Journal of Physiology (1993), **468**, *pp.*641–667
With 16 *figures*
Printed in Great Britain

$[Ca^{2+}]_i$ TRANSIENTS AND $[Ca^{2+}]_i$-DEPENDENT CHLORIDE CURRENT IN SINGLE PURKINJE CELLS FROM RABBIT HEART

BY KARIN R. SIPIDO, GEERT CALLEWAERT AND EDWARD CARMELIET

From the Laboratory of Physiology, Katholieke Universiteit Leuven, Belgium

(*Received 29 June 1992*)

SUMMARY

1. Single Purkinje cells, enzymatically isolated from rabbit ventricle, were studied under whole-cell voltage clamp and internally perfused with the fluorescent Ca^{2+} indicator, indo-1 (100 μM).

2. Fast $[Ca^{2+}]_i$ transients were elicited by brief depolarizations from a holding voltage of −45 mV and by repolarization from very positive potentials. The peak $[Ca^{2+}]_i$–voltage relation was bell-shaped with a peak around +10 mV.

3. $[Ca^{2+}]_i$ transients were completely blocked by the Ca^{2+} channel antagonist, nisoldipine (10 μM) and were very small when Ca^{2+} release from the sarcoplasmic reticulum (SR) was prevented by superfusion of cells by caffeine (1 mM) or ryanodine (10 μM). A fast application of caffeine induced a transient increase in $[Ca^{2+}]_i$. These results suggest $[Ca^{2+}]_i$ transients are due to Ca^{2+}-induced Ca^{2+} release from the SR.

4. Rate of decline of the $[Ca^{2+}]_i$ transient was voltage dependent, suggesting contribution of the Na^+–Ca^{2+} exchanger to Ca^{2+} efflux. At very positive potentials ($> +60$ mV), Ca^{2+} influx through the Na^+–Ca^{2+} exchanger could be observed.

5. A transient outward current was observed at potentials positive to +10 mV, but only if depolarizing pulses were accompanied by a $[Ca^{2+}]_i$ transient.

6. When the amplitude of the $[Ca^{2+}]_i$ transient was changed by (1) changes in $[Ca^{2+}]_o$, (2) changes in frequency of depolarization or (3) conditioning prepulses, the amplitude of the outward current changed in the same direction. This suggests activation of the current is dependent on and graded by $[Ca^{2+}]_i$.

7. The outward current was observed in K^+-free solutions, in the presence of Cs^+ and TEA^+, and was not blocked by 4-aminopyridine (10 mM). In contrast, DIDS (100 μM) decreased the outward current by 70 ± 20 % (mean ± S.D., $n = 9$), without affecting $[Ca^{2+}]_i$.

8. When external Cl^- was lowered, the amplitude of the outward current decreased; when internal Cl^- was replaced by aspartate, it became apparent at more negative potentials. These interventions strongly suggest the current was carried by Cl^-; it can therefore be referred to as a $[Ca^{2+}]_i$-activated Cl^- current or $I_{Cl(Ca)}$.

9. When $I_{Cl(Ca)}$ was maximally activated during a conditioning step, steps to negative potentials revealed inward currents through $I_{Cl(Ca)}$ (in symmetrical Cl^- solutions). The fully activated I–V relation was linear.

10. $I_{Cl(Ca)}$ could be activated at membrane potentials between −80 and +80 mV by a fast application of caffeine (10 mM), inducing Ca^{2+} release from the SR, demonstrating that $I_{Cl(Ca)}$ does not require membrane depolarization or Ca^{2+} influx through the Ca^{2+} channel for its activation. However, at negative potentials the amplitude of $I_{Cl(Ca)}$ decreased.

11. The time course of $I_{Cl(Ca)}$ did not follow the time course of the $[Ca^{2+}]_i$ transient. $I_{Cl(Ca)}$ declined early, before $[Ca^{2+}]_i$ reached its peak. Study of inward tail currents, and of $I_{Cl(Ca)}$ activated by caffeine-induced $[Ca^{2+}]_i$ transients, confirmed early decline.

12. These observations cannot be reconciled with a simple ligand-operated channel. The transient nature of $I_{Cl(Ca)}$ might be due to an inactivation process, or, alternatively, the differences in time course between $I_{Cl(Ca)}$ and $[Ca^{2+}]_i$ could reflect the existence of important gradients for Ca^{2+} between the cytoplasm and the subsarcolemmal space.

INTRODUCTION

Purkinje fibres are the specialized conduction tissue of the ventricle. Their contribution to contraction is limited, but study of intracellular calcium homeostasis in these cells is of special interest, from two points of view.

Purkinje cells lack a T-tubular system as typically seen in ventricular cells (Sommer & Jennings, 1986) and therefore signal transduction between the sarcolemma and the sarcoplasmic reticulum (SR) might be different. Previous studies of excitation–contraction coupling in canine Purkinje fibres with aequorin (Wier, 1980; Wier & Isenberg, 1982; Marban & Wier, 1985) have revealed the existence of a biphasic $[Ca^{2+}]_i$ transient, possibly allowing separation of two sources of increase in cytoplasmic calcium. Earlier experiments ascribed the first component to the calcium current, I_{Ca}, and the second to calcium release from the sarcoplasmic reticulum, SR (Wier, 1980; Wier & Isenberg, 1982). Later experiments suggested, however, that the first component was also related to Ca^{2+} release from a superficially located SR compartment, while the second component was ascribed to Ca^{2+} release from the more centrally located SR (Marban & Wier, 1985). Such a type of excitation–contraction coupling was recently suggested for atrial cells, another cardiac cell type without T-tubuli (Lipp, Pott, Callewaert & Carmeliet, 1990).

Second, calcium-activated currents may play an important role in the genesis of arrhythmias during calcium overload as seen under pathological conditions. A calcium-activated outward current, I_{to}, has been described previously in calf and sheep Purkinje fibres (Siegelbaum & Tsien, 1980; Carmeliet & Coraboeuf, 1982) and more recently a calcium-activated current, most probably carried by Cl^-, has also been identified in rabbit ventricular and atrial myocytes (Zygmunt & Gibbons, 1991, 1992). The charge carrier of the current in Purkinje fibres and the exact relation between this current and $[Ca^{2+}]_i$ have not yet been established.

Part of the work that is presented has been published in abstract form (Sipido, Callewaert, Vereecke & Carmeliet, 1992*a*, *b*).

METHODS

Cell isolation

Single Purkinje cells from rabbit heart were obtained by a method previously described (Scamps & Carmeliet, 1989). Rabbits of either sex, weighing 2·0–2·5 kg, were killed by a blow on the neck, the heart was quickly excised and washed in a high-K^+ solution to arrest the heartbeat. The heart was then mounted on a Langendorff set for retrograde perfusion and briefly washed with a Tyrode solution containing (mM): NaCl, 137; KCl, 5·4; Hepes, 11·8; glucose, 5; $MgCl_2$, 0·5; $CaCl_2$, 1·8; pH 7·40 with NaOH). The heart was then perfused with a nominally Ca^{2+}-free Tyrode solution containing (mM): NaCl, 130; KCl, 5·4; Hepes, 6; KH_2PO_4, 1·2; $MgSO_4$, 1·2; glucose, 50; pH 7·2 with NaOH) for 7–8 min. This was followed by perfusion with enzyme solution, i.e. nominally Ca^{2+}-free Tyrode containing 0·7 mg/ml collagenase A (Boehringer Mannheim, FRG) for 10 min, and with the same solution with 0·12 mg/ml protease Type XIV (Sigma, UK) added, for 15 min. The Purkinje fibres were then carefully dissected from both left and right ventricle and further incubated in the enzyme solution until they were dissociated into single cells as seen on the microscope (Zeiss, IM35). The cells were then washed repeatedly with a Tyrode solution containing 180 μM $CaCl_2$, resuspended in normal Tyrode solution and stored at room temperature.

Voltage clamp and calcium measurements

Cells were studied at room temperature (22 °C) with the whole-cell patch clamp technique (Marty & Neher, 1983) using a List EPC-7 amplifier. Pipettes were made from borosilicate (Jencons Scientific) and resistance was typically between 1·8 and 3 MΩ, when the filling solution contained 120 mM CsCl. Stimulation and data acquisition were controlled by a PC using pCLAMP 5·0 (Axon Instruments). Cells were stimulated at a frequency of 0·1 Hz, except when noted otherwise. Sampling frequency was 2 kHz. Currents were filtered at 5 kHz, fluorescence signals were filtered at 200 Hz (low-pass Bessel filters).

The experimental set-up was built around a Nikon inverted microscope and has been described in detail previously (Callewaert, Lipp, Pott & Carmeliet, 1991). The pipette solution contained 100 μM indo-1. Calcium was calculated from the ratio of fluorescence (R) measured at two emission wavelengths, i.e. 405 and 485 nm, with excitation at 360 nm, according to Grynckiewicz *et al.* (Grynkiewicz, Poenie & Tsien, 1985).

$$[Ca^{2+}]_i = K_d B (R - R_{min}) / (R_{max} - R),$$

where K_d is the dissociation constant and B is a constant. R_{max} and R_{min} for calibration of fluorescence were obtained by perfusing the cell with metabolic inhibitors (2 μM rotenone and 5 μM carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP)) and exposing to, respectively, high calcium (in the presence of 10 μM Bromo-A 23187,) and zero calcium (with 10 mM EGTA) (Beuckelmann & Wier, 1988). The K_d was 230 nM and B was 3 as determined during *in vitro* calibration.

Solutions

The standard external solution was a modified Tyrode solution (mM): NaCl, 130; CsCl, 10; Hepes, 10; glucose, 10; $MgCl_2$, 1; $CaCl_2$, 1·8 or 5·4 (pH 7·35 with NaOH). In some experiments Cl^- was lowered by substituting 90 mM NaCl with 90 mM of sodium aspartate. When caffeine was used, it was dissolved directly into the solution (1 or 10 mM) and applied to the cell through a fast solution exchange system that was positioned within 100 μm of the cell, as described previously (Callewaert, Cleeman & Morad, 1989).

The standard pipette solution contained (mM): CsCl, 110; Hepes, 10; TEACl, 20; NaCl, 10; $MgCl_2$, 1; MgATP, 4 (pH 7·2 with CsOH). In some experiments a low-Cl^- solution was used, either one with 32 mM $[Cl^-]_i$ (containing (mM): caesium aspartate, 110; TEACl, 20; Hepes, 10; NaCl, 10; $MgCl_2$, 1; Mg_2ATP, 4; pH 7·2 with TEAOH) or one with 20 mM $[Cl^-]_i$ (containing (mM): caesium aspartate, 120; TEACl, 10; Hepes, 10; $MgCl_2$, 5; Na_2ATP, 5; pH 7·2 with TEAOH). With this low $[Cl^-]_i$ solution, a substantial junction potential was seen between pipette tip and bath solution.

However, it could be compensated for completely by the amplifier and the potential was zeroed just before sealing onto the cell. During external Cl^- substitution, no correction was made for a shift in junction potential. However, because of the bath configuration, this shift was expected to be small, as the solution exchange occurred around the cell from a perfusing pipette 150 μm in diameter, while the ground electrode remained in control solution, 150–200 mm away from the cell. Neither the I–V relation of the inward current, representing current through the calcium channel, nor the $[Ca^{2+}]_i$–voltage relation were shifted appreciably, indicating that the shift in potential was small indeed.

RESULTS

The Results section has two major parts. In the first part we will examine the characteristics of the $[Ca^{2+}]_i$ transient, in the second part we will study the

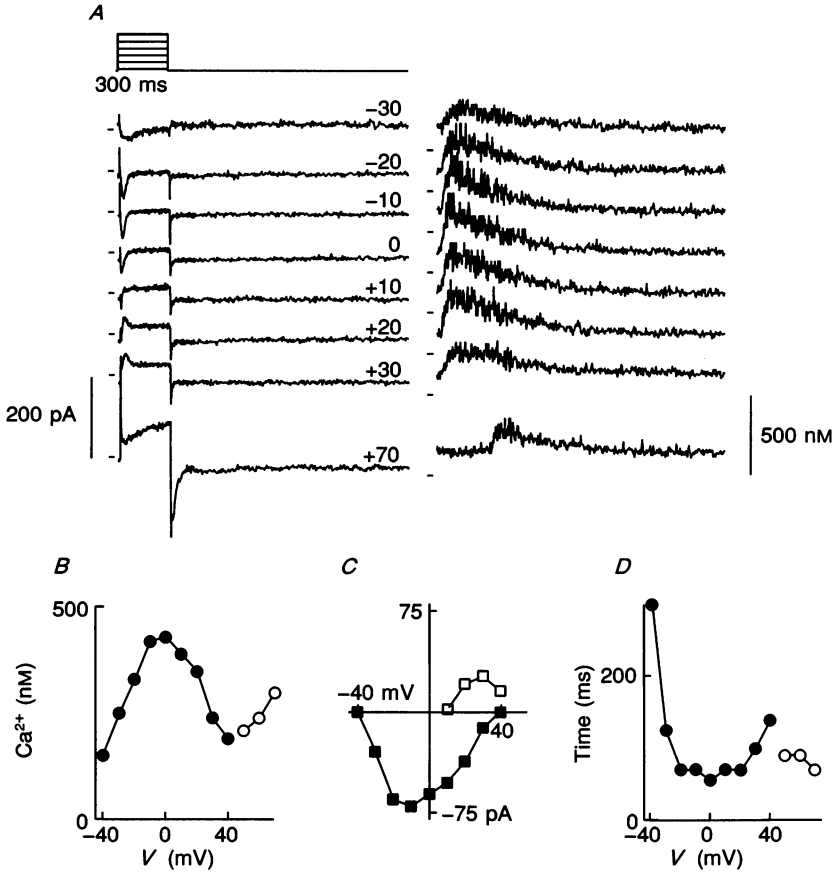

Fig. 1. Characteristics of membrane currents and $[Ca^{2+}]_i$ transients in standard solutions, 1·8 mM $[Ca^{2+}]_o$. *A*, currents (left panel) and $[Ca^{2+}]_i$ transients (right panel) during and following 300 ms depolarizing steps from a holding potential of −45 mV to the indicated potentials. The zero level is indicated by a short dash at the beginning of each trace. *B*, peak $[Ca^{2+}]_i$ (measured as the average of eleven data points) *versus* voltage during the depolarizing pulse (●) and during tail transients on repolarization (○). *C*, current–voltage relation of peak inward (■) and peak outward (□) currents, each measured as the difference between peak current and current at the end of the pulse. *D*, time to peak $[Ca^{2+}]_i$ during the depolarizing pulse (●) and during tail transients on repolarization (○).

transient outward current, its ionic nature, its $[Ca^{2+}]_i$ dependence and its kinetics. In the first part, some characteristics of this current are already pointed out, but reference is given to later paragraphs for a more extensive description.

$[Ca^{2+}]_i$ transients in single Purkinje cells

In order to identify the calcium current, I_{Ca} and the relation between I_{Ca} and the $[Ca^{2+}]_i$ transient, cells were studied in the absence of K^+ currents and Na^+ currents

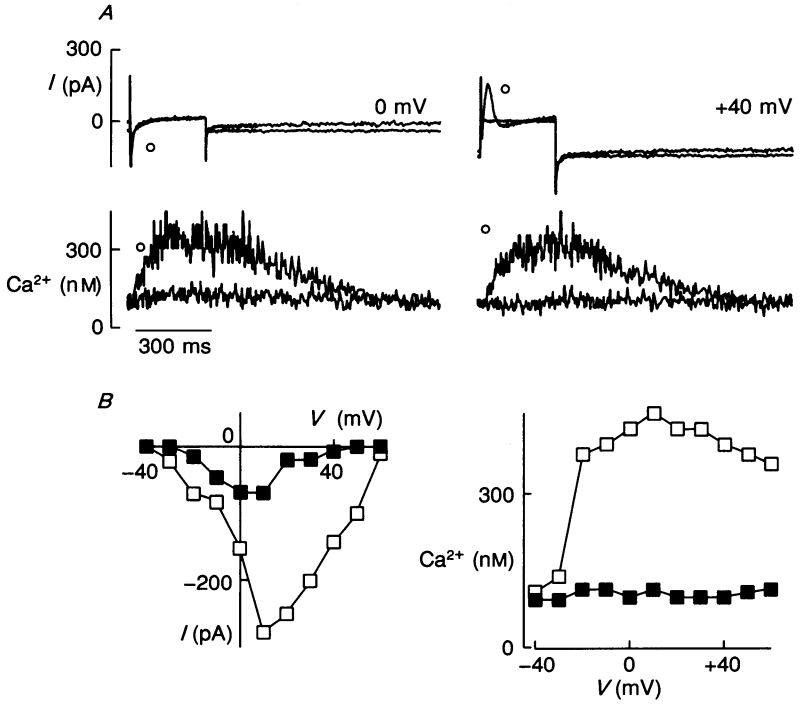

Fig. 2. Effects of 10 μM nisoldipine. Standard solutions, 5·4 mM $[Ca^{2+}]_o$. *A*, currents and $[Ca^{2+}]_i$ transients during 300 ms pulses from a holding potential of −45 mV to the indicated potential. Note at 0 mV the absence of an outward current and the block of the inward current by nisoldipine. At +40 mV, the inward current is immediately followed by an outward current component, both of which are blocked by nisoldipine (○, control records). These records were preceded by 5 prepulses to +5 mV to ensure adequate block. *B*, peak inward currents *versus* voltage and peak $[Ca^{2+}]_i$–*V* relation, before and during superfusion with 10 μM nisoldipine. Open symbols are control values, filled symbols are values obtained in the presence of nisoldipine.

(all K^+ replaced by Cs^+ and with 10–20 mM TEA^+ in the pipette solution, holding potential −45 mV to inactivate I_{Na}) (Beuckelmann & Wier, 1988; Callewaert, Cleeman & Morad, 1988). For the present study we only used cells that showed no spontaneous activity in the standard Tyrode solution.

From the holding potential of −45 mV, depolarizing steps larger than −30 mV elicited transient inward currents, accompanied by $[Ca^{2+}]_i$ transients that were

maximal at 0 to +10 mV (Fig. 1*A*). For potentials larger than +40 mV, no inward current or $[Ca^{2+}]_i$ transient was seen during depolarization, but a tail $[Ca^{2+}]_i$ transient was seen on repolarization (Fig. 1*A*). The peak $[Ca^{2+}]_i$–voltage relation was bell-shaped and followed the expected I_{Ca}–voltage relation (Fig. 1*B*). Tail $[Ca^{2+}]_i$ transients on repolarization from potentials above +40 mV (Fig. 1*B*, open symbols) can be ascribed to I_{Ca} tail currents. The maximal peak $[Ca^{2+}]_i$ was between 210 and 340 nM (295 ± 57 nM , 6 cells, mean ± S.D.).

I_{Ca} was difficult to identify since a transient outward current became apparent at potentials above +10 mV and overlapped with I_{Ca} (Fig. 1*C*). The inward current on repolarization from +70 mV should not be regarded as a tail of I_{Ca}, but is related to the transient outward current, as will be discussed later.

Time to peak $[Ca^{2+}]_i$ was between 60 and 120 ms for $[Ca^{2+}]_i$ transients recorded during depolarization to +10 mV (98 ± 20 ms, mean ± S.D.). For smaller or larger depolarizing steps, time to peak was longer (Fig. 1*D*). The $[Ca^{2+}]_i$ transient had only one component. A second peak, as seen with aequorin in multicellular canine Purkinje fibres, could not be identified.

To determine more clearly the relation between I_{Ca} and the $[Ca^{2+}]_i$ transient, we attempted to quantify the inward Ca^{2+} current as the dihydropyridine-sensitive current. Nisoldipine (10 μM) blocked both the inward current and $[Ca^{2+}]_i$ transients (Fig. 2), demonstrating that $[Ca^{2+}]_i$ transients during depolarization required Ca^{2+} influx through the Ca^{2+} channel. Under these conditions, however, the transient outward current also disappeared, thereby preventing quantification of I_{Ca} as the nisoldipine-sensitive current.

To examine the relative contribution of Ca^{2+} influx through the Ca^{2+} channel and Ca^{2+} release from an internal store, we studied the effects of caffeine (1 mM), known to prevent Ca^{2+}-induced Ca^{2+} release. During steady-state superfusion of the cells with caffeine, $[Ca^{2+}]_i$ transients on depolarization were slow and small (Fig. 3*A*), indicating $[Ca^{2+}]_i$ transients under normal conditions resulted mainly from release from a caffeine-sensitive store. The amplitude of the $[Ca^{2+}]_i$ transients in the presence of caffeine did not exceed 100 nM (Fig. 3*B*, same observations in 4 other cells). The transient outward current disappeared, but the inward currents at potentials below −10 mV also decreased (Fig. 3*A*). This could partly be ascribed to run-down of I_{Ca}, but was also related to the decrease of another inward current component (see paragraph on the nature of the transient outward current).

The absence of fast $[Ca^{2+}]_i$ transients when Ca^{2+} release from the SR is prevented, was also confirmed by examination of the effects of ryanodine (results not shown). Because of the slow onset of action of ryanodine, cells were pretreated for 30–45 min with 10 μM ryanodine before they were patched ($n = 3$). During depolarizing steps from −45 mV, we observed maximal I_{Ca} at +10 to +20 mV. $[Ca^{2+}]_i$ transients were very small, as expected, not exceeding 100 nM, similar to the values seen with caffeine.

The existence of an internal Ca^{2+} store was also confirmed by the observation that a fast application of caffeine (1 mM) at the holding potential of −45 mV, induced a large $[Ca^{2+}]_i$ transient ($n = 4$, Fig.3*C*). This $[Ca^{2+}]_i$ transient was accompanied by a large transient inward current. These results obtained with nisoldipine, caffeine and

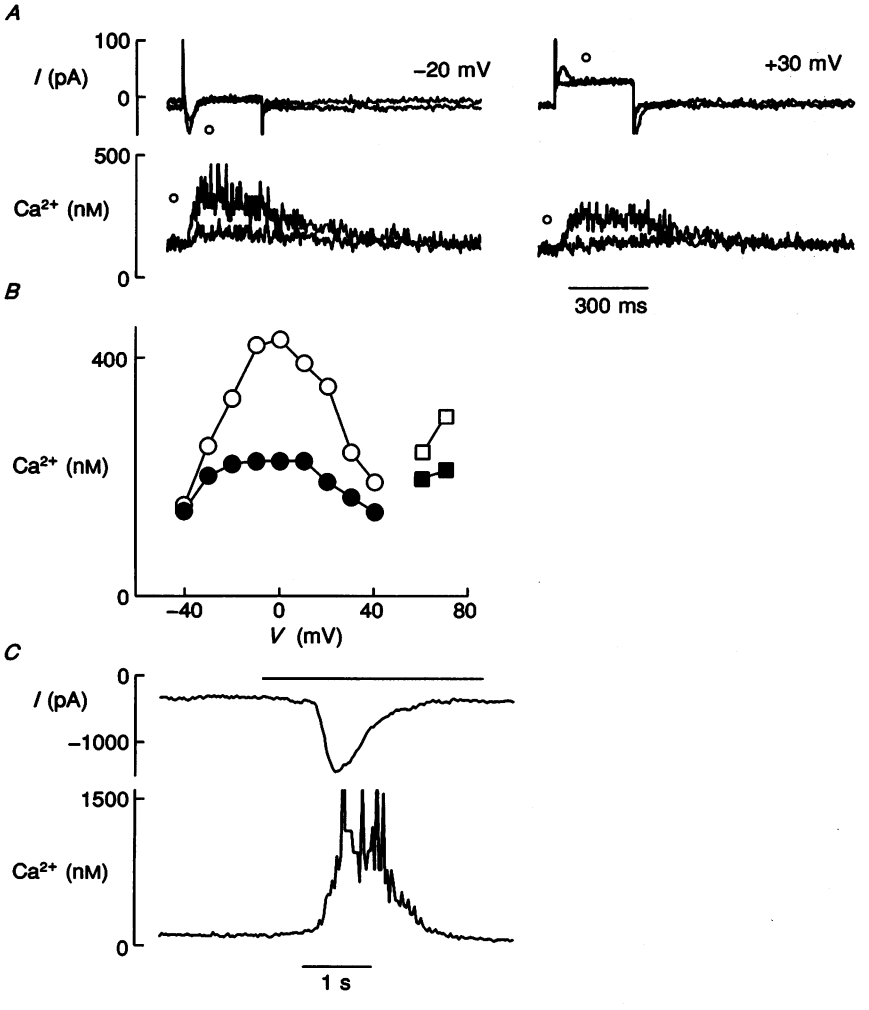

Fig. 3. Effects of 1 mM caffeine. *A*, currents and $[Ca^{2+}]_i$ transients before (○) and during steady-state perfusion with 1 mM caffeine, during a 300 ms pulse from −45 mV to the indicated potential, standard solutions, 1·8 mM $[Ca^{2+}]_o$. *B*, peak $[Ca^{2+}]_i$–*V* relation, open symbols are control values, filled symbols are values obtained in the presence of caffeine; circles are values obtained during depolarization, squares are values from tail transients. *C*, current and $[Ca^{2+}]_i$ transient during fast application of 1 mM caffeine at a holding potential of −45 mV (caffeine exposure indicated by line at top of figure). Standard solutions, 5·4 mM $[Ca^{2+}]_o$. In the same cell, maximal $[Ca^{2+}]_i$ during a depolarizing pulse to +20 mV was 520 nM.

ryanodine, indicate that $[Ca^{2+}]_i$ transients on depolarization were the result of Ca^{2+}-induced release of Ca^{2+} from the SR.

In ventricular cells, $[Ca^{2+}]_i$ transients are also modulated by Na^+–Ca^{2+} exchange. We therefore examined whether this was also true in Purkinje cells.

The decline of the $[Ca^{2+}]_i$ transient was dependent on membrane voltage as illustrated in Fig. 4*A*. At more negative potentials, decline was faster. This is compatible with a voltage-dependent Ca^{2+} extrusion system, such as the Na^+–Ca^{2+} exchanger (Bridge, Spitzer & Ershler, 1988). When extracellular $[Ca^{2+}]$

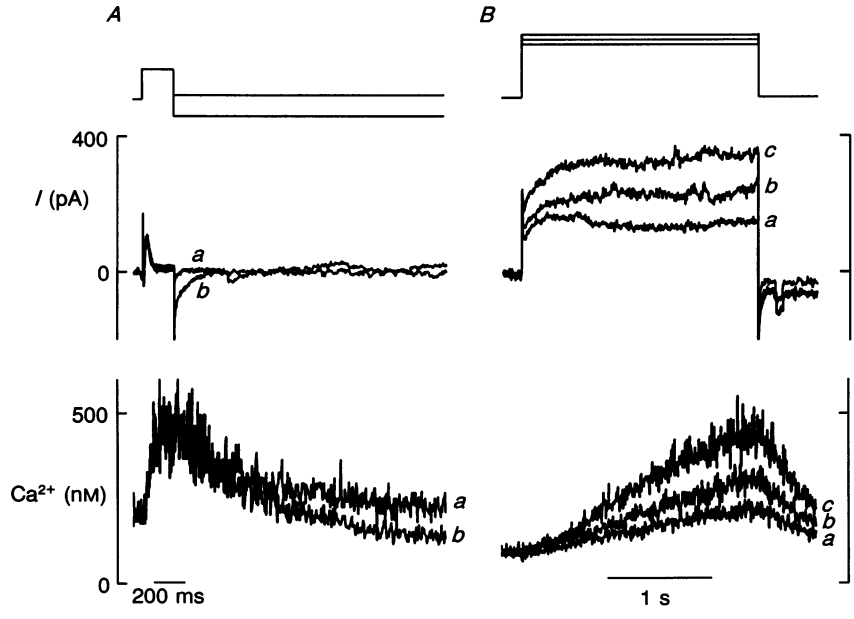

Fig. 4. Modulation of $[Ca^{2+}]_i$ by Na^+–Ca^{2+} exchange. *A*, rate of decline of $[Ca^{2+}]_i$. Currents and $[Ca^{2+}]_i$ transients during 200 ms depolarization to +20 mV followed by repolarization to −40 mV (*a*) or −80 mV (*b*). Standard solutions, 1·8 mM $[Ca^{2+}]_o$. *B*, increase in $[Ca^{2+}]_i$ during long depolarizations to +60 (*a*), +70 (*b*), +80 mV (*c*). Standard solutions, 5·4 mM $[Ca^{2+}]_o$. The currents have been leak subtracted, assuming linear leak currents and extrapolating from the holding current at −45 mV.

was increased from 1·8 to 5·4 mM, long depolarizing pulses to potentials above +50 mV were accompanied by a slow increase in $[Ca^{2+}]_i$ (Fig. 4*B*) . This slow increase in $[Ca^{2+}]_i$ was largest for the most positive potentials, suggesting this increase was due to calcium influx via the Na^+–Ca^{2+} exchanger (Beuckelmann & Wier, 1989).

The above results suggest that $[Ca^{2+}]_i$ in rabbit Purkinje cells is also modulated by Na^+–Ca^{2+} exchange as was reported for rabbit ventricular cells (Bers & Bridge, 1989). In the example illustrated in Fig. 4*A*, the peak transient inward current carried by the exchanger on repolarization to −80 mV is around 100 pA. However, on repolarization to −40 mV, the current was less than 25 pA (see also Fig. 1, transient inward current on repolarization from steps to +10 and +20 mV with largest $[Ca^{2+}]_i$ transients). This observation is in contrast to the large transient inward currents seen at −45 mV, during the $[Ca^{2+}]_i$ transients induced by fast application of caffeine (Fig. 3*C*). Possible causes of these apparent discrepancies are presented in the Discussion.

The transient outward current

In the previous paragraphs we already noted the appearance of a transient outward current during depolarizing steps larger than +10 mV. In the following paragraphs we will further characterize this current, at first referring to it as I_{to}.

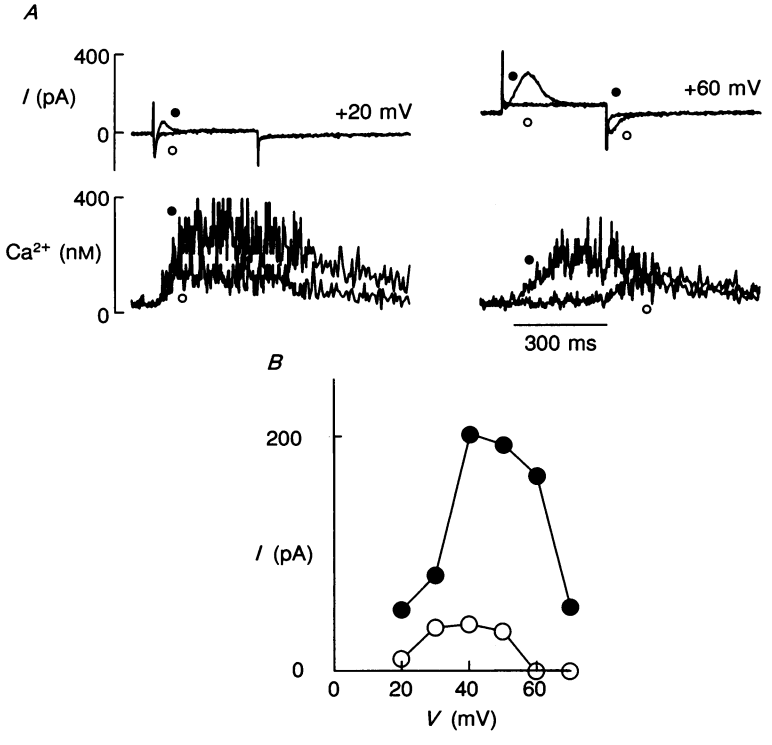

Fig. 5. Effects of increasing $[Ca^{2+}]_o$ from 1·8 to 5·4 mM, standard solutions. *A*, currents and $[Ca^{2+}]_i$ transients in low (1·8 mM, ●) and high (5·4 mM, ○) external calcium during 300 ms depolarizing pulses from −45 mV to the indicated potentials. Both the inward and outward current components on depolarization increase, as well as peak $[Ca^{2+}]_i$. The inward current on repolarization from +60 mV, present in 1·8 mM $[Ca^{2+}]_o$, is not seen in the higher $[Ca^{2+}]_o$. *B*, *I–V* relation of the outward current, measured as the difference between peak outward current and steady-state current at the end of the pulse. Open symbols are values in 1·8 mM, filled symbols are values in 5·4 mM $[Ca^{2+}]_o$.

$[Ca^{2+}]_i$ dependence of the transient outward current

Activation of I_{to} was strongly dependent on $[Ca^{2+}]_i$ as it could only be observed during voltage steps that were accompanied by a $[Ca^{2+}]_i$ transient. Brief depolarizations to positive potentials that did not elicit a $[Ca^{2+}]_i$ transient, had no I_{to} (Fig. 1*A*, step to +70 mV). Depolarizations in the presence of 10 μM nisoldipine (Fig. 2) or during steady-state perfusion with 1 mM caffeine (Fig. 3) or 10 μM ryanodine (results not shown) abolished $[Ca^{2+}]_i$ transients and I_{to} was no longer seen, indicating I_{to} required a rise in $[Ca^{2+}]_i$ for its activation.

I_{to} was also graded by $[Ca^{2+}]_i$ as evident during interventions that changed the amplitude of the $[Ca^{2+}]_i$ transient. Elevating $[Ca^{2+}]_o$ from 1·8 to 5·4 mM increased the amplitude of the inward current, related to I_{Ca}, and of the $[Ca^{2+}]_i$ transient (Fig. 5*A*, pulse to +20 mV). The amplitude of I_{to} concomitantly increased. In

Fig. 6. Effects of increasing the stimulation interval from 10 to 30 s. Records were obtained after 7 pulses at the indicated frequency (duration of pulse was 300 ms, from −45 mV to +10 mV). Standard solutions, 1·8 mM $[Ca^{2+}]_o$.

elevated $[Ca^{2+}]_o$, I_{to} could also be observed during pulses to more positive potentials, as these were now also accompanied by an increase in $[Ca^{2+}]_i$ (see pulse to +60 mV in Fig. 5*A*; the disappearance of the inward current on repolarization from +60 mV in 5·4 mM $[Ca^{2+}]_o$ will be discussed later). Because of the overlap of I_{Ca} and I_{to} the absolute magnitude of the latter could not be determined. Measured as the difference between peak outward current and steady-state current at the end of the pulse, I_{to} was increased by a factor of 5·7 (mean, range 3–7·7, $n = 5$, Fig. 5*B*).

To exclude the possibility that the previous observations were related to an effect of $[Ca^{2+}]_o$, rather than $[Ca^{2+}]_i$, we manipulated the amplitude of the $[Ca^{2+}]_i$ transient in other ways. First, the amplitude of the $[Ca^{2+}]_i$ transient was decreased by lowering the stimulation frequency (Fig. 6). Concomitant with the decrease of the $[Ca^{2+}]_i$ transient, I_{to} also decreased, indicating it was dependent on $[Ca^{2+}]_i$ and not $[Ca^{2+}]_o$, as was reported for the transient outward K^+ current in rat ventricular myocytes (Dukes & Morad, 1991).

In another experiment, we applied conditioning prepulses that inactivated I_{Ca} and (thus) Ca^{2+} release from the SR. From a holding potential of −45 mV, pulses of 1 s to −80 mV through +40 mV were applied, followed by a test pulse to +30 mV for 300 ms (Fig. 7*A*). Conditioning pulses larger than −40 mV elicited I_{Ca} and a $[Ca^{2+}]_i$ transient during this conditioning pulse. Concomitantly, the amplitude of the $[Ca^{2+}]_i$ transient during the test pulse declined and I_{to} during the test pulse declined in parallel (Fig. 7*A*). This is illustrated in the plot of relative amplitude of I_{to} and of $[Ca^{2+}]_i$ during the test pulse, *versus* voltage of the conditioning pulse (Fig. 7*B*), showing that both I_{to} and $[Ca^{2+}]_i$ during the test pulse behave identically. This decline of the $[Ca^{2+}]_i$ transient during the test pulse is the result of activation and subsequent inactivation of I_{Ca} and of Ca^{2+} release from the SR during the conditioning pulse. This is illustrated in Fig. 7*C*. The plot of decline of $[Ca^{2+}]_i$ during the test pulse (□, same plot as in Fig. 7*B*) is the mirror image of the plot of $[Ca^{2+}]_i$

during the conditioning pulse (■). The apparent voltage dependence of I_{to} is thus the consequence of the voltage dependence of $[Ca^{2+}]_i$ transients. This result is in sharp contrast to the voltage dependence of the transient outward K^+ current in rats, which is inactivated more than 75 % at −50 mV (Dukes & Morad, 1991).

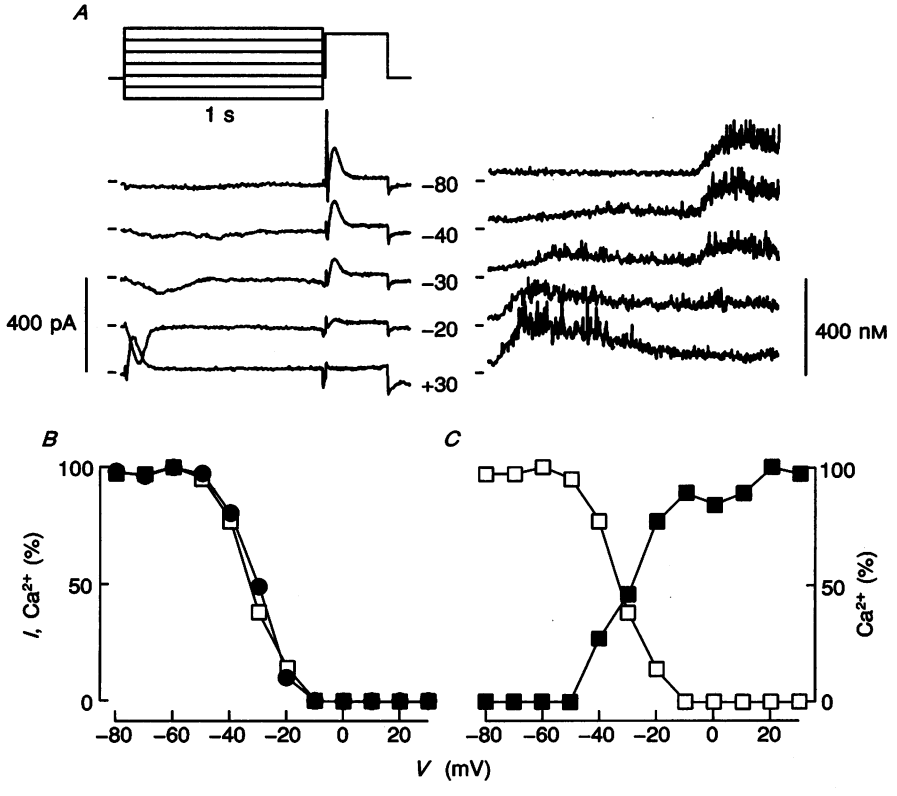

Fig. 7. Inactivation of the transient outward current by conditioning prepulses. Standard solutions, 5·4 mM $[Ca^{2+}]_o$. *A*, currents and $[Ca^{2+}]_i$ transients during 1 s conditioning pulses to the indicated potentials, followed by a 300 ms test pulse to + 30 mV, all from a holding potential of −45 mV. The zero levels are indicated by a short dash at the beginning of each trace. *B*, normalized peak outward current (●) and peak $[Ca^{2+}]_i$ (□) during the test pulse *versus* voltage of the conditioning pulse, showing the same relation. *C*, normalized peak $[Ca^{2+}]_i$ during the test pulse (□, same data as in *B*) and normalized peak $[Ca^{2+}]_i$ during the conditioning pulse (■) *versus* voltage during the conditioning pulse, showing the inverse relationship.

Ionic nature of the transient outward current

I_{to} apparently was not carried by K^+ as it was seen in the absence of K^+ (replaced by Cs^+ in both internal and external solutions) and in the presence of TEA^+ (20 mM, present in the pipette solution). To exclude the possibility of I_{to} being a Cs^+ current through the transient outward K^+ channel, described for rat ventricular myocytes by Dukes & Morad (1991), we examined the effect of a high concentration of 4-aminopyridine (4-AP), as suggested by these authors. Ten millimolar 4-AP ($n = 4$,

results not shown) did not affect I_{to}. We then hypothesized that the current was carried by chloride and looked at the effects of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) and of chloride substitutions.

Effects of a Cl^- channel blocker. We studied the effects of 100 μM DIDS (Sigma; stock solution of 50 mM in dimethyl sulphoxide), a blocker of anion transport. DIDS was autofluorescent, but this increase in fluorescence was constant

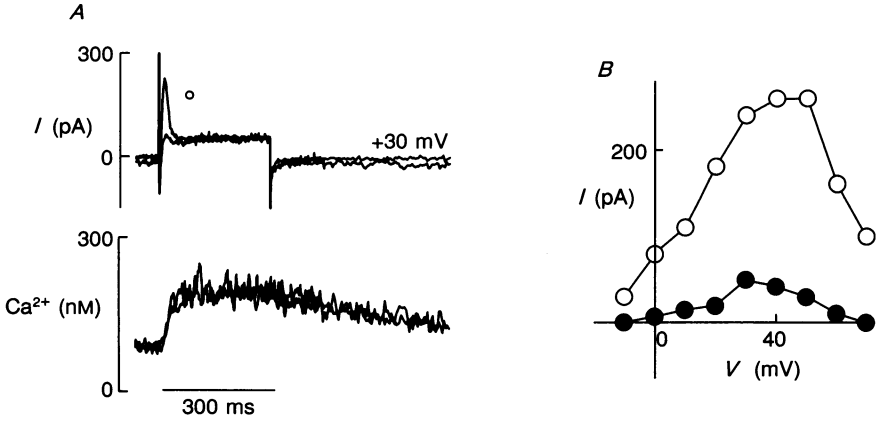

Fig. 8. Effects of 100 μM DIDS. *A*, currents before (○) and during superfusion with 100 μM DIDS. $[Ca^{2+}]_i$ transients were not affected. The pipette contained 90 mM aspartate and 32 mM Cl^-; standard external solution, 5·4 mM $[Ca^{2+}]_o$; holding potential, −45 mV; 300 ms depolarizing step to + 30 mV. *B*, outward current, measured as the difference between peak outward current and current at the end of the pulse, *versus* voltage, before (○) and during superfusion of the cell with 100 μM DIDS (●).

throughout the experiment (steady-state superfusion) and could be subtracted as background. $[Ca^{2+}]_i$ transients were not altered by DIDS, therefore DIDS did not affect the $[Ca^{2+}]_i$-dependent activation of I_{to}. Nevertheless, I_{to} decreased (Fig. 8*A*). The amplitude of the outward current component, measured as the difference between peak outward current and steady-state current decreased by 45 to 100 % (total range for 9 cells, mean ± S.D. was 70 ± 20 %) (Fig. 8*B*). This block was reversible. However, a full block as described for ventricular cells (Zygmunt & Gibbons, 1991), was seen in only one out of nine experiments. The low efficiency of DIDS is possibly related to degradation of DIDS during the UV illumination for $[Ca^{2+}]_i$ measurements, as this drug is known to be light sensitive, or it may be related to low specificity of DIDS for I_{to} in Purkinje cells.

Effects of Cl^- substitution on the outward current and on $[Ca^{2+}]_i$ transients. On the hypothesis that I_{to} was carried by Cl^-, we examined whether I_{to} shifted according to the Nernst potential for Cl^-, during substitution experiments.

We lowered *internal* $[Cl^-]$ by equimolar replacement with aspartate. In six cells perfused with 20 mM $[Cl^-]_i$ (chloride reversal potential, E_{Cl}, −52 mV), an outward current component was already observed on depolarization to −20 mV (Fig. 9), in contrast to the standard solution, where outward currents were never seen below +10 mV (E_{Cl} −2 mV). In two other cells, perfused with 32 mM Cl^-, I_{to} was

seen on depolarization to −10 mV (E_{Cl}, −38 mV) (original traces not shown, but see Fig. 10). The appearance of an outward current component at more negative potentials could theoretically also be explained by an effect on I_{Ca}, if this inward current would decrease in amplitude. As $[Ca^{2+}]_o$ is not affected by the internal $[Cl^-]$

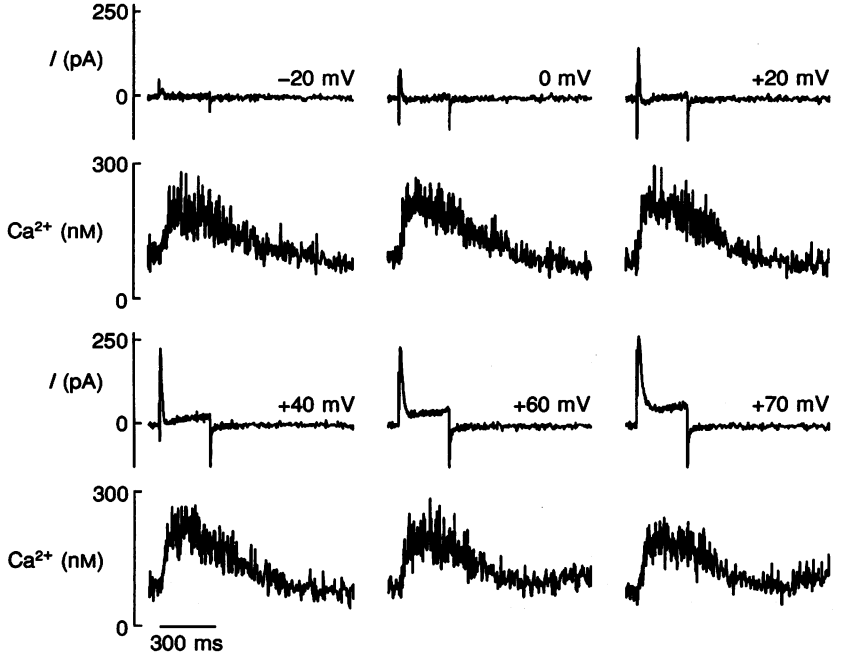

Fig. 9. Currents and $[Ca^{2+}]_i$ transients recorded with low internal Cl^-. $[Cl^-]_i$ was 20 mM; standard external solution, 5·4 mM $[Ca^{2+}]$. The calculated E_{Cl} was −52 mV. Holding potential was −45 mV; traces obtained during 300 ms depolarizing pulses to the indicated potentials.

substitution, this is very unlikely. Moreover, inward currents under these conditions were, if anything, larger. An increase in I_{Ca} may be related to buffering of $[Ca^{2+}]_i$ by intracellular aspartate, which would reduce Ca^{2+}-dependent inactivation. The observation that $[Ca^{2+}]_i$ transients were somewhat smaller (Fig. 9) would support this hypothesis. Time course of $[Ca^{2+}]_i$ transients also varied less with voltage, an observation that has also been made in atrial cells perfused with citrate, another low-affinity Ca^{2+} buffer (G. Callewaert, K. R. Sipido, P. Lipp, L. Pott & R. Carmeliet, unpublished observations).

In Fig. 10, the peak $[Ca^{2+}]_i$–voltage and I_{to}–voltage relation for different $[Cl^-]_i$ have been plotted; the calculated E_{Cl} is marked (✳). The amplitude of I_{to} was measured as the difference between the peak outward current and the steady-state current at the end of the pulse. This measurement is only an approximation because of the overlap with I_{Ca} and absolute values are therefore not considered here. The lowest potential at which I_{to} became apparent, shifted to the left in accordance with the Nernst equation for Cl^-. The absence of I_{to} at potentials below

−30 mV, despite a lower calculated E_{Cl}, could be related to the absence of a $[Ca^{2+}]_i$ transient. The decline of outward current at potentials above +40 mV also parallels decline of $[Ca^{2+}]_i$ transients at these potentials.

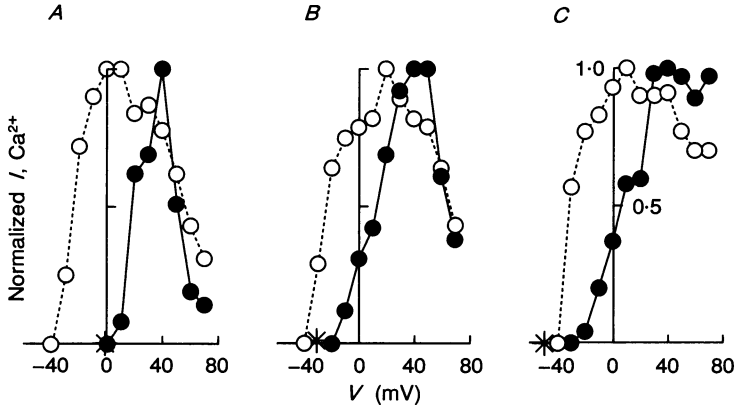

Fig. 10. Normalized peak outward current (●) and peak $[Ca^{2+}]_i$ (○) *versus* voltage in the presence of different $[Cl^-]_i$. *A*, $[Cl^-]_i = 142$ mM, calculated $E_{Cl} = -5$ mV, marked by ✳. *B*, $[Cl^-]_i = 32$ mM, calculated $E_{Cl} = -38$ mV, marked by ✳. *C*, $[Cl^-]_i = 20$ mM, calculated $E_{Cl} = -52$ mV, marked by ✳.

With a low $[Cl^-]_i$ solution, we lowered *external* Cl^- from 152 to 62 mM by equimolar replacement with aspartate. Calculated E_{Cl} values were −52 and −27 mV respectively. Lowering external $[Cl^-]$ did not affect $[Ca^{2+}]_i$ transients, and thus did not interfere with the $[Ca^{2+}]_i$-dependent activation of I_{to} (Fig. 11*A*). This is in accordance with our calculations, showing the addition of 90 mM aspartate lowered free $[Ca^{2+}]_o$ only by 20 μM (total $[Ca^{2+}]_o$ 5·4 mM). As predicted for a Cl^- current, the outward current component decreased (Fig. 11*A*). Measured as the difference between peak outward current and steady-state current, the amplitude of I_{to} diminished at all potentials (Fig. 11*B*, $n = 3$). As I_{to} was activated only at potentials above E_{Cl} in both high and low $[Cl^-]_o$, we could not observe possible inward currents.

Can we measure a reversal potential? The experiments described in the two previous paragraphs strongly suggested that the outward current component, activated by $[Ca^{2+}]_i$, was carried by chloride. We will therefore from now on refer to it as $I_{Cl(Ca)}$. As demonstrated in the experiments in low $[Cl^-]_i$, $I_{Cl(Ca)}$ could be observed at potentials below 0 mV, if they were accompanied by a $[Ca^{2+}]_i$ transient. This implies that under our standard conditions ($E_{Cl} = 0$ mV) *inward* currents are activated at negative potentials, unless $I_{Cl(Ca)}$ is strongly outwardly rectifying as described for ventricular cells (Zygmunt & Gibbons, 1991).

We tested this hypothesis by examining tail currents at potentials between −50 and +50 mV, following a 25 ms conditioning pulse to +30 mV (Fig. 12*A*). The amplitude and duration of this conditioning pulse were sufficient to fully activate $I_{Cl(Ca)}$. Since $[Ca^{2+}]_i$ transients were minimally disturbed by changes in membrane potential after this conditioning pulse, the degree of activation of $I_{Cl(Ca)}$ would be

the same at all potentials. Analysis of the tail currents remained tricky though, since they might be contaminated by tails of I_{Ca}. Theoretical considerations led us to conclude this contamination must be small. During a 25 ms pulse to +30 mV, I_{Ca}

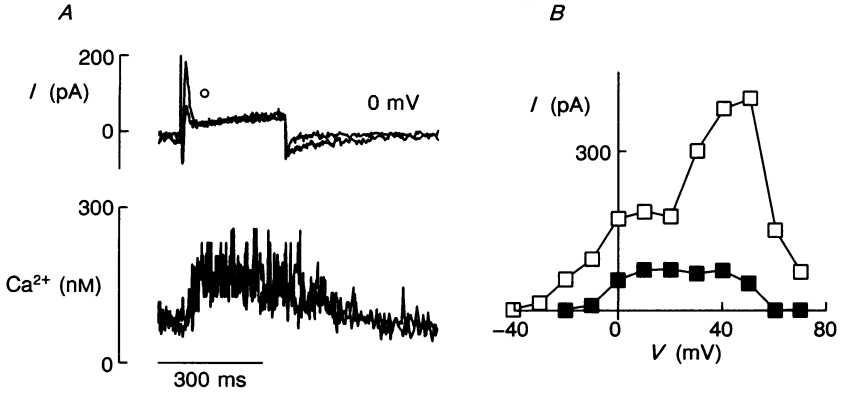

Fig. 11. Effects of lowering external Cl^-. $[Cl^-]_o$ was reduced from 152 to 62 mM by substitution with aspartate. $[Cl^-]_i = 20$ mM, calculated E_{Cl} shifted from −52 to −27 mV. *A*, currents and $[Ca^{2+}]_i$ transients during 300 ms depolarizing pulses from −45 mV to the indicated potentials; ○, records in high $[Cl^-]_o$). *B*, transient outward current measured as the difference between peak outward current and current at the end of the pulse, *versus* voltage in high $[Cl^-]_o$ (□) and low $[Cl^-]_o$ (■).

is already partly inactivated. On further depolarization to potentials > +40 mV, a possible I_{Ca} component will be small. On repolarization, I_{Ca} deactivates quickly and tails of I_{Ca} are generally brief. At potentials around 0 mV, a contribution of I_{Ca} to the tail current cannot be excluded though. However, experimental evidence supported the idea that the time-dependent currents were indeed mostly due to $I_{Cl(Ca)}$. First, these slow tail currents were not seen in the rare cells that had no $I_{Cl(Ca)}$ (5 out of a total of 42 cells in this study, protocol tested in 2 cells). Second, they were largely decreased by DIDS, although the absence of complete block prevented calculation of a DIDS-sensitive current.

In standard solutions, we expected to see time-dependent outward currents at positive potentials and inward currents at negative potentials as the calculated E_{Cl} was around 0 mV. This was indeed the case as shown in Fig. 12*A*. In Fig. 12*B* we have plotted the amplitude of the tail currents *versus* voltage. Although in this particular protocol contamination with I_{Ca} was reduced, we emphasize that the absolute values may still show some offset. Nevertheless, the reversal occurred at the expected potential and there was no apparent rectification in this voltage range. Similar results were obtained in two other cells.

Since it is evident from these experiments that $I_{Cl(ca)}$ will be inward at negative potentials (in standard solutions), we can now also explain why the inward current at −20 mV was decreased during continuous superfusion of the cells with caffeine, as seen in Fig. 3*A*. It also explains the slower component of inward current seen during the conditioning pulses to −30 and −20 mV in Fig. 7*A*.

We can now also re-examine the transient inward currents accompanying tail $[Ca^{2+}]_i$ transients on repolarization from very positive potentials (see e.g. pulse to +70 mV in Fig. 1*A* and pulse to +60 mV in Fig. 5*A*). One could interpret these currents either as tails of I_{Ca} or as $I_{Na/Ca}$. The first possibility is excluded by the fact

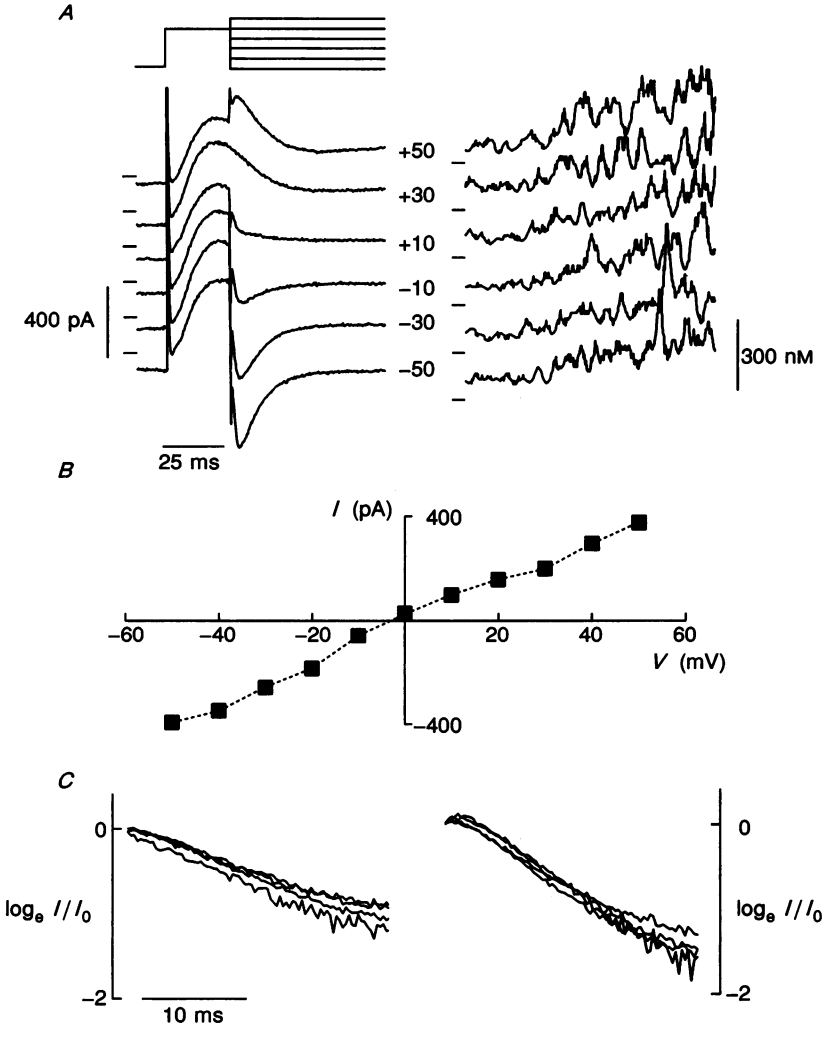

Fig. 12. Amplitude and decline of $I_{Cl(Ca)}$ at different membrane potentials after fully activating the current by a 25 ms conditioning pulse to +30 mV. Standard solutions, 1·8 mM $[Ca^{2+}]_o$, calculated E_{Cl} −5 mV. *A*, currents and $[Ca^{2+}]_i$ transients during the conditioning pulse to + 30 mV and the following step to the test potentials indicated in the figure. The short dash before each trace indicates the zero level. *B*, amplitude of time-dependent current at the various test potentials (measured as the difference between the current 5 ms after the step, and at the end of the pulse) as a function of the test potential. *C*, logarithmic plot of the decline of the tail current; I_0 is the amplitude of the initial tail current at 2 ms. Left panel, steps to +50, +40, +30 and +20 mV. Right panel, currents during steps to −50, −40, −30 and −20 mV.

that these currents were not seen when Ca^{2+} release from the SR was inhibited (ryanodine, 10 μM, $n = 3$), a condition which would rather enhance tails of I_{Ca}. The second possibility is excluded from examination of the currents and $[Ca^{2+}]_i$ transients on repolarization from a 300 ms pulse to +60 mV in Fig. 5*A*, and

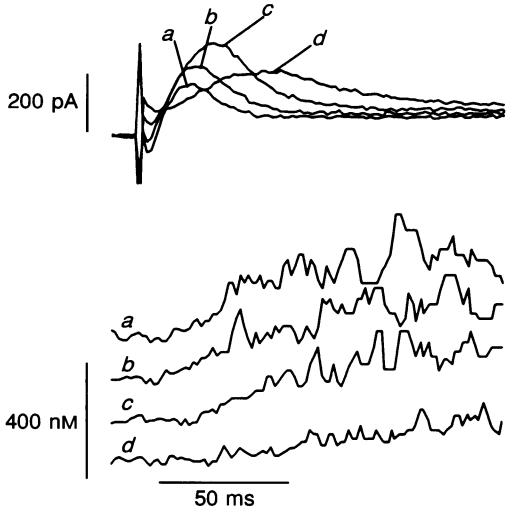

Fig. 13. Relation between time course of $I_{Cl(Ca)}$ and $[Ca^{2+}]_i$. Currents and $[Ca^{2+}]_i$ transients on expanded time scale, during 300 ms depolarizations from −45 to +20 mV (*a*), +30 (*b*), +40 (*c*) and +50 mV (*d*), offset of 150 nM between $[Ca^{2+}]_i$ traces. Standard solutions, 5·4 mM $[Ca^{2+}]_o$.

comparing results obtained in 1·8 and 5·4 mM $[Ca^{2+}]_o$. In 1·8 mM $[Ca^{2+}]_o$, a tail $[Ca^{2+}]_i$ transient on repolarization was accompanied by a transient inward current, as in the cell illustrated in Fig. 1*A*. The time course of this current does not follow the time course of $[Ca^{2+}]_i$, a first argument against $I_{Na/Ca}$. In 5·4 mM external calcium, $[Ca^{2+}]_i$ increased during the depolarizing step, was still elevated at the time of repolarization, but no further increase in $[Ca^{2+}]_i$ occurred on repolarization. Concomitantly, $I_{Cl(Ca)}$ was activated during depolarization and no tail current was seen. If this tail current had been $I_{Na/Ca}$ it would still have been present. We therefore interpret the current on repolarization in 1·8 mM $[Ca^{2+}]_o$ as being an inward current through $I_{Cl(Ca)}$.

Gating and kinetics of $I_{Cl(Ca)}$

In the first section we have established that $I_{Cl(Ca)}$ was strongly dependent on $[Ca^{2+}]_i$ for its activation. In the simplest model for this channel, it would be activated by an increase in $[Ca^{2+}]_i$ and deactivated by a decrease in $[Ca^{2+}]_i$. Membrane potential may modulate this $[Ca^{2+}]_i$-dependent gating. These hypotheses will be examined in the next paragraphs.

Time course of $I_{Cl(Ca)}$. Inspection of the time course of $I_{Cl(Ca)}$ *versus* the time course of the $[Ca^{2+}]_i$ transient indicated that the current declined early, before $[Ca^{2+}]_i$ reached its peak value.

This is illustrated in Fig. 13, showing on an expanded time scale, the time course of the membrane currents and the time course of the $[Ca^{2+}]_i$ transient for depolarizing steps to +20, +30, +40, and +50 mV (labelled *a*, *b*, *c* and *d*, respectively). Although $I_{Cl(Ca)}$ overlaps with the inactivating, inward I_{Ca}, the decline of the total outward

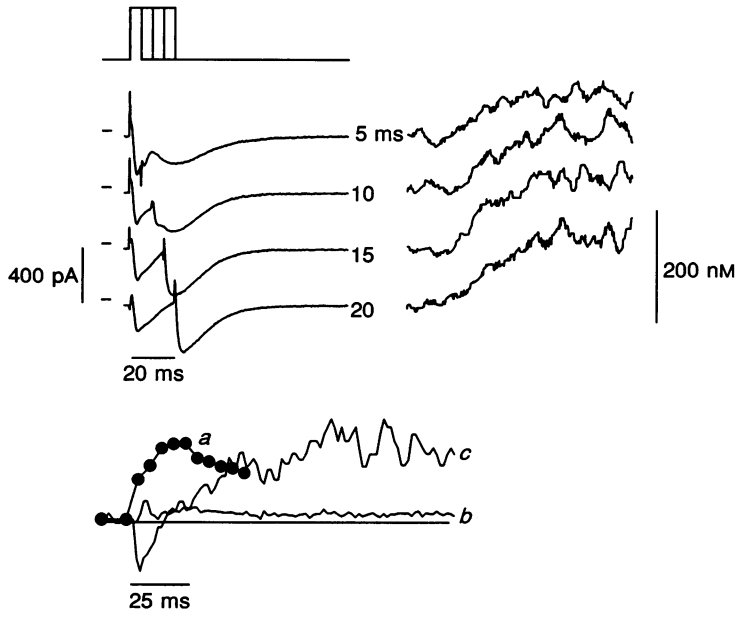

Fig. 14. Time course of activation of $I_{Cl(Ca)}$ during a depolarizing step from −45 to +10 mV. Standard solutions, 1·8 mM $[Ca^{2+}]_o$. *A*, currents and $[Ca^{2+}]_i$ transients, when the membrane is repolarized after 5, 10, 15 or 20 ms of depolarization, as indicated in the figure. *B*, plot on the same time scale of (*a*) amplitude of tail currents from *A*, as a function of duration of depolarization (●; this amplitude was measured as the difference between the current 3 ms after repolarization and steady-state current, the sign was inverted as the current is outward during depolarization), (*b*) total membrane current and (*c*) $[Ca^{2+}]_i$ transient during 300 ms depolarization. Vertical scale is arbitrary; the continuous line indicates the baseline value of $[Ca^{2+}]_i$ and membrane current.

current, can only be explained by decline of $I_{Cl(Ca)}$. It is clear that $I_{Cl(Ca)}$ was declining before $[Ca^{2+}]_i$ reached its peak.

We further explored the time course of activation and inactivation of $I_{Cl(Ca)}$ during a depolarizing pulse by applying a conditioning pulse of variable duration, from −45 to +10 mV, and examining the inward currents on repolarization to the holding potential (Fig. 14*A*). As discussed above, these currents may be less contaminated by I_{Ca}. On repolarization from a 5 ms pulse, two inward currents were seen: a small and very brief one, probably related to I_{Ca}, and a slower component, related to $I_{Cl(Ca)}$. $I_{Cl(Ca)}$ was not yet fully activated on repolarization and reached its peak at 20 ms (from the onset of depolarization, see traces at 5, 10 and 15 ms in Fig. 14*A*). For depolarizing steps of 20 and 25 ms, the amplitude of the inward $I_{Cl(Ca)}$ was maximal; the amplitude declined with longer depolarizations. In Fig. 14*B* we have plotted the amplitude of the tail current, 3 ms after repolarization from +10 mV,

versus duration of depolarization and on the same time scale, the time course of the total membrane current and of the $[Ca^{2+}]_i$ transient during an undisturbed depolarizing pulse (vertical scale is arbitrary). This plot shows that $I_{Cl(Ca)}$ is activated early during the rise of $[Ca^{2+}]_i$, reaches its peak at 20–25 ms and is declining before the peak of $[Ca^{2+}]_i$ (here 98 ms).

It is worth noting that the peak amplitude of the inward $I_{Cl(Ca)}$, occurring at 20 ms after onset of depolarization, was smaller for conditioning pulses of 5, 10 and 15 ms. This is probably related to the fact that these brief pulses were typically accompanied by an abbreviation of the $[Ca^{2+}]_i$ transient (Beuckelmann & Wier, 1988; Callewaert *et al.* 1988; Lederer, Cannell, Cohen & Berlin, 1989), which may lead to a lesser degree of $[Ca^{2+}]_i$-dependent activation. This does not affect, however, the interpretation of the time course of $I_{Cl(Ca)}$.

These observations on the relation between the time course of $I_{Cl(Ca)}$ and the time course of $[Ca^{2+}]_i$ suggest that an inactivation mechanism may be responsible for the early decline of the current. The possibility that $[Ca^{2+}]_i$ at the channel is different from what we are measuring with indo-1, will be considered in the Discussion.

We next examined whether the rate of decline of $I_{Cl(Ca)}$ was voltage dependent. We return to the experiment presented in Fig. 12. With a conditioning pulse of 25 ms to +30 mV we fully activated $I_{Cl(Ca)}$ and then stepped to various potentials without affecting the $[Ca^{2+}]_i$ transient. Using this approach we do not affect the $[Ca^{2+}]_i$-dependent activation of $I_{Cl(Ca)}$ and we can study the decline of $I_{Cl(Ca)}$ at various potentials. Differences in rate of decline must then result from voltage-dependent processes. In Fig. 12*C* we have plotted the decline of $I_{Cl(Ca)}$ during the test potential on a logarithmic scale. For the steps to positive potentials, time constants were 20, 24, 24 and 20 ms for +50, +40, +30 and +20 mV respectively. For the negative potentials, decay was slightly faster with time constants of 13, 13, 13 and 16 ms for −20, −30, −40 and −50 mV respectively (R^2 for all fits was between 0·89 and 0·99). These results do not indicate a major contribution of voltage to inactivation.

Voltage dependence of activation of $I_{Cl(Ca)}$. From the experiment illustrated in Fig. 7, it is apparent that the voltage dependence of $I_{Cl(Ca)}$ during a depolarizing step follows the voltage dependence of the $[Ca^{2+}]_i$ transient.

We next examined whether membrane potential could modulate the Ca^{2+}-dependent activation of $I_{Cl(Ca)}$. To test rigorously for pure voltage dependence of activation, one has to design an experiment where one can activate $I_{Cl(Ca)}$ at different potentials with the same $[Ca^{2+}]_i$-dependent degree of activation.

We therefore attempted to activate $I_{Cl(Ca)}$ by inducing $[Ca^{2+}]_i$ transients by fast application of caffeine, 10 mM, at various steady-state membrane potentials. We eliminated the current through the Na^+–Ca^{2+} exchanger by substituting Na^+, in the internal solution with Cs^+, in the external solution with Li^+ or TEA^+. Fast caffeine pulses, applied for 100 ms at the holding potential indicated in Fig. 15*A*, induced large $[Ca^{2+}]_i$ transients, accompanied by a transient current, reversing slightly below 0 mV, as predicted from the E_{Cl}, which was −5 mV. This current inactivated early, similar to $I_{Cl(Ca)}$ during a depolarizing pulse. We can exclude the possibility that this is a current through the Ca^{2+}-activated non-specific cation channel, I_{ns}. First, I_{ns} would follow closely the time course of the $[Ca^{2+}]_i$ transient,

as this channel does not show inactivation (Ehara, Noma & Ono, 1988). Second, with TEA^+ as Na^+ substitute, the current still reversed at 0 mV, while E_{ns} under these conditions is $-\infty$.

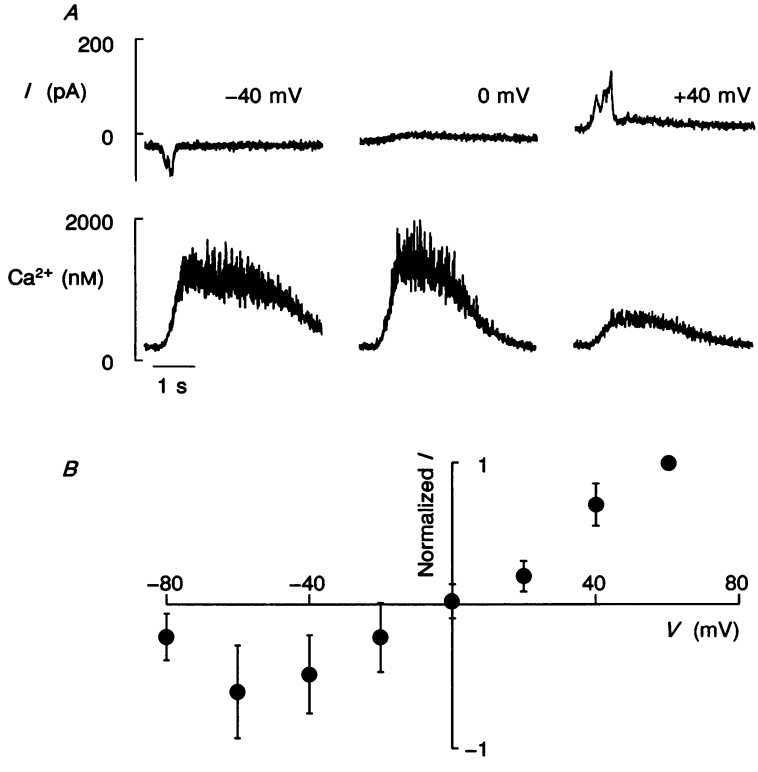

Fig. 15. Activation of Ca^{2+} release from the SR and of $I_{Cl(Ca)}$ by fast application of 10 mM caffeine (during 100 ms at the beginning of the records) in Na^+-free solutions. *A*, currents and $[Ca^{2+}]_i$ transients induced by caffeine application at a steady membrane potential as indicated in the figure (NaCl in the external solution replaced by LiCl). *B*, plot of normalized peak current *versus* holding potential. Data from 6 cells were pooled, each point represents the mean ± S.D. of 3–6 measurements (external NaCl replaced with TEACl in 5 cells, with LiCl in 1 cell, of which the original traces are shown in *A*.

The amplitude of the caffeine-induced $[Ca^{2+}]_i$ transients could vary during the course of an experiment, as in the example of Fig. 15*A*, but the variation was not related to a particular membrane potential. Therefore, in Fig. 15*B* pooled data from six cells are plotted, as the amplitude of the normalized peak current *versus* the membrane potential. From these results we can conclude that $I_{Cl(Ca)}$ can be activated by Ca^{2+} at all potentials, and does not require Ca^{2+} influx through the Ca^{2+} channel. At negative membrane potentials, however, the amplitude of $I_{Cl(Ca)}$ decreases despite comparable $[Ca^{2+}]_i$-dependent activation. In three other cells, in addition to the transient current, a second, much slower, component of Ca^{2+}-activated current was observed. The nature of this latter current is still under investigation.

DISCUSSION

The discussion will focus on three points, i.e. characteristics of the $[Ca^{2+}]_i$ transients, nature of the transient outward current and its physiological role, and kinetics of this current, to which we will refer as $I_{Cl(Ca)}$. Methodological problems and caveats for interpretation have been discussed in the Results section and will not be treated again.

$[Ca^{2+}]_i$ transients in single Purkinje cells from rabbit heart

One of the aims of this study was to examine whether excitation–contraction coupling in single rabbit cardiac Purkinje cells is different from what is known for single ventricular cells, and whether we could identify in a single cell the two components in the $[Ca^{2+}]_i$ transients as described for multicellular canine Purkinje fibres (Wier, 1980; Wier & Isenberg, 1982; Marban & Wier, 1985).

The $[Ca^{2+}]_i$ transient observed with fura-2 in single rabbit Purkinje cells had only one component. The absence of two components may be a species difference or it may be related to the specific experimental conditions. In dog Purkinje fibres the existence of two components was most clearly revealed by comparing different inotropic conditions. In the rabbit Purkinje cell, neither lowering the frequency of stimulation, nor increasing $[Ca^{2+}]_o$ changed the time course of $[Ca^{2+}]_i$ but other inotropic interventions, such as application of digitalis, have not been tested yet. We were unable to examine frequency effects in depth, as a stable patch could only be maintained for a limited time (usually less than 15 min). The differences may also be related to the use of a different Ca^{2+} indicator. It is tempting to speculate that the first component detected by aequorin may result from a more localized increase in cytoplasmic $[Ca^{2+}]$, related to Ca^{2+} release from the subsarcolemmal SR compartment (Marban & Wier, 1985). Because of the non-linear properties of aequorin, this increase might be detected by aequorin, but not by fura-2.

The $[Ca^{2+}]_i$ transient appeared to be due to triggered release of calcium from the sarcoplasmic reticulum. Compared to ventricular cells, transients were slower and of somewhat lower amplitude. Time to peak was about twice as long as described for guinea-pig or rat ventricular cells (Cannell, Berlin & Lederer, 1987; Beuckelmann & Wier, 1988; Cleemann & Morad, 1991). This was not inherent to the set-up or the dye concentration. Time to peak for guinea-pig ventricular cells under exactly the same conditions was between 30 and 50 ms ($n = 3$). We also examined the time to peak on a different set-up, using 100 μM fura-2. Again time to peak of Purkinje cells was between 60 and 100 ms, while it was between 30 and 50 ms for guinea-pig ventricular myocytes. The slow rise in $[Ca^{2+}]_i$ may be related to the absence of an extensive T-tubular system, delaying signal transduction (Sommer & Jennings, 1986). It cannot be excluded that these rabbit Purkinje cells also have two SR compartments, and that the slower time to peak reflects the time it takes to activate release from the more centrally located compartment, as was postulated for canine Purkinje fibres (Marban & Wier, 1985). The absence of T-tubules will also lead to a smaller ratio of sarcolemmal surface area to cytoplasmic

volume, compared to ventricular cells. This could be associated with a smaller Ca^{2+} influx to trigger Ca^{2+} release. A possible indication that I_{Ca} may be insufficient to trigger a large calcium release comes from experiments where we induced $[Ca^{2+}]_i$ transients by fast application of caffeine. The amplitude of these transients reached between 800 nM and 1·2 μM, usually two to four times larger than the maximal $[Ca^{2+}]_i$ observed during depolarization in the same cell ($n = 5$). This is in contrast to observations in rat or guinea-pig ventricular cells, where the caffeine-induced $[Ca^{2+}]_i$ transient was only slightly larger than the depolarization-induced $[Ca^{2+}]_i$ transient (Callewaert *et al.* 1989)

Influx through the Na^+–Ca^{2+} exchanger probably contributes little to the increase in cytoplasmic $[Ca^{2+}]$ during a depolarizing pulse. In the absence of a triggered calcium release, very long depolarizing pulses to potentials above +60 mV were needed to significantly increase $[Ca^{2+}]_i$. Modulation of calcium release by influx through the Na^+–Ca^{2+} exchanger and changes in Ca^{2+} in a limited space has been suggested for ventricular cells (Leblanc & Hume, 1990), but was not examined in this study. Efflux through the Na^+–Ca^{2+} exchanger contributes to decline of the $[Ca^{2+}]_i$ transient, as this rate of decline was increased at more negative potentials. Transient inward currents on repolarization from standard depolarizing pulses, were small, i.e. less than 25 pA at −45 mV. In contrast to the small transient inward currents on repolarization from a depolarizing pulse, in rabbit Purkinje cells large transient inward currents were seen during caffeine-induced $[Ca^{2+}]_i$ transients (more than 500 pA, at −45 mV). In the presence of caffeine, sequestration of calcium by the SR is inhibited, which would induce a larger fraction of Ca^{2+} to be extruded by the Na^+–Ca^{2+} exchanger. Another possibility is that this transient inward current is partly due to the Ca^{2+}-activated non-specific cation channel (Cannell & Lederer, 1986). In excised patches, K_m (the Michaelis–Menten constant) for $[Ca^{2+}]$ is 1·2 μM (Ehara *et al.* 1988). It is therefore likely that it is activated during these large $[Ca^{2+}]_i$ transients. A third possibility is a contribution of the Ca^{2+}-activated Cl^- current, $I_{Cl(Ca)}$, to this inward current.

A $[Ca^{2+}]_i$-dependent transient outward current, in rabbit heart Purkinje cells, carried by chloride

We have identified a transient outward current in single rabbit Purkinje cells, that is activated and graded by the $[Ca^{2+}]_i$ transient. We conclude that it is carried by chloride, based on its sensitivity to chloride substitutions and to the anion blocker DIDS, and on the observation of a reversal around 0 mV in symmetrical chloride solutions. Therefore this current can be referred to as $I_{Cl(Ca)}$. We will discuss the relation of this current to other transient outward currents, first in Purkinje fibres, and then in ventricular and atrial cells.

$I_{Cl(Ca)}$ may be related to the Ca^{2+}-activated 'brief' outward current, I_{bo}, described in sheep Purkinje fibres (Carmeliet & Coraboeuf, 1982), that is also sensitive to caffeine, and to the Ca^{2+}-activated transient outward current in calf Purkinje fibres (Siegelbaum & Tsien, 1980). Sheep Purkinje fibres also have a second component of outward current, I_{lo}, sensitive to 4-aminopyridine, and carried by K^+. Calf Purkinje fibres do not have a 4-aminopyridine-sensitive current. The early decline of the transient outward current in calf Purkinje fibres, as compared to the time course of contraction, strongly resembles the early decline of $I_{Cl(Ca)}$ in rabbit

Purkinje cells, compared to the time course of the $[Ca^{2+}]_i$ transient. In calf Purkinje fibres, the Ca^{2+}-activated transient outward current may also contain a component of Ca^{2+}-activated K^+ current, as observed in single channel recordings (Callewaert, Vereecke & Carmeliet, 1986).

In rat ventricular myocytes, the transient outward current has only one component, a 4-aminopyridine-sensitive K^+-current. Like I_{to} in sheep Purkinje fibres, this current is apparently not sensitive to $[Ca^{2+}]_i$. It is inactivated at more negative potentials, with a 75 % inactivation at −50 mV (Dukes & Morad, 1991). This current has no relation to $I_{Cl(Ca)}$ described in the present study.

Zygmunt and Gibbons have recently described a transient outward current carried by chloride and activated by $[Ca^{2+}]_i$ in rabbit ventricular and atrial cells (Zygmunt & Gibbons, 1991, 1992). They measured this current as the DIDS-sensitive current. In Purkinje cells, $I_{Cl(Ca)}$ could not be blocked as efficiently. In ventricular cells, the authors did not see any current at potentials below 0 mV, and could not identify inward currents, suggesting the current was outwardly rectifying (Zygmunt & Gibbons, 1991), while in atrial cells the current did not show rectification (Zygmunt & Gibbons, 1992). In rabbit Purkinje cells, we could clearly identify $I_{Cl(Ca)}$ as an outward current at potentials as low as −20 mV by lowering E_{Cl}, a clear distinction with ventricular cells. We also could identify inward currents, distinct from the findings in ventricular cells, but resembling $I_{Cl(Ca)}$ in atrial cells. At more negative potentials, though, the chord conductance of $I_{Cl(Ca)}$ activated by caffeine-induced Ca^{2+} release from the SR, decreased, an observation that may be related to a decrease in Ca^{2+} binding to the activation site.

$I_{Cl(Ca)}$ has been commonly referred to as a transient outward current. Is it a true outward current? Under physiological conditions (37 °C, 25 mM $[Cl^-]_i$ and 114 mM $[Cl^-]_o$), E_{Cl} is −40 mV. This means that during phase 1 of the action potential, when the current is activated by the accompanying $[Ca^{2+}]_i$ transient, currents will be outward indeed. Because of its transient nature, $I_{Cl(Ca)}$ will induce early repolarization during phase 1 of the action potential. Since $I_{Cl(Ca)}$ will increase with increases in amplitude of the $[Ca^{2+}]_i$ transient, this current will contribute to modulation of the action potential during inotropic interventions. However, its effect on total duration of the action potential cannot be easily predicted, as more pronounced repolarization would affect all currents during the plateau phase. Under pathological conditions, one can speculate that inward $I_{Cl(Ca)}$ may be activated during Ca^{2+} oscillations at more negative potentials and thus may contribute to triggered depolarizations and the initiation of arrhythmias.

Is $I_{Cl(Ca)}$ a simple ligand-operated channel?

In the present study we could clearly establish that (1) $I_{Cl(Ca)}$ in single rabbit Purkinje cells was activated and graded by $[Ca^{2+}]_i$ and (2) $I_{Cl(Ca)}$ was activated early during the rising phase of a $[Ca^{2+}]_i$ transient, but declined long before $[Ca^{2+}]_i$ declined. Membrane potential apparently has little role in either activation or rate of decline of $I_{Cl(Ca)}$. In the following paragraphs we will first discuss some characteristics of the Ca^{2+}-dependent activation and next examine several hypotheses to explain the discrepancy in the time course of $I_{Cl(Ca)}$ and of $[Ca^{2+}]_i$.

The experiments with caffeine and ryanodine indicate that Ca^{2+} influx through the Ca^{2+} channel alone is insufficient to activate $I_{Cl(Ca)}$, and that Ca^{2+} release from

the SR is required. While the peak amplitude of $[Ca^{2+}]_i$ may be a measure of the amount of Ca^{2+} released from the SR, the rate of rise of $[Ca^{2+}]_i$ may be a more appropriate indicator of the flux of Ca^{2+} release from the SR (Sipido & Wier, 1991). We have confirmed that in all conditions where $I_{Cl(Ca)}$ was increased (i.e. increase in

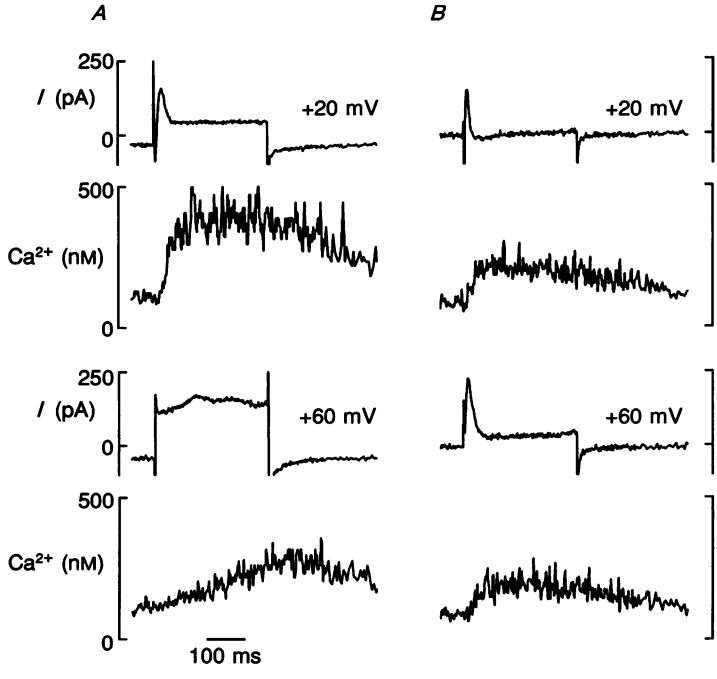

Fig. 16. $I_{Cl(Ca)}$ during slow and fast $[Ca^{2+}]_i$ transients. *A*, current and $[Ca^{2+}]_i$ transient during a 300 ms depolarizing step from −45 mV to the indicated potentials in standard solution, 5·4 mM $[Ca^{2+}]_o$; pipette contained 142 mM Cl^-. *B*, current and $[Ca^{2+}]_i$ transient during similar depolarizing steps, but now the pipette contained 20 mM Cl^-. The external solution was the same as in *A*, but the records are from a different cell.

external $[Ca^{2+}]$, increase in frequency of stimulation), the rate of rise of $[Ca^{2+}]_i$ was also increased, indicating an increase in the flux of Ca^{2+} release from the SR.

When comparing the time course of $I_{Cl(Ca)}$ with the time course of $[Ca^{2+}]_i$, we have to consider the possibility that $[Ca^{2+}]_i$ at the channel may be different from the bulk cytoplasmic concentration as measured with indo-1. This has already been suggested by other groups who observed discrepancies between the behaviour of the Na^+–Ca^{2+} exchanger, a known Ca^{2+}-activated current, and cytoplasmic $[Ca^{2+}]_i$ (Lipp *et al.* 1990). A similar subsarcolemmal gradient has been suggested for Na^+ (Wendt-Gallitelli & Isenberg, 1990; Leblanc & Hume, 1990; Bielen, Glitsch & Verdonck, 1991; Carmeliet, 1992). In vascular smooth muscle, recent experiments have demonstrated that $[Ca^{2+}]_i$ underneath the sarcolemma can be quite different from the bulk cytoplasmic concentration (Stehno-Bittel & Sturek, 1992). Such gradients in smooth muscle have been ascribed to the existence of a 'superficial barrier', consisting of the superficially located SR compartment (van Breemen &

Saida, 1989; Stehno-Bittel & Sturek, 1992). The existence of a subsarcolemmal gradient in Purkinje cells could explain the discrepancy in the quantitative relation between $I_{Cl(Ca)}$ and $[Ca^{2+}]_i$.

The existence of this mechanism in Purkinje cells, however, remains hypothetical. Therefore we also have to consider the possibility that the early decline of $I_{Cl(Ca)}$ is due to an inactivation mechanism, in the presence of elevated $[Ca^{2+}]_i$. One might propose hypotheses that have been formulated for other Ca^{2+}-activated channels. In the case of the large Ca^{2+}-activated K^+ (BK) channels of rat skeletal muscle, slow inactivation has been associated with block of the channel by Ca^{2+} entry into the pore (Barrett, Magleby & Pallotta, 1982; Vergara & Latorre, 1983; Pallotta, 1985). For BK channels of chromaffin cells, a rapid and trypsin-sensitive inactivation mechanism has recently been described (Solaro & Lingle, 1992).

The existence of a Ca^{2+}-dependent inactivation mechanism was proposed by Fabiato for the Ca^{2+} release channel from the SR, to explain his observations that (1) the release process was activated by Ca^{2+}, but apparently shut down early despite the presence of an elevated $[Ca^{2+}]_i$ and (2) that activation of Ca^{2+} release was dependent on the rate of change of Ca^{2+} at the channel (Fabiato, 1985). Fabiato specified that the binding site responsible for activation, would have a high affinity for Ca^{2+}, while the one responsible for inactivation would have low affinity. According to this hypothesis, the channel would become inactivated at high $[Ca^{2+}]_i$, and slow changes in $[Ca^{2+}]_i$ would simultaneously activate and inactivate the channel. Therefore the current would be smaller during slow changes in $[Ca^{2+}]_i$. Although Fabiato could demonstrate the existence of such a mechanism in a skinned cardiac cell, his hypothesis remains unproven in intact cardiac cells. We have applied it as a working hypothesis to explain the following observations on $I_{Cl(Ca)}$ in Purkinje cells. During depolarization to potentials of +40 to +60 mV, $[Ca^{2+}]_i$ transients in standard solutions were slow, and concomitantly, $I_{Cl(Ca)}$ became smaller, although eventually $[Ca^{2+}]_i$ increased significantly (compare trace *c* and *d* in Fig. 13). In contrast, for reasons as yet unclear, $[Ca^{2+}]_i$ transients in low $[Cl^-]_i$ tended to remain fast even at more positive potentials. Under those conditions, $I_{Cl(Ca)}$ could still be clearly observed during a pulse to +60 mV. In Fig. 16, we have plotted for comparison, currents and $[Ca^{2+}]_i$ transients under standard conditions (*A*) and low $[Cl^-]_i$ (*B*). $I_{Cl(Ca)}$ is evident during the fast, but not during the slow $[Ca^{2+}]_i$ transient. These observations could be explained by the existence of a Ca^{2+}-dependent inactivation.

The fast decline of the current could theoretically also be explained by desensitization, which could occur at any point along the chain of events between Ca^{2+} binding and channel opening.

In conclusion, the Ca^{2+}-dependent transient outward current of rabbit cardiac Purkinje cells, is a chloride current that is activated during the $[Ca^{2+}]_i$ transient. Its transient nature could be due to early inactivation, by an as yet unknown mechanism, before the decline of $[Ca^{2+}]_i$, or could reflect the existence of important differences between bulk cytoplasmic $[Ca^{2+}]$ and subsarcolemmal $[Ca^{2+}]$.

The authors acknowledge the expert technical assistance of Jean Prenen with the cell isolation. We thank Professor J. Vereecke and Professor F. Verdonck for their helpful comments.

REFERENCES

BARRETT, J. N., MAGLEBY, K. L. & PALLOTTA, B. S. (1982). Properties of single calcium-activated potassium channels in cultured rat muscle. *Journal of Physiology* **331**, 211–230.

BERS, D. M. & BRIDGE, J. H. B. (1989). Relaxation of rabbit ventricular muscle by Na–Ca exchange and sarcoplasmic-reticulum calcium-pump – ryanodine and voltage sensitivity. *Circulation Research* **65**, 334–342.

BEUCKELMANN, D. J. & WIER, W. G. (1988). Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *Journal of Physiology* **405**, 233–255.

BEUCKELMANN, D. J. & WIER, W. G. (1989). Sodium–calcium exchange in guinea-pig cardiac cells: exchange current and changes in intracellular Ca^{2+}. *Journal of Physiology* **414**, 499–520.

BIELEN, F. V., GLITSCH, H. G. & VERDONCK, F. (1991). Changes of the subsarcolemmal Na^+ concentration in internally perfused cardiac cells. *Biochimica et Biophysica Acta* **1065**, 269–271.

BRIDGE, J. H. B., SPITZER, K. W. & ERSHLER, P. R. (1988). Relaxation of isolated ventricular myocytes by a voltage-dependent process. *Science* **241**, 823–825.

CALLEWAERT, G., CLEEMANN, L. & MORAD, M. (1988). Epinephrine enhances Ca^{2+}-current regulated Ca^{2+} release and Ca^{2+} reuptake in rat ventricular myocytes. *Proceedings of the National Academy of Sciences of the USA* **85**, 2009–2013.

CALLEWAERT, G., CLEEMANN, L. & MORAD, M. (1989). Caffeine-induced Ca^{2+} release activates Ca^{2+} extrusion via Na^+–Ca^{2+} exchanger in cardiac myocytes. *American Journal of Physiology* **257**, C147–152.

CALLEWAERT, G., LIPP, P., POTT, L. & CARMELIET, E. (1991). High-resolution measurement and calibration of Ca^{2+}-transients using indo-1 in guinea-pig atrial myocytes under voltage clamp. *Cell Calcium* **12**, 269–277.

CALLEWAERT, G., VEREECKE, J. & CARMELIET, E. (1986). Existence of a calcium-dependent potassium channel in the membrane of cow cardiac Purkinje cells. *Pflügers Archiv* **406**, 424–426.

CANNELL, M. B., BERLIN, J. R. & LEDERER, W. J. (1987). Effect of membrane potential changes on the calcium transient of single rat cardiac muscle cells. *Science* **238**, 1419–1423.

CANNELL, M. B. & LEDERER, W. J. (1986). The arrhythmogenic current I_{TI} in the absence of electrogenic sodium–calcium exchange in sheep cardiac Purkinje fibres. *Journal of Physiology* **374**, 201–219.

CARMELIET, E. (1992). A fuzzy subsarcolemmal space for intracellular Na^+ in cardiac cells? *Cardiovascular Research* **26**, 433–442.

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

CLEEMANN, L. & MORAD, M. (1991). Role of Ca^{2+} channel in cardiac excitation–contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *Journal of Physiology* **432**, 283–312.

DUKES, I. D. & MORAD, M. (1991). The transient K^+ current in rat ventricular myocytes: evaluation of its Ca^{2+} and Na^+ dependence. *Journal of Physiology* **435**, 395–420.

EHARA, T., NOMA, A. & ONO, K. (1988). Calcium-activated non-selective cation channel in ventricular cells isolated from adult guinea-pig hearts. *Journal of Physiology* **403**, 117–133.

FABIATO, A. (1985). Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology* **85**, 247–289.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence. *Journal of Biological Chemistry* **260**, 3440–3450.

LEBLANC, N. & HUME, J. R. (1990). Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science* **248**, 372–376.

LEDERER, W. J., CANNELL, M. B., COHEN, N. M. & BERLIN, J. R. (1989). Excitation–contraction coupling in heart muscle. *Molecular and Cellular Biochemistry* **89**, 115–119.

LIPP, P., POTT, L., CALLEWAERT, G. & CARMELIET, E. (1990). Simultaneous recording of Indo-1 fluorescence and Na^+/Ca^{2+} exchange current reveals two components of Ca^{2+}-release from sarcoplasmic reticulum of cardiac atrial myocytes. *FEBS Letters* **275**, 181–184.

MARBAN, E. & WIER, W. G. (1985). Ryanodine as a tool to determine the contributions of calcium entry and calcium release to the calcium transient and contraction of cardiac Purkinje fibers. *Circulation Research* **56**, 133–138.

MARTY, A. & NEHER, E. (1983). Tight-seal whole-cell recording. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 107–122. Plenum Press, New York.

PALLOTTA, B. S. (1985). Calcium-activated potassium channels in rat muscle inactivate from a short-duration open state. *Journal of Physiology* **363**, 501–516.

SCAMPS, F. & CARMELIET, E. (1989). Delayed K^+ current and external K^+ in single cardiac Purkinje cells. *American Journal of Physiology* **257**, 1086–1092.

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

SIPIDO, K. R. & WIER, W. G. (1991). Flux of Ca^{2+} across the sarcoplasmic reticulum of guinea-pig cardiac cells during excitation–contraction coupling. *Journal of Physiology* **435**, 605–630.

SIPIDO, K. R., CALLEWAERT, G., VEREECKE, J. & CARMELIET, E. (1992*a*). $[Ca^{2+}]_i$ transients in isolated Purkinje cells from rabbit heart. *Journal of Physiology* **446**, 332*P*.

SIPIDO, K. R., CALLEWAERT, G., VEREECKE, J. & CARMELIET, E. (1992*b*). $[Ca^{2+}]_i$-dependence of a transient outward current in isolated rabbit Purkinje cells. *FASEB Journal* **6**, A509.

SOLARO, C. R. & LINGLE, C. J. (1992). Trypsin-sensitive, rapid inactivation of a calcium-activated potassium channel. *Science* **257**, 1694–1698.

SOMMER, B. & JENNINGS, R. B. (1986). Ultrastructure of cardiac muscle. In *The Heart and Cardiovascular System*, ed. FOZZARD, H. A., pp. 61–100. Raven Press, New York.

STEHNO-BITTEL, L. & STUREK, M. (1992). Spontaneous sarcoplasmic reticulum calcium release and extrusion from bovine, not porcine, coronary artery smooth muscle. *Journal of Physiology* **451**, 49–78.

VAN BREEMEN, C. & SAIDA, K. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ in smooth muscle. *Annual Review of Physiology* **51**, 315–329.

VERGARA, C. & LATORRE, R. (1983). Kinetics of Ca^{2+}-activated K^+ channels from rabbit skeletal muscle incorporated into planar lipid bilayers: evidence for a Ca^{2+} and Ba^{2+} blockade. *Journal of General Physiology* **82**, 543–568.

WENDT-GALLITELLI, M. F. & ISENBERG, G. (1990). X-ray microprobe analysis of sodium concentration reveals large transverse gradients from the sarcolemma to the centre of voltage-clamped guinea-pig ventricular myocytes. *Journal of Physiology* **420**, 86*P*.

WIER, W. G. (1980). Calcium transients during excitation–contraction coupling in mammalian heart: aequorin signals of canine Purkinje fibers. *Science* **207**, 1085–1087.

WIER, W. G. & ISENBERG, G. (1982). Intracellular $[Ca^{2+}]$-transients in voltage clamped cardiac Purkinje fibers. *Pflügers Archiv* **392**, 284–290.

ZYGMUNT, A. C. & GIBBONS, W. R. (1991). Calcium-activated chloride current in rabbit ventricular myocytes. *Circulation Research* **68**, 424–437.

ZYGMUNT, A. C. & GIBBONS, W. R. (1992). Properties of the calcium-activated chloride current in the heart. *Journal of General Physiology* **99**, 391–414.