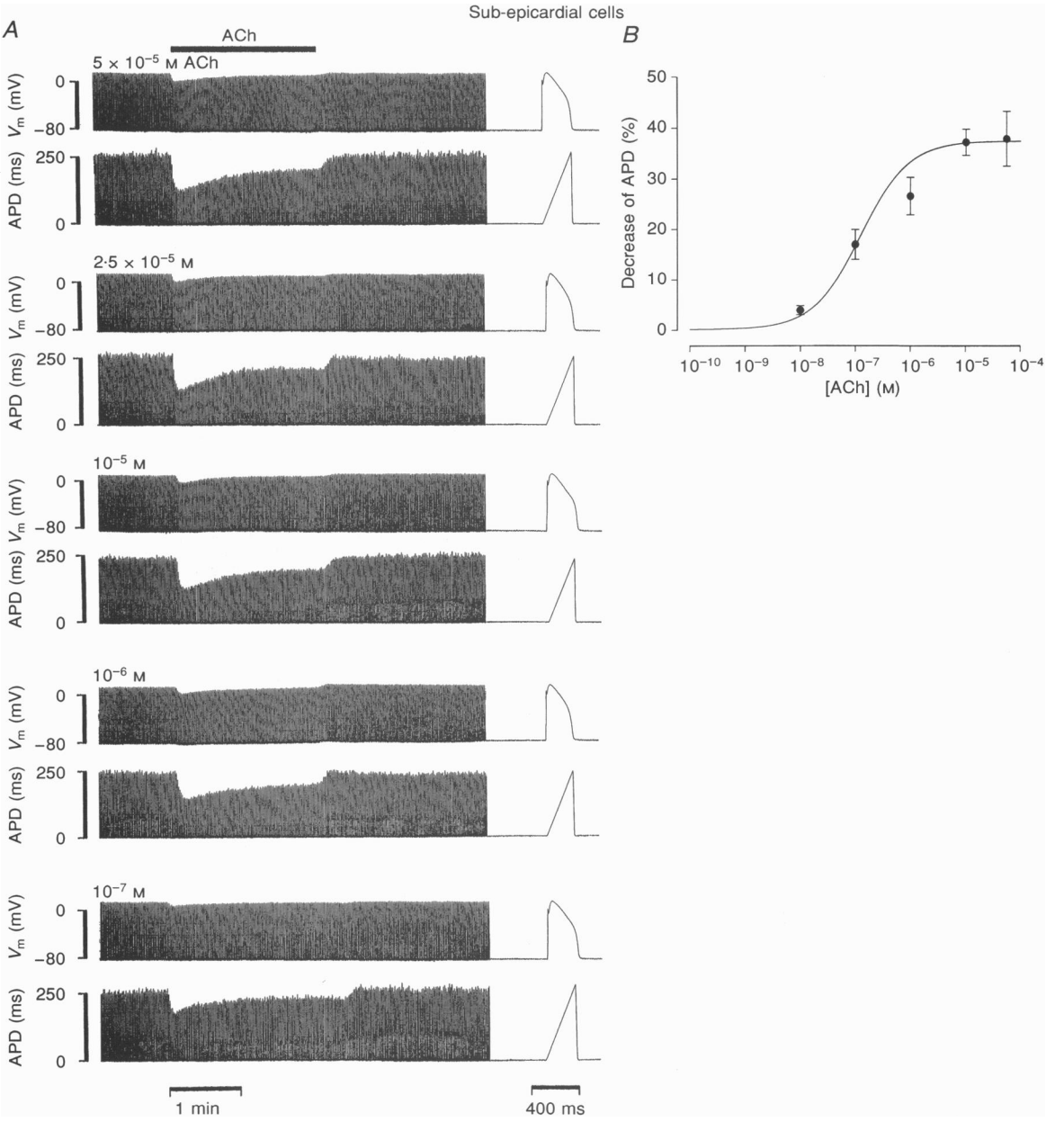

Figure 4. Dose-dependent action of ACh on the action potential in sub-epicardial cells

A, slow time base chart records of membrane potential (V_m, to show the action potential; upper record of each pair) and action potential duration (APD, lower record of each pair) before, during and after the application of various concentrations of ACh (shown on the left). *B*, dose–response curve for the action of ACh on action potential duration. The decrease of action potential duration (as a percentage of the action potential duration under control conditions) early during an exposure to ACh is plotted against the ACh concentration. Means ± S.E.M. are shown. Value for n, lowest concentration given first: 5, 9, 7, 14 and 5. The data were fitted by eqn (1). $K_{½} = 1{\cdot}3 \times 10^{-7}$ and $r_{max} = 37{\cdot}6\%$.

inotropic effects of ACh during trains of either action potentials or voltage clamp pulses of constant duration were compared. In Fig. 5*A*, the upper trace shows the action potential, the middle trace shows action potential duration and the lower trace shows the contraction of a sub-epicardial cell. On application of 10^{-6} M ACh, there was an immediate reduction of action potential duration of 32% and a decrease of contraction of 71%. During the remainder of the exposure to ACh, both effects faded with time over a similar time course. On wash-off of ACh, there was a rebound increase of contraction as usual (cf. Fig. 1). In contrast, there was no rebound increase of action potential duration which returned to its control value monotonically. This can also be seen in Figs 3 and 4.

In the experiment illustrated in Fig. 5*B*, the same cell was stimulated with voltage clamp pulses (from −80 to 0 mV for 200 ms) rather than action potentials. During the train of voltage clamp pulses of constant duration, the immediate decrease of contraction on application of 10^{-6} M ACh was much reduced. The contraction was decreased by just 24% in this example (there was a 71% decrease during the train of action potentials). However, the rebound increase of contraction on wash-off of ACh was similar in amplitude to that observed during the train of action potentials (Fig. 5*A*). Data from seven sub-epicardial cells are summarized in Fig. 6*A* and *B*, which shows the mean twitch shortening under control conditions, early and late during a 3 min exposure to 10^{-6} M ACh, and early and late after wash-off of ACh. During a train of action potentials (Fig. 6*A*) there was a significant decrease of contraction of 37·3 ± 5·7% early during the exposure to ACh, and a smaller decrease of contraction of 18·4 ± 4·4% late during the exposure to ACh. However, during a train of voltage clamp pulses of constant duration there was no significant decrease of contraction either early or late during the

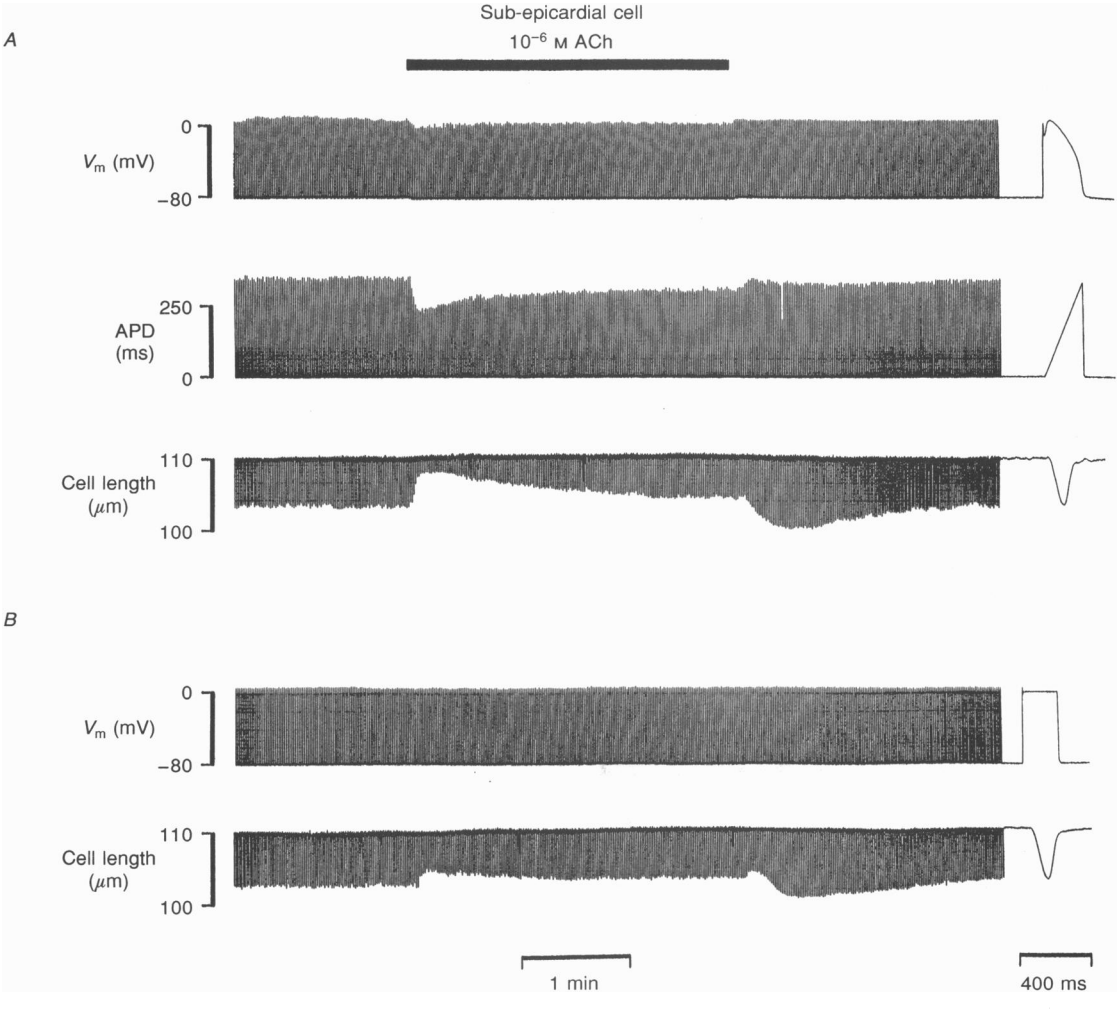

Figure 5. Comparison of the effects of 10^{-6} M ACh on contraction during trains of either action potentials or voltage clamp pulses in a sub-epicardial cell

A, the effect of ACh during a train of action potentials. Slow time base chart records of membrane potential (V_m, to show action potentials; upper record), action potential duration (APD, middle record) and cell length (lower record) are shown. *B*, the effect of ACh on the same cell during a train of 200 ms voltage clamp pulses from −80 to 0 mV. Slow time base chart records of membrane potential (to show the voltage clamp pulses; upper record) and cell length (lower record) are shown.

exposure to ACh – although 10^{-6} M ACh had a small inotropic effect under voltage clamp control in the cell in Fig. 5, it had no effect on the six other cells (a different result was obtained when a higher ACh concentration was used as explained below). These results suggest that the negative inotropic effect on application of 10^{-6} M ACh and its subsequent fade during the remainder of the exposure to ACh were primarily the result of the changes in action potential duration. During a train of voltage clamp pulses there was a rebound increase of contraction (of 12·2 ± 7·6%) on wash-off of ACh; this is not significantly different ($P = 0·449$) from the rebound increase during a train of action potentials (21·1 ± 4·1%). It is concluded that at least part of the rebound increase in contraction cannot be attributed to changes in action potential duration and, therefore, must be the result of another mechanism.

The possible role of I_{Ca} in the negative inotropic effect of ACh

It is possible that the negative inotropic effect of ACh (and the shortening of the action potential) could be the result of a decrease in I_{Ca}. However, if I_{Ca} were important, ACh would be expected to exert a negative inotropic effect under voltage clamp control and, although an example of this is shown in Fig. 5*B*, it was not routinely observed with 10^{-6} M ACh (Fig. 6*B*). Nevertheless, the effect of 10^{-5} M ACh on I_{Ca} in sub-epicardial cells was investigated using the whole cell patch clamp technique which allowed K^+ currents to be blocked by substitution of intracellular K^+ by Cs^+ and by intracellular perfusion with TEA (see Methods for details; in addition 20 mM CsCl was added to the extracellular solution to help block K^+ currents). It is conventional to measure I_{Ca} at a holding potential of −40 mV and the top panel of Fig. 7*A* shows an example of the effect of ACh on I_{Ca} recorded during 200 ms voltage clamp pulses from −40 to 0 mV. A plot of the amplitude of I_{Ca} before, during and after the application of ACh is shown on the left and selected records of I_{Ca} are superimposed on the right. In the example shown ACh reduced I_{Ca} by 33%. At a holding potential of −40 mV, ACh decreased I_{Ca} in all cells studied; the mean decrease of 48·6 ± 2·0% ($n = 5$) is shown in Fig. 7*C*. In eight cells, GTP was omitted from the patch pipette solution and ACh had no effect on I_{Ca} (recorded during 200 ms pulses from −40 to 0 mV); this suggests that the effect of ACh on I_{Ca} is mediated by a G protein. I_{Ca} was also measured using a holding potential of −80 mV; during each pulse the membrane potential was first stepped to −40 mV for 20 ms to inactivate the Na^+ current and then to 0 mV for 200 ms to activate I_{Ca}. At a holding potential of −80 mV, ACh had little or no effect on I_{Ca}. In the example shown in the bottom panel of Fig. 7*A*, ACh decreased I_{Ca} by ~11% and in four cells it decreased I_{Ca} by 8·2 ± 0·8% as shown in Fig. 7*C*. The substantial ACh-induced decrease in I_{Ca} at a holding potential of −40 mV is expected to result in a significant decrease in contraction under voltage clamp control. To test this, the effect of 10^{-5} M ACh on contraction was measured at a holding potential of −40 mV; contractions were triggered by 200 ms voltage clamp pulses from −40 to 0 mV. The discontinuous switch clamp technique was used once again (to avoid cell dialysis and allow measurement of contraction). Figure 7*B* shows an example of the effect of ACh on contraction at holding potentials of −40 and −80 mV; slow time base records are shown on the left and selected fast time base records are superimposed on the right. As expected, ACh decreased contraction in all cells studied at a holding potential of −40 mV; the mean decrease of 56·5 ± 7·0% ($n = 4$) is shown in Fig. 7*C*. At a holding potential of −80 mV, ACh resulted in a smaller inotropic effect in all cells studied (10·8 ± 3·8% decrease, $n = 5$) as shown in Fig. 7*B* and *C*.

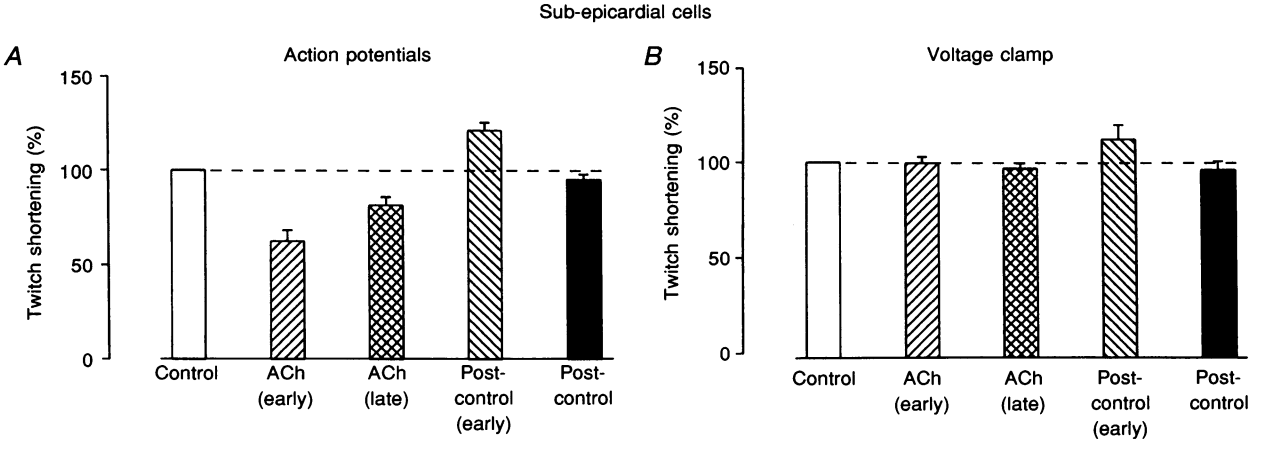

Figure 6. Comparison of the effects of 10^{-6} M ACh on contraction during trains of either action potentials or voltage clamp pulses in sub-epicardial cells

A and *B*, twitch shortening (as a percentage of twitch shortening under control conditions) during trains of either action potentials (*A*) or voltage clamp pulses (*B*) under control conditions, early and late during a 3 min exposure to ACh and early and late after wash-off of ACh. Means ± S.E.M. are shown ($n = 7$).

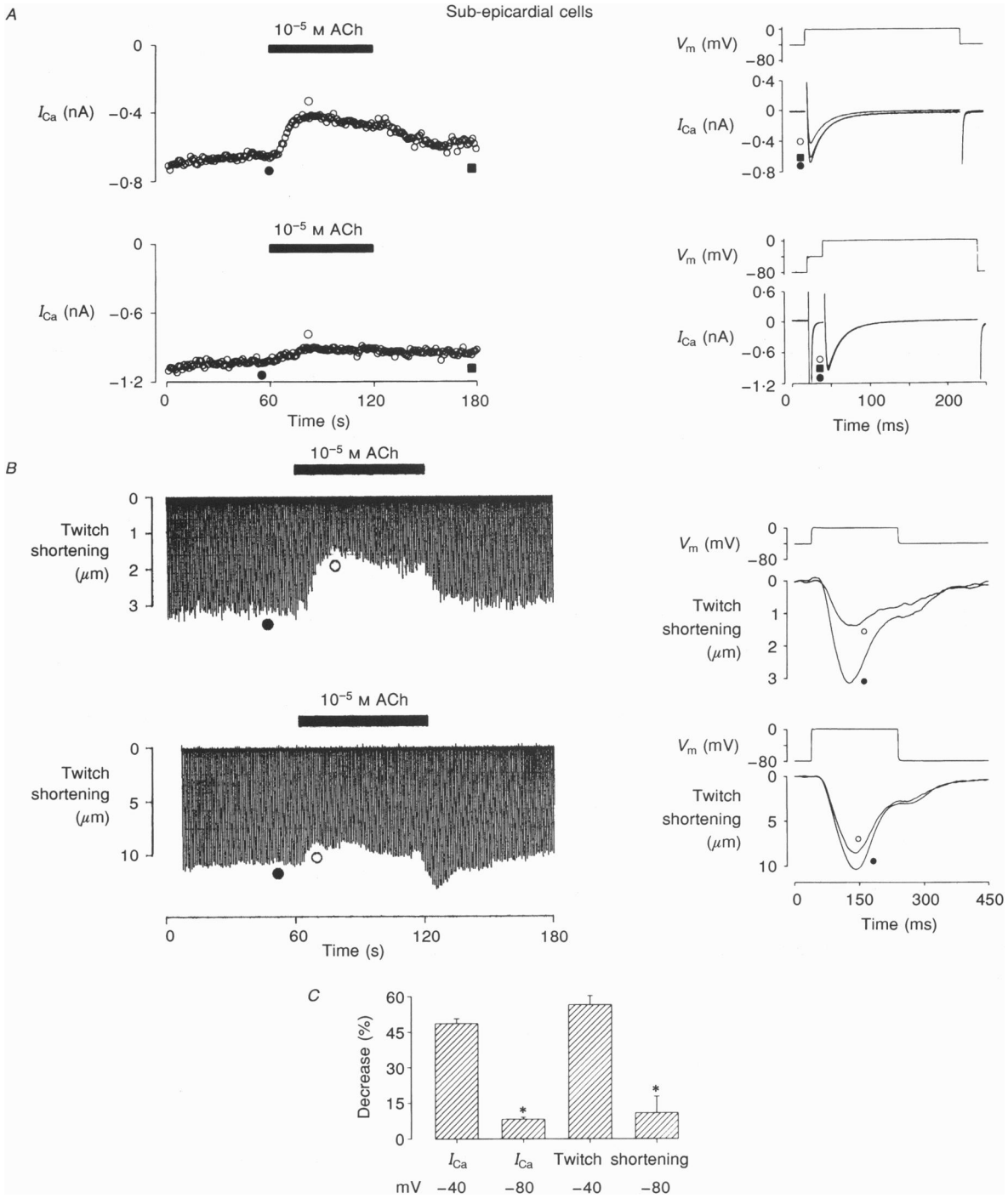

Figure 7. Effect of 10^{-5} M ACh on I_{Ca} in sub-epicardial cells

A, effect of 10^{-5} M ACh on I_{Ca} at holding potentials of −40 (top) and −80 mV (bottom). The amplitude of I_{Ca} (measured as the difference between the peak inward current and the current at the end of 200 ms pulses to 0 mV) before, during and after the exposure to ACh is shown on the left. Membrane potential (V_m, to show the protocol) and superimposed records of membrane current under control conditions (●), early during the exposure to ACh (○) and after wash-off of ACh (■) are shown on the right at a fast time base. All records are from the same cell. *B*, effect of 10^{-5} M ACh on twitch shortening at holding potentials of −40 (top) and −80 mV (bottom). Slow time base records of twitch shortening are shown on the left. Membrane potential (to show the protocol) and superimposed records of twitch shortening under control conditions (●) and early during the exposure to ACh (○) are shown on the right at a fast time base. All records are from the same cell. *C*, maximum decrease (as a percentage of the control in the absence of ACh) of I_{Ca} and twitch shortening in response to 10^{-5} M ACh at holding potentials of −40 and −80 mV. Means ± S.E.M. are shown; $n = 5$, 4, 4 and 5 (left to right). * Significantly different ($P < 0.05$) from data at a holding potential of +40 mV.

In the experiments summarized in Fig. 6*B*, 10^{-6} M ACh failed to decrease contraction significantly during a train of pulses at a holding potential of −80 mV, whereas, using the same protocol (as summarized in Fig. 7*C*), 10^{-5} M ACh did decrease contraction significantly. The difference is presumably the result of a significant decrease in I_{Ca} seen only at the higher ACh concentration. Although 10^{-5} M ACh produced a significant decrease in contraction that can be attributed to a decrease in I_{Ca}, the decrease was still small: during a train of pulses at a holding potential of −80 mV, 10^{-5} M ACh decreased contraction by 10·8 ± 3·8% ($n = 5$), whereas in the same series of experiments, during a train of action potentials, 10^{-5} M ACh decreased contraction by 74·6 ± 4·0% ($n = 12$). It is concluded that ACh can decrease I_{Ca}, although at the normal resting potential of ventricular cells (~−80 mV) the decrease is small which thus leads to a small decrease in contraction.

Possible role of I_{to} in the negative inotropic effect of ACh

The spike and dome morphology of the sub-epicardial action potential (e.g. Fig. 3*A*) is known to be the result of a prominent 4-AP-sensitive I_{to}. This I_{to} is substantially smaller in sub-endocardial cells, thus explaining the absence of this type of morphology in the sub-endocardial action potential. In the right ventricle, Litovsky & Antzelevitch (1990) have suggested that the prominent I_{to} and spike and dome morphology of the action potential in the sub-epicardium sensitizes the sub-epicardium to ACh. To test this possibility, the effect of block of I_{to} by 4-AP was investigated in sub-epicardial cells

In the first series of experiments, the effect of partial block of I_{to} by 1 mM 4-AP on the response of both the action potential and the contraction to ACh was investigated in seven sub-epicardial cells. Figure 8*A* and *B* shows superimposed action potentials under control conditions, early and late during a 2 min exposure to 10^{-5} M ACh and early and late after wash-off of ACh. Under normal conditions (Fig. 8*A*) the usual changes in the action potential were observed (cf. Fig. 3A). Figure 8*B* shows that 1 mM 4-AP was sufficient to abolish the spike and dome morphology of the sub-epicardial action potential as shown previously (Litovsky & Antzelevitch, 1988): in the presence of 4-AP the action potential was similar to that recorded in sub-endocardial cells (Fig. 3*C*) – there was no fast phase of repolarization after the action potential peak. Despite the

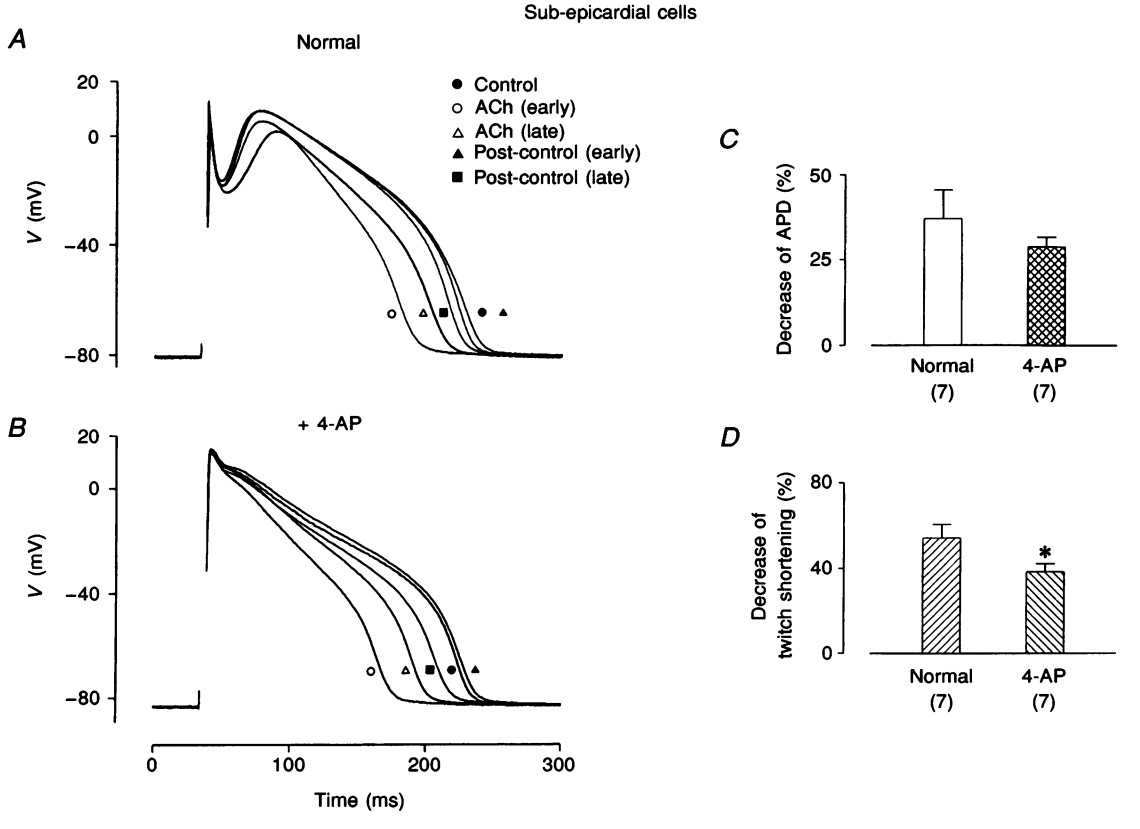

Figure 8. Effect of 1 mM 4-AP on the response to 10^{-5} M ACh in sub-epicardial cells

A and *B*, superimposed action potentials from the same cell under control conditions (●), early (○) and late (△) during a 3 min exposure to ACh and early (▲) and late (■) after wash-off of ACh under normal conditions (*A*) and in the presence of 4-AP (*B*). *C* and *D*, percentage decrease in action potential duration (*C*) and twitch shortening (*D*) early during exposures to ACh under normal conditions and in the presence of 4-AP. Means ± S.E.M. are shown ($n = 7$). * Significantly different from the decrease under normal conditions ($P < 0·05$, paired t test).

loss of the characteristic configuration, ACh still had a similar effect on the sub-epicardial action potential (Fig. 8*B*). Data from seven sub-epicardial cells are summarized in Fig. 8*C* and *D*. Early during an exposure to 10^{-5} M ACh, the duration of the action potential was decreased by 36·9 ± 8·5% under normal conditions and by 28·7 ± 2·8% in the presence of 4-AP (Fig. 8*C*). The accompanying contraction was decreased by 54·2 ± 6·3% under normal conditions and by 38·5 ± 3·8% in the presence of 4-AP. The decrease in action potential duration in the presence of 4-AP, is not significantly different from that under normal conditions ($P = 0{\cdot}267$), whereas the decrease in contraction, although small, is ($P < 0{\cdot}05$).

In a second series of experiments 5 mM 4-AP was used (5 mM 4-AP abolishes I_{to} completely); under control conditions 10^{-5} M ACh decreased the contraction by 68·1 ± 4·5%, whereas in the presence of 5 mM 4-AP it decreased the contraction by 66·6 ± 3·1% ($n = 8$); the difference is not significant. These results confirm that block of I_{to} by 4-AP does not affect the response to ACh substantially.

The possibility that ACh may have a direct action on I_{to} in sub-epicardial cells was investigated using the whole cell patch clamp technique. I_{Ca} was abolished by the application of 10 μM nifedipine. I_{to} was recorded during 50 ms pulses from −80 to +60 mV. An example is shown in Fig. 9; a plot of the amplitude of I_{to} before, during and after application of 10^{-5} M ACh is shown in Fig. 9*A* and selected records of I_{to} are superimposed in Fig. 9*B*. In four cells, 1 min after the application of 10^{-5} M ACh, there was no detectable effect on I_{to}. This is confirmed by Fig. 9*C*, which shows current–voltage relationships for I_{to} in three of the cells before, during and after the application of ACh. In Fig. 9*A* it can be seen that the pedestal current on which I_{to} is superimposed was shifted in the outward direction in the presence of ACh (by ~155 pA maximally in this example).

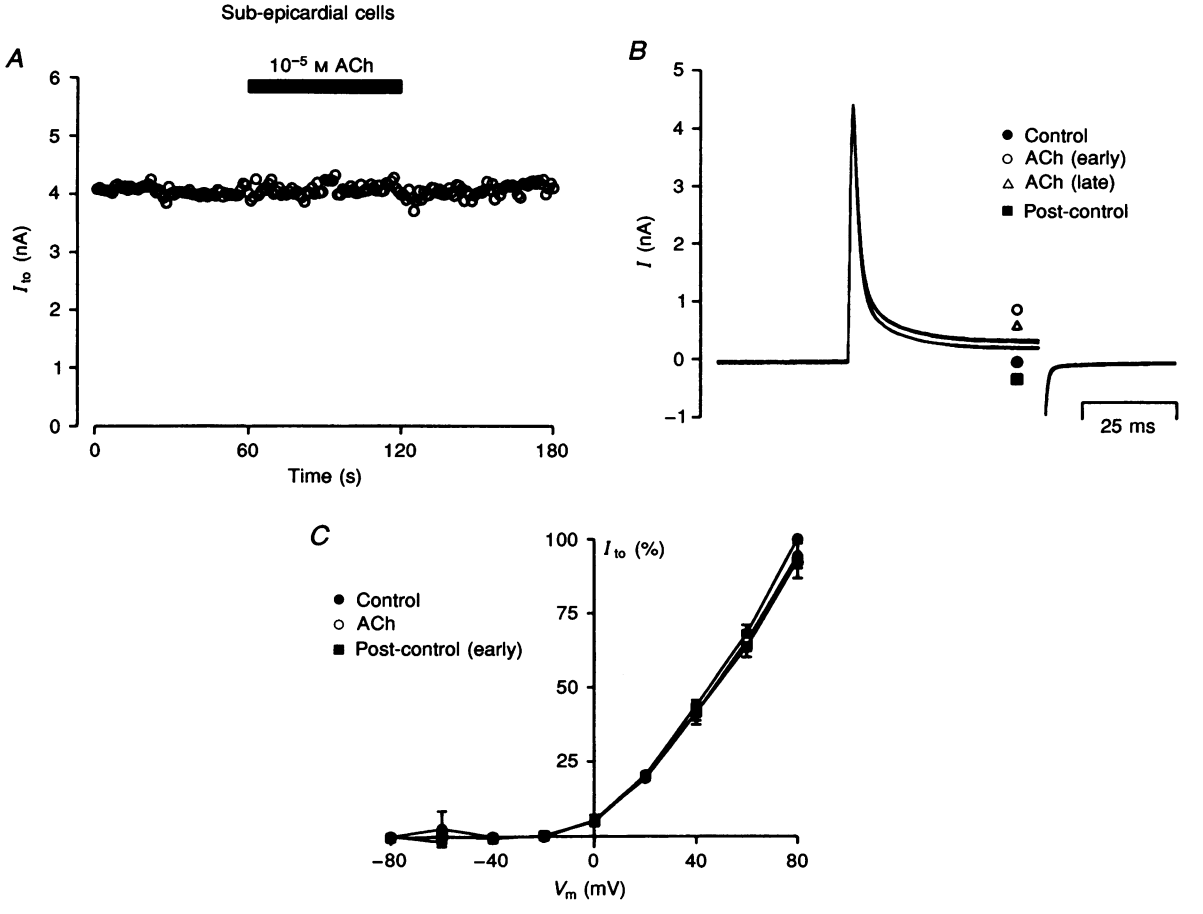

Figure 9. Effect of 10^{-5} M ACh on I_{to} in sub-epicardial cells

A, amplitude of I_{to} (measured as the difference between the peak outward current and the current at the end of 50 ms pulses from −80 to +60 mV) before, during and after the exposure to ACh. *B*, superimposed records of membrane current under control conditions (●), early (○) and late (△) during the exposure to ACh and after wash-off of ACh (■) at a fast time base. From the same cell as *A*. *C*, current–voltage relationships for I_{to} before, during and after the exposure to ACh. I_{to} was measured as the difference between the peak outward current and the current at the end of 50 ms pulses (holding potential, −80 mV). Currents were expressed as a percentage of the current during pulses to +80 mV under control conditions. Means ± S.E.M. are shown ($n = 3$).

This outward shift was observed in all cases and is considered in the next section.

In summary, these experiments suggest that I_{to} does not play a major role in the electrical and mechanical responses of left ventricular sub-epicardial cells to ACh.

Activation of K^+ current by ACh in sub-epicardial cells

To investigate the mechanisms underlying the shortening of the action potential on application of ACh, the effect of ACh on membrane current in both sub-epicardial and sub-endocardial cells was measured during 200 ms voltage clamp pulses from −80 to 0 mV. Figure 10 shows superimposed records of membrane current before, during and after a 3 min exposure to 10^{-5} M ACh in a sub-epicardial cell (Fig. 10*A*) and a sub-endocardial cell (Fig. 10*B*). Membrane current under control conditions is identified by filled circles. As expected, prominent I_{to} at the start of the pulse was present in the sub-epicardial cell, but not in the sub-endocardial cell. Membrane current recorded early during the exposure to ACh is identified by the open circles. Figure 10*A* shows that, at this time, there was an outward shift of current during the pulse to 0 mV in the sub-epicardial cell; there was no change in the holding current at −80 mV. In contrast, Fig. 10*B* shows that there was no change in membrane current in the presence of ACh in the sub-endocardial cell. Membrane current recorded late during the exposure to ACh is identified by the open triangles. Figure 10*A* shows that, at this time, the current had partly recovered towards control in the sub-epicardial cell. Membrane current recorded early and late after the wash-off of ACh is identified by the filled triangles and filled squares, respectively. Figure 10*A* shows that in the sub-epicardial cell, on wash-off of ACh, the current during the pulse first became slightly more inward than control and then returned towards control. The hatched bars in

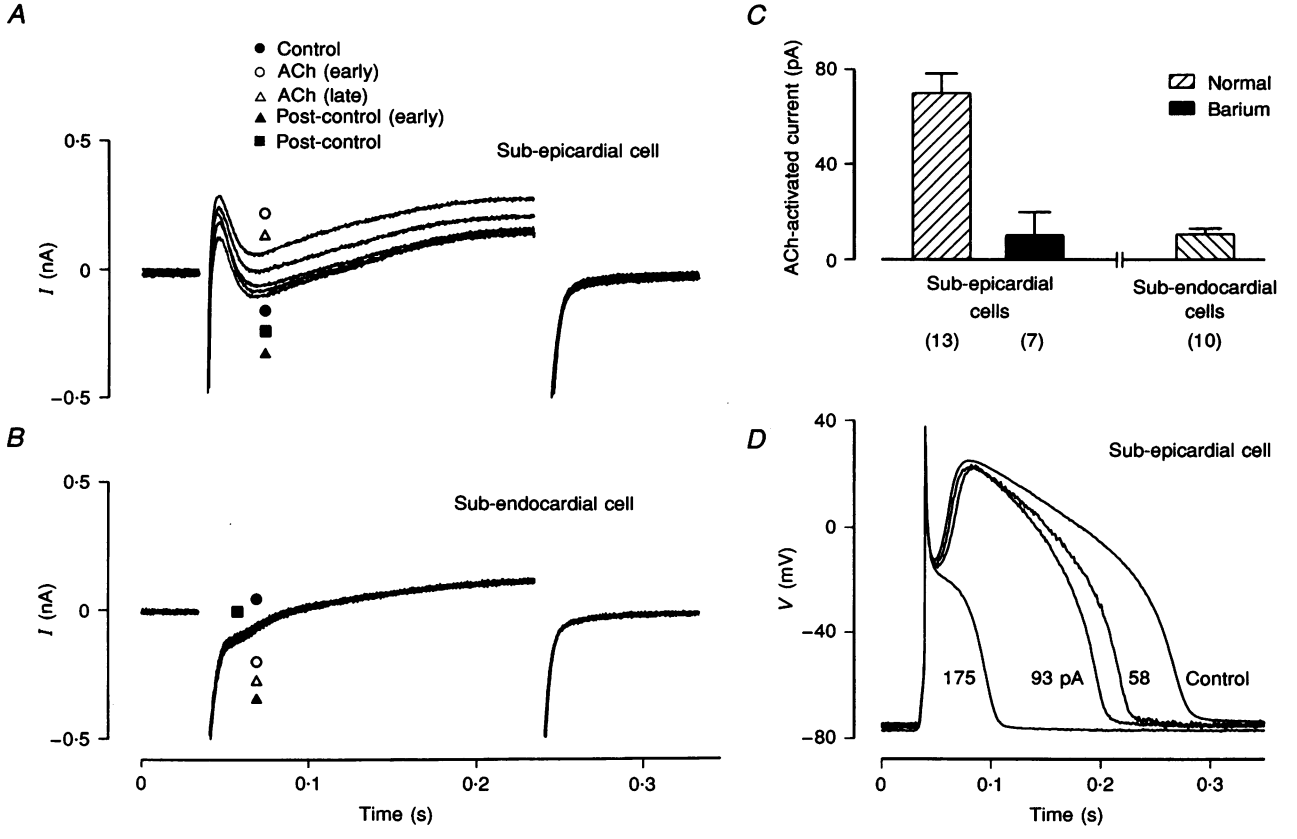

Figure 10. ACh-induced changes in membrane current in sub-epicardial and sub-endocardial cells

A and *B*, superimposed records of membrane current under control conditions (●), early (○) and late (△) during a 3 min exposure to 10^{-5} M ACh and early (▲) and late (■) after wash-off of ACh in a sub-epicardial (*A*) and a sub-endocardial cell (*B*). Membrane current was recorded during 200 ms voltage clamp pulses from −80 to 0 mV. *C*, mean amplitude of the ACh-induced change in membrane current at 0 mV (measured at the end of the pulse) early during an exposure to 10^{-5} M ACh in 13 sub-epicardial cells under normal conditions, 7 sub-epicardial cells (a sub-group of the 13 sub-epicardial cells) in the presence of 2 mM Ba^{2+} and 10 sub-endocardial cells. Means ± S.E.M. are shown. *D*, superimposed action potentials recorded from a sub-epicardial cell under control conditions and during the injection of outward currents, 58, 93 and 175 pA in amplitude.

Fig. 10*C* show the mean amplitude of the ACh-activated outward current at 0 mV in thirteen sub-epicardial cells and ten sub-endocardial cells (70·0 ± 8·2 and 10·6 ± 2·5 pA, respectively). The difference in current between the sub-epicardial and sub-endocardial cells is significant ($P < 0.001$) and this confirms that ACh activated a substantial current in sub-epicardial cells, but not in sub-endocardial cells. It is possible that the ACh-activated outward current in sub-epicardial cells is muscarinic K^+ current ($I_{K,ACh}$). To test this possibility, seven of the thirteen sub-epicardial cells were exposed to 2 mM Ba^{2+}, a blocker of K^+ currents, including $I_{K,ACh}$ (Zang, Yu & Boyett, 1995). In the presence of Ba^{2+}, the ACh-activated outward current in the sub-epicardial cells was decreased to 10·1 ± 9·9 pA. This suggests that the ACh-activated outward current is a K^+ current.

To test whether an outward current of the magnitude of the ACh-activated current (mean ± S.D., 70·0 ± 29·6 pA at 0 mV as explained above) could be responsible for the shortening of the sub-epicardial action potential in the presence of ACh, the effect of the injection of constant outward current on the sub-epicardial action potential was investigated. A constant outward current is a reasonable simulation of $I_{K,ACh}$, because, over the range of potentials encountered during the action potential, $I_{K,ACh}$ is relatively constant. In the example shown in Fig. 10*D*, the effect of currents ranging from 58 to 175 pA in amplitude was investigated. The 58 and 93 pA currents (similar to the mean amplitude of the ACh-activated current at 0 mV), produced 22 and 31% decreases in action potential duration; this is comparable with the ACh-induced shortening of the action potential (Fig. 4*B*). Similar results were obtained from a total of three cells.

Inhibition of $I_{K,1}$ by ACh in sub-epicardial cells

To confirm that the ACh-activated outward current in sub-epicardial cells is a K^+ current, we endeavoured to measure the reversal potential of the current. However, we were unsuccessful as shown in Fig. 11. Membrane current was measured at the holding potential of −80 mV and during a 200 ms pulse to 0 mV followed by a 50 ms pulse to −100 mV and a 50 ms pulse to −120 mV (Fig. 11*A*). On application of 10^{-4} M ACh, there was little change in the holding current, but the current during the pulse to 0 mV was shifted outwards as usual (Fig. 11*A*; cf. Fig. 10*A*). At the more negative potentials, we were expecting the

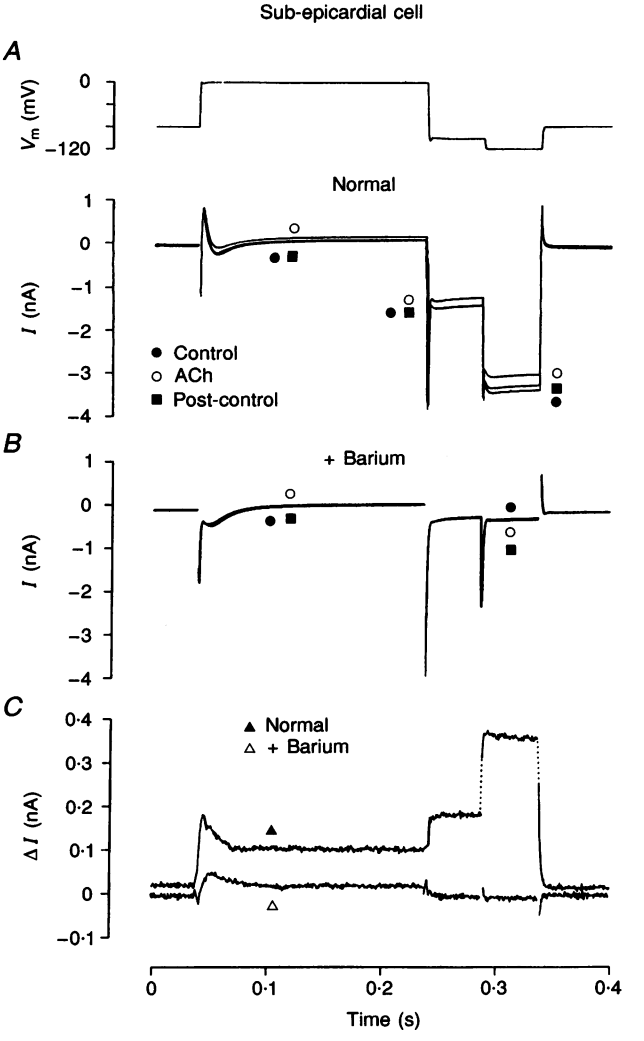

Figure 11. Simultaneous activation of $I_{K,ACh}$ and inhibition of $I_{K,1}$ in a sub-epicardial cell

A and *B*, superimposed records of membrane current under control conditions (●), early during an exposure to 10^{-4} M ACh (○) and after wash-off of ACh (■) under normal conditions (*A*) and in the presence of 2 mM Ba^{2+} (*B*). The records in *A* and *B* were obtained from the same cell. Membrane potential is shown in the upper record to illustrate the experimental protocol. *C*, the ACh-induced change in membrane current early during an exposure to ACh under normal conditions (▲) and in the presence of 2 mM Ba^{2+} (△). The records were obtained by subtracting the current records in *A* and *B* under control conditions from those in the presence of ACh.

current to be shifted in the inward direction, but a more substantial outward shift of current was observed. The change in membrane current induced by ACh was calculated by subtracting the current under control conditions from the current in the presence of ACh and is shown in Fig. 11*C*; the filled triangle identifies the difference current under normal conditions at the different potentials. The figure shows the time dependence of the ACh-induced change in membrane current. At 0 mV, but not at the other potentials, there was a time-dependent 'relaxation' of the ACh-activated current – this possibly represents relaxation of $I_{K,ACh}$ (Noma & Trautwein, 1978).

Current–voltage relationships were constructed from experiments like that illustrated in Fig. 11*A* carried out on eight sub-epicardial cells and are shown in Fig. 12. Current at the end of each pulse is plotted against the membrane potential in Fig. 12*A*. The ACh-activated current (calculated by subtracting the current under control conditions from the current in the presence of ACh) is plotted against the membrane potential in Fig. 12*B*; the circles show data collected under normal conditions. Figure 12*A* and *B* confirms that ACh had little effect on current at −80 mV, but shifted current in the outward direction at both more negative and more positive potentials. This shows that we were unsuccessful in identifying a reversal potential in the conventional way. It is possible that ACh simultaneously activated $I_{K,ACh}$ and inhibited $I_{K,1}$. This would explain why ACh had little effect on membrane current at −80 mV (close to or equal to the expected equilibrium potential for K^+, E_K). There is a plausible reason why the activation of $I_{K,ACh}$ should dominate at potentials positive to E_K and the inhibition of $I_{K,1}$ should dominate at potentials negative to E_K (see Discussion). Figure 11*B* shows the effect of ACh on membrane current at the various membrane potentials in the presence of 2 mM Ba^{2+}. The data in Fig. 11*A* and *B* were obtained from the same cell. As expected (see Fig. 10*C*), the majority of the ACh-activated outward current at 0 mV was abolished in the presence of Ba^{2+} (Fig. 11*B*) – this is confirmed by the difference current in the presence of Ba^{2+} labelled by the open triangle in Fig. 11*C*. In this example a small ACh-activated outward current at 0 mV remained in the presence of Ba^{2+} and it is not known whether this is residual $I_{K,ACh}$ or another current. However, this was not observed routinely. (Note that Ba^{2+} was not used as a substitute for Ca^{2+} – Ba^{2+} and Ca^{2+} were present together – and, therefore, no significant change in I_{Ca} at 0 mV was expected or observed in records like that in Fig. 11*B*.) In the presence of Ba^{2+}, the large inward currents at −100 and −120 mV were absent; the large inward currents at potentials negative to E_K are known to be largely $I_{K,1}$ and Ba^{2+} is known to block $I_{K,1}$ (Carmeliet & Mubagwa, 1986). The ACh-induced inhibition of inward current at −100 and −120 mV was also abolished. This is again confirmed by the difference current in the presence of Ba^{2+} in Fig. 11*C*. It is also confirmed by the current–voltage relationships for the difference current in the presence of Ba^{2+} shown by the triangles in Fig. 12*B*. These results are consistent with our interpretation of the ACh-induced changes in membrane current.

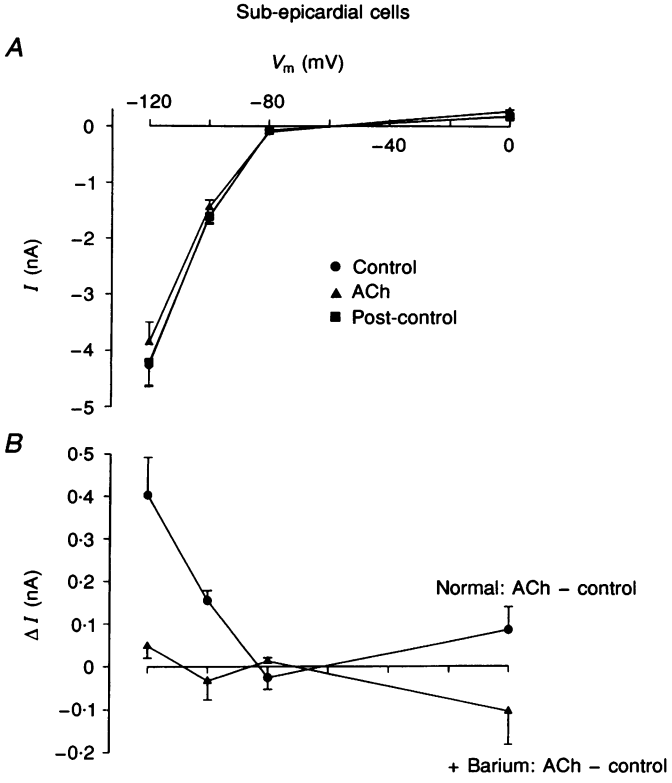

Figure 12. Effect of 10^{-4} M ACh on the current–voltage relationship of sub-epicardial cells

A, current–voltage relationships before (●) and early during an exposure to ACh (▲) and after wash-off of ACh (■). Membrane current was measured at the end of each pulse in experiments like that in Fig. 11. *B*, current–voltage relationships for the ACh-induced change in membrane current under normal conditions (●) and in the presence of 2 mM Ba^{2+} (▲). The ACh-induced currents were obtained by subtracting currents under control conditions from those early during an exposure to 10^{-4} M ACh. The data in *A* and *B* were obtained from the same cells. Means ± S.E.M. are shown ($n = 8$).

DISCUSSION

The principal finding from the present study is that ACh has a direct negative inotropic effect on ventricular cells from the left ventricle of the dog and the effect varies according to the site of origin of the cells: it is prominent in sub-epicardial cells and it is small or absent in sub-endocardial cells. These results add to the increasing body of evidence that ACh has a direct effect on ventricular muscle in the absence of β-adrenergic receptor stimulation.

Mechanism underlying the inotropic effect of ACh in sub-epicardial cells

ACh-induced changes in membrane current. G.-N. Tseng found that 10^{-5} M ACh decreased I_{Ca} in dog ventricular mid-wall cells by 20–30% (S.H. Litovsky & C. Antzelevitch, personal communication, 1990). In the present study 10^{-5} M ACh was observed to decrease I_{Ca} in dog left ventricular sub-epicardial cells (Fig. 7). However, the effect was voltage dependent and, whereas 10^{-5} M ACh decreased I_{Ca} by 48·6 ± 2·0% at a holding potential of −40 mV, at a holding potential of −80 mV it decreased it by just 8·2 ± 0·8% (Fig. 7*C*). The voltage dependence of the action of ACh on I_{Ca} has not been reported before and the underlying mechanism is not known.

In sub-epicardial cells, ACh activated an outward current at 0 mV. This current may be the muscarinic K^+ current ($I_{K,ACh}$) for several reasons: (i) it was activated by ACh; (ii) it was blocked by 2 mM Ba^{2+} (Figs 10–12) – this concentration of Ba^{2+} is known to block $I_{K,ACh}$ fully (Zang *et al.* 1995); and (iii) during an exposure to ACh, the ACh-activated outward current faded with time (Fig. 10*A*) – fade during an exposure to ACh is a characteristic feature of $I_{K,ACh}$ and is the result of desensitization to ACh (e.g. Zang, Yu, Honjo, Kirby & Boyett, 1993). The current at 0 mV is unlikely to be contaminated by $I_{K,1}$ as explained below. It is well known that $I_{K,ACh}$ is present in the sinoatrial node and atrial muscle (Honjo, Kodama, Zang & Boyett, 1992; Zang *et al.* 1993). However, it has also been reported in rabbit Purkinje fibres and ferret and rat ventricular cells (Carmeliet & Mubagwa, 1986; Boyett *et al.* 1988; McMorn *et al.* 1993). In rabbit sinoatrial node cells at −55 mV, $I_{K,ACh}$ (10^{-5} M ACh) is ~614 pA in amplitude (Honjo *et al.* 1992) and in rat atrial and ventricular cells at −53 mV $I_{K,ACh}$ (10^{-6} M ACh) is ~1980 and ~320 pA in amplitude, respectively (McMorn *et al.* 1993); in the present study, in dog left ventricular sub-epicardial cells at 0 mV $I_{K,ACh}$ (10^{-5} M ACh) was 70·0 ± 8·2 pA in amplitude.

In the present study, ACh also inhibited inward current at potentials negative to E_K (Figs 11 and 12). This may be the result of inhibition of $I_{K,1}$, because: (i) $I_{K,1}$ is a dominant current in this range of potentials, (ii) the ACh-induced change in current was greater at −120 than −100 mV (Fig. 12*B*) and this is appropriate for $I_{K,1}$, and (iii) the ACh-induced change in current was abolished in the presence of 2 mM Ba^{2+} – $I_{K,1}$ is known to be abolished under these conditions (Carmeliet & Mubagwa, 1986). The voltage dependence of the ACh-induced changes in membrane current (Fig. 12*B*, circles) can be explained by a dual effect of ACh on membrane currents. At −80 mV, there was no change in membrane current – this is expected, because this potential is close to or equal to E_K and at this potential both $I_{K,ACh}$ and $I_{K,1}$ are small or zero and, furthermore, it is possible that at this potential any effects of ACh on $I_{K,ACh}$ and $I_{K,1}$ are equal and opposite. At 0 mV the data indicate that the activation of $I_{K,ACh}$ is more important than the inhibition of $I_{K,1}$. Again this is expected, because $I_{K,1}$ shows greater rectification than $I_{K,ACh}$; $I_{K,1}$ is approximately zero at 0 mV, whereas $I_{K,ACh}$ is still substantial. At potentials negative to E_K, the data indicate that the inhibition of $I_{K,1}$ is more important than $I_{K,ACh}$; again this is to be expected, if the activation of $I_{K,ACh}$ is smaller than the inhibition of $I_{K,1}$. In other cell types, ACh results in a hyperpolarization of the resting membrane, whereas inspection of Figs 3, 4, 5 and 8 shows that there was little or no change in the resting potential in the sub-epicardial cells in the present study. This corresponds to the lack of change in membrane current at −80 mV and is possibly because the membrane potential was already close to E_K under control conditions or, alternatively, the effects of ACh on $I_{K,ACh}$ and $I_{K,1}$ were equal and opposite.

ACh-induced changes in the action potential. The shortening of the sub-epicardial action potential in the presence of ACh is likely to be the result of the decrease in I_{Ca} and the activation of $I_{K,ACh}$. The fade of the action potential shortening in the presence of ACh (Figs 3*A* and 4*A*) can again be explained by changes in I_{Ca} and $I_{K,ACh}$, because the effects of ACh on both currents faded with time during an exposure to ACh (Figs 7*A* and 10*A*). The activation of $I_{K,ACh}$ is likely to be more important than the reduction in I_{Ca}, because, at a holding potential of −80 mV, the effect of ACh on I_{Ca} was small or absent. In guinea-pig atrial cells (Iijima, Irisawa & Kameyama, 1985), rabbit Purkinje fibres (Mubagwa & Carmeliet, 1983), ferret ventricular cells (Boyett *et al.* 1988) and rat ventricular cells (McMorn *et al.* 1993) the ACh-dependent shortening of the action potential has also been largely attributed to the activation of $I_{K,ACh}$, rather than inhibition of I_{Ca}. Figure 10*D* shows that an outward current comparable in amplitude with the ACh-activated outward current can produce a shortening of the action potential similar to that observed in the presence of ACh.

The dose dependence of the change in action potential duration by ACh in dog *left* ventricular sub-epicardial cells obtained in the present study (Fig. 4) is very different from the dose dependence in dog *right* ventricular sub-epicardium reported by Litovsky & Antzelevitch (1990). Litovsky & Antzelevitch (1990) showed that low ACh concentrations ($<10^{-6}$ M) *prolonged* the action potential; only high concentrations of ACh ($>10^{-6}$ M) shortened the action potential. The prolongation of the action potential at low

ACh concentrations was abolished after the block of I_{to}. In contrast, block of I_{to} in the present study had no significant effect on the ACh-induced changes in the action potential (Fig. 8). This difference may possibly be the result of a more marked spike and dome morphology of the sub-epicardial action potential in the study of Litovsky & Antzelevitch (1990). The spike and dome morphology of left ventricular sub-epicardial cells is reported to be less marked than that of right ventricular sub-epicardial cells (Di Diego, Sicouri, Litovsky & Antzelevitch, 1991). When the spike and dome morphology is marked (as in the right ventricle), the dome has the properties of an all-or-none slow response type action potential superimposed on another action potential. In the study of Litovsky & Antzelevitch (1990) the low ACh concentrations prolonged the action potential by delaying the appearance of the dome. Thus, when I_{to} was abolished, the spike and dome morphology of the action potential was lost, and ACh no longer prolonged the action potential in this manner. At high ACh concentrations, the dome abruptly failed and the action potential was shortened. It is possible that the different pattern of changes in the present study was the result of a less marked spike and dome morphology of the action potential (in the present study the changes in the action potential were graded and the dome did not show the properties of an all-or-none slow response type action potential). In the study of Litovsky & Antzelevitch (1990), both the delay of the dome at low ACh concentrations and the failure of the dome at high ACh concentrations can still be explained by the activation of $I_{K,ACh}$ (and reduction in I_{Ca}) and, therefore, the results obtained in the two studies are not inconsistent.

ACh-induced changes in contraction. In sub-epicardial cells, the negative inotropic effect of ACh is likely to be the result of the decrease in I_{Ca} and the shortening of the action potential. The contribution of I_{Ca} to the inotropic effect of ACh was assessed during trains of voltage clamp pulses of constant duration at a holding potential of −80 mV. With 10^{-6} M ACh a negative inotropic effect was only observed in one out of seven cells, whereas with 10^{-5} M ACh a negative inotropic effect (accompanied by a decrease in I_{Ca}) was observed in four out of five cells (Figs 5–7). With both ACh concentrations the inotropic effect of ACh during trains of voltage clamp pulses (at a holding potential of −80 mV) was small compared with that during trains of action potentials. This is presumably the result of the small decrease in I_{Ca} under these conditions (Fig. 7). At a holding potential of −40 mV, 10^{-5} M ACh had a greater inotropic effect (and a greater depressant effect on I_{Ca}), but this is not a normal physiological situation (Fig. 7). Two lines of evidence support the hypothesis that the negative inotropic effect of ACh on sub-epicardial cells is primarily the result of the shortening of the action potential. (i) On application of ACh, the time course of the initial decrease in contraction and the subsequent increase in contraction was similar to the initial shortening of the action potential and the subsequent prolongation (Fig. 5*A*). As explained above, the changes in the action potential can be explained by the changes in $I_{K,ACh}$ (and I_{Ca}). (ii) The dose dependencies of the initial decreases in the contraction and action potential duration on application of ACh are roughly similar (Figs 2*B* and 4*B*). The negative inotropic effect of ACh on ferret and rat ventricular cells has also been shown to be the result of the shortening of the action potential caused by the activation of $I_{K,ACh}$ (Boyett *et al.* 1988; McMorn *et al.* 1993). Like ACh, adenosine is known to activate $I_{K,ACh}$ and recently it has been shown that the negative inotropic effect of adenosine on guinea-pig atrial cells is the result of the activation of $I_{K,ACh}$ and a shortening of the action potential (Wang & Belardinelli, 1994). Shortening of the action potential will indirectly decrease the Ca^{2+} influx via I_{Ca} by decreasing the time that Ca^{2+} flows into the cell. Furthermore, the shortening of the action potential is expected to increase Ca^{2+} efflux via the Na^+–Ca^{2+} exchanger. Both the decrease of Ca^{2+} influx and the increase of Ca^{2+} efflux are expected to reduce the Ca^{2+} loading of the sarcoplasmic reticulum (SR). Consequently, less Ca^{2+} will be released from the SR on stimulation and this will result in a smaller contraction.

During an exposure to ACh, we have attributed the slow increase in contraction to desensitization to ACh (see above). However, there is another possible explanation: high concentrations of muscarinic agonist have been shown to result in an increase in contraction in guinea-pig and rat papillary muscles (Korth & Kühlkamp, 1985; Korth, Sharma & Sheu, 1988). This has been suggested to be the result of a slow increase of the intracellular Na^+ concentration (Korth & Kühlkamp, 1985), perhaps as a result of the activation of a background Na^+ current (Matsumoto & Pappano, 1989), and a slow increase in intracellular Ca^{2+} (Korth *et al.* 1988). However, if this mechanism were involved in the responses to ACh in the present study, a slow increase in contraction should have been observed during a train of voltage-clamp pulses, and this was not the case in the cells studied (Fig. 6*B*).

On wash-off of ACh, there was a rebound increase in the contraction beyond its control value (e.g. Fig. 1). Unlike the negative inotropic effect of ACh, it is unlikely that the rebound increase in contraction was the result of a change in the action potential for two reasons: firstly, there was no rebound increase in action potential duration and action potential duration always returned to its control value monotonically after wash-off of ACh (e.g. Fig. 4) and, secondly, a rebound increase in contraction occurred during trains of voltage-clamp pulses of constant duration (Figs 5 and 6). The mechanism underlying the rebound increase in contraction is unclear. A rebound increase in the contraction also occurs in ferret and rat ventricular cells (Boyett *et al.* 1988; McMorn *et al.* 1993). In ferret ventricular cells there is no rebound increase in action potential duration (Boyett *et al.* 1988). In rat ventricular

cells there is a rebound increase in the duration of the low plateau of the action potential (McMorn *et al.* 1993). However, this could be a consequence rather than a cause of the rebound increase in contraction – it could be the result of an increase in Na^+–Ca^{2+} exchange current caused by a potentiation of the Ca^{2+} transient at this time (it is surprising that a similar rebound increase in action potential duration does not occur in dog and ferret ventricular cells for the same reason). In ferret ventricular muscle, the rebound increase in the contraction has been shown to be the result of a rebound increase in the amplitude of the Ca^{2+} transient (Boyett *et al.* 1988). It is possible that the rebound increase in contraction is the result of the rise in intracellular Na^+ noted by Korth & Kühlkamp (1985). As already discussed, this may explain the increase in contraction during an exposure to ACh (because it will lead to an increase in the Ca^{2+} content of the SR). Despite this, it is possible that, during the exposure to ACh, Ca^{2+} release from the SR may continue to be less than normal because of a depletion of the SR as a result of the shortened action potential. However, on wash-off of ACh the duration of the action potential is rapidly restored and this may lead to a greater Ca^{2+} release from the SR than normal and thus the rebound increase in contraction (because the restored action potential rapidly increases the Ca^{2+} content of the SR and intracellular Na^+ remains high). Another possibility is that the rebound increase in contraction is the result of a rebound increase in I_{Ca} as recently demonstrated by Wang & Lipsius (1995) in cat atrial cells. However, a rebound increase in I_{Ca} is expected to result in a prolongation of the action potential; in the present study and in ferret ventricular cells (Boyett *et al.* 1988) this was not observed. Furthermore, a rebound increase in I_{Ca} was not observed in the present study (Fig. 7).

Regional differences in the response to ACh within the left ventricle

In addition to the marked heterogeneity in the electrophysiological properties of the sub-epicardium, mid-wall and sub-endocardium of the left ventricle (Antzelevitch *et al.* 1991), there are regional differences in metabolism (see Figueredo, Brandes, Weiner, Massie & Camacho, 1993), wall stress (Yin, 1981), myosin isoform (Eisenberg, Edwards & Zak, 1985), sarcomere length (see Eisenberg *et al.* 1985) and excitation–contraction coupling (Figueredo *et al.* 1993; Chamunorwa & O'Neill, 1995; Main, Bryant & Hart, 1995). The present study is the first to demonstrate a regional variation in the regulation of contraction by an agonist. The regional variation in the negative inotropic effect can be explained by the activation of $I_{K,ACh}$ in the sub-epicardium, but not the sub-endocardium (and possibly a regional variation in the regulation of I_{Ca} by ACh, although this was not studied). The absence of $I_{K,ACh}$ in the sub-endocardium could be the result of the absence of the muscarinic ACh receptor or the muscarinic K^+ channel in the sub-endocardium. In tissue from the dog right ventricle, Litovsky & Antzelevitch (1990) observed a response of the sub-endocardial action potential as well as the sub-epicardial action potential to ACh *in the presence of a β-agonist*. It is likely that this involves the muscarinic ACh receptor and a decrease in β-stimulated I_{Ca}. This experiment suggests that the muscarinic ACh receptor is present in the sub-endocardium and, therefore, the most likely explanation of our data at present is that there is a regional variation in the expression of the muscarinic K^+ channel. Recently, the expression of Kv4.2 (a transient outward K^+ channel) in the rat ventricle has been demonstrated to be eight times higher in the sub-epicardium than in papillary muscle (Dixon & McKinnon, 1994) – this confirms electrophysiological data concerning regional variation in I_{to} in the ventricular wall (Antzelevitch *et al.* 1991; Liu *et al.* 1993). By analogy, it will be of interest to measure the expression of GIRK1 (the muscarinic K^+ channel) in the left ventricular wall. $I_{K,ACh}$ can be activated by a wide range of agonists, such as adenosine (Kurachi, Nakajima & Sugimoto, 1987) and an albumin-associated phospholipid factor (Bünemann, Ferrebee, Tigyi & Pott, 1994), and this raises the possibility that there could be regional variations within the left ventricle in the response to these agonists.

The physiological importance of the negative inotropic effect of ACh on the ventricles and the regional difference in the effect can only be speculated on. The ventricles of the heart receive parasympathetic innervation (Löffelholz & Pappano, 1985) and, therefore, it can be assumed that they are exposed to ACh. Because the negative inotropic effect of ACh fades with time, the effect of vagal stimulation is expected to be transient. Why the sub-epicardium should be selectively suppressed by ACh is not known.

In summary, in sub-epicardial cells from the left ventricle of the dog, ACh activates $I_{K,ACh}$. This results in a shortening of the action potential and, therefore, a negative inotropic effect. An ACh-induced decrease in I_{Ca} may also contribute to the changes. In sub-endocardial cells, ACh activates little or no $I_{K,ACh}$ and, therefore, it has little or no negative inotropic effect.

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