Two types of action potential configuration in single cardiac Purkinje cells of sheep

ARIE O. VERKERK, MARIEKE W. VELDKAMP, FABIO ABBATE, GUDRUN ANTOONS, LENNART N. BOUMAN, JAN H. RAVESLOOT, AND ANTONI C. G. VAN GINNEKEN

Department of Physiology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands

Verkerk, Arie O., Marieke W. Veldkamp, Fabio Abbanate, Gudrun Antoons, Lennart N. Bouman, Jan H. Ravesloot, and Antoni C. G. van Ginneken. Two types of action potential configuration in single cardiac Purkinje cells of sheep. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1299–H1310, 1999.—Membrane potentials and currents of isolated sheep Purkinje and ventricular cells were compared using patch-clamp and microelectrode techniques. In ~50% of Purkinje cells, we observed action potentials that showed a prominent phase 1 repolarization and relatively negative plateau (LP cells). Action potential configuration of the remaining Purkinje cells was characterized by little phase 1 repolarization and relatively positive plateau (HP cells). Microelectrode impalement of Purkinje strands also revealed these two types of action potential configuration. In LP cells, the density of L-type Ca2+ current (I_{Ca,L}) was lower, whereas the density of transient outward K+ current was higher, than in HP cells. Action potentials of HP cells strongly resembled those of ventricular cells. Densities of inward rectifier current and I_{Ca,L} were significantly higher in ventricular cells compared with densities in both LP and HP Purkinje cells. Differences in current densities explain the striking differences in action potential configuration and the stimulus frequency dependency thereof that we observed in LP, HP, and ventricular cells. We conclude that LP Purkinje cells, HP Purkinje cells, and ventricular cells of sheep each have a unique action potential configuration.

Electrical properties of Purkinje cells have been mostly inferred from current-clamp studies on multicellular Purkinje strand preparations. These studies have been instrumental in our understanding of Purkinje cell electrophysiology (for reviews, see Refs. 38 and 46). However, working with multicellular preparations has several inherent drawbacks. Interpretation of voltage-clamp results is often hindered by the accumulation or depletion of ions in the extracellular space, the size of the preparation, and the extent of the cell coupling (5, 17, 36). Most of these problems can be avoided by using a single-cell preparation. Indeed, in the past decade interest in single Purkinje cell electrophysiology has grown, but the amount of information obtained from single Purkinje cell experiments still contrasts sharply with the large body of data available on the electrophysiological properties of single atrial, ventricular, or sinoatrial cells. This is explained by the laborious and painstaking isolation procedure. A thick collagen sheet that surrounds the Purkinje strands severely hampers the isolation of single cells.

Sheep Purkinje strands often have been used for electrophysiological measurements, and quite a bit of data are available on the current-clamp properties of this preparation (for reviews, see Refs. 38 and 46 and the primary references therein). Because of their abundance, size, and color, Purkinje strands in the heart in this species can be recognized with relative ease, yet sheep Purkinje strands are seldom used for single-cell experiments (5). Therefore, single Purkinje cells of the sheep have not been subjected to a systematic electrophysiological investigation. In this paper we address this issue. The aim of our study was to characterize action potentials and the principal transmembrane currents in single Purkinje cells of the sheep and to compare these with those of sheep ventricular cells. We modified the method of Glitsch et al. (18) to obtain single Purkinje cells and applied patch-clamp methodology as well as microelectrode impalement techniques.

We have shown that sheep Purkinje cells are capable of producing two types of action potential configuration. The first type is characterized by a prominent phase 1 repolarization and a relatively negative plateau level. The second type resembles the action potential elicited in ventricular cells isolated from the same hearts. It shows little phase 1 repolarization and a relatively positive plateau level. We attribute the two varieties of Purkinje action potentials to differences in both L-type Ca2+ current (I_{Ca,L}) and transient outward K+ current (I_{to}). Portions of this work have been published in abstract form elsewhere (43, 44).

Materials and Methods

Cell Isolation Procedure

Isolation of cardiac Purkinje cells. Single cardiac Purkinje cells were isolated from sheep hearts by an enzymatic dissociation procedure modified from Glitsch et al. (18). Cells were isolated from a total of 21 hearts obtained from the slaughterhouse immediately after exsanguination of the animals and from 5 hearts obtained from animals that were anesthetized with intravenously injected Nesdonal (10 mg/kg thiopental sodium; Rhone-Merieux, Lyon, France). The hearts were transported to the laboratory in cold, oxygenated “normal” Tyrode solution (4°C). Large and medium-size free-running Purkinje strands, free from ventricular tissue, were excised from both ventricles and placed for 20 min in “Ca2+-free” Tyrode solution containing 1 mg/ml protease (220 U/l type XIV; Sigma, St. Louis, MO) and 1 mg/ml BSA (Behring, Marburg, Germany). Next, strands were placed for 20 min in Ca2+-free Tyrode solution containing 1 mM EGTA, followed by an additional 45–60 min in Ca2+-free Tyrode solution.
containing 0.2 mg/ml protease, 0.5 mg/ml collagenase (59 U/l type B; Boehringer Mannheim, Mannheim, Germany), 0.5 mg/ml BSA, and 0.2 mM EGTA. Subsequently, the Purkinje strands were cut into pieces ∼2 mm long. These pieces were agitated for 10–20 min by a magnetic stirring bar at 120–150 rpm in Kraft-Brühe (KB) solution (24) to obtain single Purkinje cells. The temperature of all solutions was maintained at 35–37°C. The KB solution containing single cells was placed in a disposable centrifuge tube in which the single cells were allowed to sediment. Next, the KB solution was replaced by normal Tyrode solution in three steps. In each step, ∼75% of the solution in the centrifuge tube was replaced by normal Tyrode solution (20–22°C). The interval between these steps was 15–20 min. The cells were stored at room temperature (20–22°C). In the minority of experiments, cells were stored at 4°C overnight in normal Tyrode solution and used the next day.

Isolation of ventricular cells. Single ventricular cells were isolated from hearts of anesthetized sheep. A part of the left ventricular wall was mounted on a Langendorff perfusion apparatus and perfused through a branch of the left anterior descending coronary artery with the following solutions: 1) normal Tyrode solution for 10 min, 2) Ca2+-free Tyrode solution for 10 min, and 3) Ca2+-free Tyrode solution with collagenase (59 U/l type B and 150 U/l type P; Boehringer Mannheim) and trypsin inhibitor (250 mg/l; Boehringer Mannheim) for 15–25 min. Parts of the ventricular wall that were visibly digested were cut into pieces and gently agitated in KB solution to obtain single cells. During the entire isolation procedure, all solutions were oxygenated and temperature was maintained at 35–37°C. The Ca2+ concentration was increased as described in isolation of cardiac Purkinje cells.

Determination of cell size. To estimate cell size, photographs were taken while the cells were exposed to normal Tyrode solution at 35–37°C. Cells were viewed and photographed under ×400 magnification.

Solutions and Drugs

Composition of solutions. The normal Tyrode solution contained (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 5.5 glucose, and 5.0 HEPES; pH was adjusted to 7.4 with NaOH. The Ca2+-free Tyrode solution contained (in mM) 140 NaCl, 5.4 KCl, 0.5 MgCl2, 1.2 KH2PO4, 5.5 glucose, and 5.0 HEPES; pH was adjusted to 7.2 with NaOH. The KB solution contained (in mM) 85 KCl, 30 KH2PO4, 5.0 MgSO4, 20 glucose, 5.0 pyruvic acid, 5.0 creatine, 5.0 taurine, 0.5 EGTA, 5.0 β-hydroxybutyric acid, 5.0 succinic acid, and 2.0 Na2ATP; pH was adjusted to 7.2 with KOH. The pipette solution used for both perforated patch-clamp and conventional whole cell recordings contained (in mM) 125 K-gluonate, 20 KCl, and 10 HEPES; pH was adjusted to 7.2 with KOH. The pipette solution used for the conventional microelectrode technique contained 3 M KCl.

Drugs. 4-Aminopyridine (4-AP; Sigma) was dissolved in normal Tyrode solution at a final concentration of 2 mM. DIDS (Sigma) and amphotericin B (Sigma) were prepared as 0.5 M and 52 mM stock solution in DMSO, respectively. The final DMSO concentration in the solutions was <0.3%.

Electrical Recordings

Recording procedure for Purkinje strands. Purkinje strands were mounted on a perforated silicon rubber block in a tissue bath (5 ml) and superfused with normal Tyrode solution at a rate of 5 ml/min. The temperature of the bathing solution was monitored continuously by a thermistor probe and was kept at 35–37°C. Resting membrane and action potentials of the Purkinje strands were intracellularly recorded by means of the conventional microelectrode technique. Electrodes were pulled from borosilicate glass with a glass fiber inside the lumen with the use of a laboratory-made two-stage puller. Electrodes were filled with 3 M KCl and had resistances of 20–30 MΩ.

Recording procedure for isolated cells. Small aliquots of cell suspension were put in a cell chamber on the stage of an inverted microscope (Nikon Diaphot). The cells were allowed to adhere for 5 min, after which continuous perfusion with normal Tyrode solution was started at a rate of 2–3 ml/min. The temperature of the bath was maintained at 35–37°C by the combination of an electrically regulated preheating system and a translucent heating plate underneath the bottom of the recording chamber (41). The temperature of the bathing solution was monitored continuously by a thermistor probe.

Membrane potentials and currents were recorded with a custom-made voltage-clamp amplifier with the use of either the conventional whole cell patch-clamp technique or the amphotericin perforated patch-clamp technique. Electrodes were pulled from borosilicate glass with a glass fiber inside the lumen with the use of a custom-made one-stage puller. For amphotericin perforated patch-clamp recordings, the very tip of the pipette was filled with amphotericin-free pipette solution. Subsequently, the electrodes were backfilled with pipette solution to which 0.2 mg/ml amphotericin B was added. For conventional whole cell recordings, electrodes were filled with amphotericin-free pipette solution. The electrodes had resistances of 3–5 MΩ. The potential between pipette and bath solution was adjusted to zero before a high-resistance seal between pipette and cell was formed. Pipette series resistance (R pip) was compensated for by ∼80% to minimize the duration of the capacitive surge in the voltage-clamp records. All potentials were corrected for the estimated 13-mV change in liquid junction potential that occurs when contact with the cells is made (2). In perforated-patch experiments, R pip rapidly decreased within 10 min after seal formation and remained stable for at least 1.5 h.

Membrane potentials and currents were filtered on-line (1 kHz), digitized by a 12-bit analog-to-digital converter (National Instruments NB-MIO-16) at a frequency of 2 kHz, and stored on the hard disk of an Apple Macintosh personal computer (Quadra 650). Data were analyzed by a custom-made data acquisition and analysis program.

Stimulation and Voltage-Clamp Protocols

Intact Purkinje strands were stimulated at a frequency of 1 Hz by a pair of Teflon-coated platinum wires placed at one end of the strand. In single Purkinje cells, action potentials were elicited at a rate between 3.33 and 0.1 Hz by current pulses of 2 ms applied via the patch pipette. The following action potential parameters were measured: action potential duration at 20 and 90% of repolarization (APD20 and APD90), resting membrane potential (V m), action potential amplitude (APA), and maximum upstroke velocity (dV/dt max). In isolated cells, cell capacitance (C m) was determined from the change in slope of the potential (ΔV m) caused by 10-ms hyper- and depolarizing pulses of 30 or 100 pA (ΔI m) applied during the plateau phase of the action potential. Cell capacitance was calculated as C m = ΔI m/ΔV m.

I CaL, the inward rectifier current (I k1), and the delayed rectifier current (I k2) were measured during 500-ms steps to voltages ranging from −113 mV to +37 mV in 10-mV increments at a frequency of 0.5 Hz. The holding potential was −53 mV to inactivate the Na+ current (I Na) and T-type Ca2+ current (I CaT). I CaL, I k1, and I k were measured in the


presence of 2 mM 4-AP to block \( I_{\text{Na}} \). \( I_{\text{Ca,L}} \) was defined as the difference between the peak inward current and the current amplitude at the end of the 500-ms voltage clamp step. \( I_{\text{K}} \) was defined as the current at the end of the 500-ms hyperpolarizing voltage steps. \( I_{\text{K}} \) was activated by depolarizing voltage steps at a frequency of 0.25 Hz from a holding potential of \(-93\) mV. It was measured as the 4-AP-sensitive current or the transient outward current that overshot the zero potential value. These action potentials and basal electrophysiological properties of our cell preparations are summarized in Table 1.

Consistent with the laborious isolation method for -tolerant Purkinje cells that were quiescent when bathed in normal Tyrode solution, both single Purkinje and ventricular cells had resting membrane potentials close to the \( K^+ \) equilibrium potential (\( E_K \)). In both cell types we could evoke action potentials that overshot the zero potential value. These action potentials were often stable for \( >30 \) min. We did not observe phase 4 depolarization in Purkinje cells bathed in normal Tyrode solution. It has been observed that lowering the extracellular \( K^+ \) concentration causes spontaneous activity in single Purkinje cells (5). However, we did not observe this phenomenon when we lowered the \( K^+ \) concentration from 5.4 mM to 3.7 (\( n = 3 \)) or 2.0 mM (\( n = 3 \)).

### Action Potentials in Single Purkinje and Ventricular Cells

In 64 single Purkinje cells, action potentials were elicited at a frequency of 1 Hz. About two-thirds of the action potentials were measured with the conventional whole cell patch-clamp technique and the remaining with the perforated patch-clamp technique. Independently of the patch-clamp technique, two types of action potential configuration were found. One type was characterized by a prominent phase 1 repolarization followed by a relatively negative plateau phase. It was found in 31 cells (48% of total). We named the Purkinje cells showing such an action potential configuration low-plateau (LP) cells (Fig. 1A). The second type showed little phase 1 repolarization and a relatively positive plateau. It was found in 33 cells (52% of total). We named the Purkinje cells showing such an action potential configuration high-plateau (HP) cells (Fig. 1B). To discriminate between these two types on more objective grounds, we computed the ratio of \( APD_{20} \) to \( APD_{90} \) (\( APD_{20}/APD_{90} \)). The frequency distribution of this ratio revealed two peaks (Fig. 1D), indicating the presence of two distinct types of action potentials.

#### RESULTS

### General Characteristics of Single Purkinje and Ventricular Cell Preparations

General data regarding the success rate, morphology, and basal electrophysiological properties of our cell preparations are summarized in Table 1.

<table>
<thead>
<tr>
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<th>Purkinje Cells</th>
<th>Ventricular Cells</th>
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<tbody>
<tr>
<td>Isolation success rate</td>
<td>14 of 21</td>
<td>5 of 5</td>
</tr>
<tr>
<td>Viable cells, %</td>
<td>5–20</td>
<td>60–80</td>
</tr>
<tr>
<td>Cell length, µm</td>
<td>90 ± 7.3</td>
<td>117 ± 5.6*</td>
</tr>
<tr>
<td>Cell width, µm</td>
<td>17 ± 1.8</td>
<td>21 ± 1.1*</td>
</tr>
<tr>
<td>( V_m ), mV</td>
<td>−78 ± 1.1</td>
<td>−80 ± 0.5</td>
</tr>
<tr>
<td>( C_m ), pF</td>
<td>70 ± 5.0</td>
<td>116 ± 7.0*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; \( n \) = no. of cells. \( V_m \), resting membrane potential; \( C_m \), capacitance. \(* P < 0.05\) vs. Purkinje cells.
action potentials. We designated this type of action potential configuration "LP-like." LP-like action potentials of eight strands had an average APD20/APD90 of 0.06 ± 0.01. This value did not differ significantly from the value obtained in 31 single Purkinje LP cells (0.07 ± 0.02). The second group showed little phase 1 repolarization and a positive plateau phase (Fig. 2B). This group resembled HP cell action potentials. We designated this type of action potential configuration "HP-like." HP-like action potentials of eight strands had an average APD20/APD90 of 0.33 ± 0.07. This value too is not different from that computed for 33 single Purkinje HP cells (0.33 ± 0.05). Thus the two types of action potential configuration that we observed in single Purkinje cells were also present in intact, untreated Purkinje strands.

In most microelectrode experiments Purkinje strands exhibited only one type of action potential configuration. However, when a strand was branching, we occa-
sionally observed LP-like and HP-like action potential in different branches of the same strand. We never observed intermediate action potential configurations. The intact Purkinje strands exhibited a very slow phase 4 depolarization. At a stimulus frequency of 1 Hz, the rate of depolarization over the first 100 ms starting at the maximal diastolic potential was $2.5 \pm 0.5$ mV/s ($n = 16$). In general, the depolarization of the membrane potential during phase 4 did not reach the threshold for excitation. Only in one case did we observe automaticity when the stand was not stimulated.

From these data we conclude that the LP and HP action potentials are not an artifact of the isolation method but in fact signify a physiological difference between two populations of Purkinje cells.

Frequency Dependency of Action Potential Configuration

We next investigated how the action potential configuration of LP, HP, and ventricular cells behaved as a function of the stimulus frequency. To this end, we varied the stimulus frequency between 0.1 and 3.33 Hz in single cells. We postulated that the frequency dependency of action potential configuration would offer additional criteria to discriminate between LP and HP cell action potentials and between HP and ventricular cell action potentials.

Figure 3A shows typical action potentials recorded from an LP cell, an HP cell, and a ventricular cell at stimulus frequencies of 0.1 Hz, 1 Hz, and 3.33 Hz. Data from these experiments are summarized in Fig. 3, B and C, which shows APD$_{90}$ and APD$_{20}$ plotted as a function of the stimulus frequency. These data are normalized for the APD$_{90}$ and APD$_{20}$ observed at 1 Hz. Action potential prolongation is the most prominent effect of the increased stimulus frequency in LP cells. Action potential shortening, on the other hand, is the most prominent effect in ventricular cells. HP cell action potential duration increased from 0.1 Hz to 1 Hz and then decreased at 3.33 Hz.

These data strengthen our conclusion that LP and HP cell action potential configurations are inherently distinct. Moreover, these data show that although the action potential configuration of HP cells resembles that of ventricular cells, the ionic currents that shape the action potential must be fundamentally different, at least in their time dependency. This indicates that the population of HP cells differs from the population of ventricular cells.

Membrane Currents in Single Purkinje and Ventricular Cells

In a concluding series of patch-clamp experiments we determined which ionic currents contribute to the observed types of action potential configuration in the three cell types. In these experiments current-damp measurements were alternated with voltage-clamp measurements. This enabled us to relate the ionic currents to the action potential shape observed in the same cell. Using the conventional whole cell patch-clamp technique, we focused on the principal cationic currents, including the transient outward current ($I_{\text{to}}$), the L-type Ca$^{2+}$ current ($I_{\text{Ca,L}}$), the delayed rectifier current ($I_{\text{K}}$), and the inward rectifier current ($I_{\text{K1}}$).

Transient outward current. Part of $I_{\text{to}}$ is carried by K$^+$ flowing through slowly inactivating, 4-AP-sensitive channels (16). This portion of $I_{\text{to}}$ is termed $I_{\text{to1}}$. The remainder of the current is carried by Cl$^-$ flowing through rapidly inactivating channels that are sensitive to inhibition by stilbene disulphonates such as DIDS (16, 47). This portion of $I_{\text{to}}$ is termed $I_{\text{to2}}$. $I_{\text{to2}}$ is activated...
by the release of Ca\(^{2+}\) from the sarcoplasmic reticulum, which is triggered by transmembrane Ca\(^{2+}\) currents (47).

We focused on \(I_{\text{to1}}\) density in LP, HP, and ventricular cells. Our current-clamp measurements suggested differences in \(I_{\text{to1}}\) density between LP and HP cells. The reason was that \(I_{\text{to1}}\) imparts a prominent phase 1 repolarization to action potentials. Furthermore, because \(I_{\text{to1}}\) recovers relatively slowly from inactivation, its influence on action potential configuration becomes less at higher stimulus frequencies. This is what we observed in LP cells (Fig. 3A). We thus hypothesized large differences in \(I_{\text{to1}}\) density between LP and HP cells but not between HP and ventricular cells.

In all three cell types \(I_{\text{to2}}\) was activated by 500-ms depolarizing voltage steps from \(-93\) mV to \(+47\) mV every 4 s while the cell was bathed in normal Tyrode solution. Next, we switched to Tyrode solution in which 2 mM 4-AP was dissolved (Fig. 4A). In Purkinje cells, \(I_{\text{to}}\) was virtually completely inhibited by the drug. This suggests that \(I_{\text{to2}}\) is the most important, if not the only, component of \(I_{\text{to}}\) in these cells. We found that \(I_{\text{to}}\) of ventricular cells was only partly suppressed by 2 mM 4-AP. This indicates that, contrary to its lack of contribution in Purkinje cells, \(I_{\text{to2}}\) makes a substantial contribution to \(I_{\text{to}}\) in ventricular cells (Fig. 4A). To further test this hypothesis, we applied 0.5 mM DIDS without 4-AP to the sheep ventricular cells. In all four cells tested, DIDS largely inhibited \(I_{\text{to1}}\) (data not shown). We conclude that \(I_{\text{to1}}\) in Purkinje cells mainly consists of \(I_{\text{to2}}\), whereas in ventricular cells \(I_{\text{to2}}\) also contributes to \(I_{\text{to}}\).

To get a measure of \(I_{\text{to1}}\) density at \(+47\) mV, we subtracted the current observed under control conditions from the current observed in the presence of 4-AP. The peak of the difference current was divided by \(C_m\) to normalize for the membrane surface area. These data from five LP cells, four HP cells, and eight ventricular cells are summarized in Table 3. Compared with its density in LP cells, \(I_{\text{to1}}\) density in both HP and ventricular cells was sixfold less. Similar to HP cells, ventricular cells showed relatively little \(I_{\text{to1}}\) activity (Table 3).

Next, the voltage dependency of \(I_{\text{to1}}\) was determined. \(I_{\text{to2}}\) currents were suppressed by exposing the cells to 1 mM CdCl\(_2\). CdCl\(_2\) blocks inward Ca\(^{2+}\) currents, thereby preventing activation of \(I_{\text{to2}}\) (47). CdCl\(_2\) also strongly inhibits inward Na\(^{+}\) currents (12, 34, 42). Suppressed inward currents favor an accurate determination of the voltage dependency of \(I_{\text{to1}}\). \(I_{\text{to1}}\) was activated by 500-ms depolarizing voltage steps from \(-93\) mV to \(+57\) mV in 10-mV increments. Figure 4B shows the raw membrane currents at \(+27, +37, +47\) mV recorded in an LP cell, an HP cell, and a ventricular cell. Figure 4C shows the averaged current-voltage (I-V) relationships of \(I_{\text{to1}}\) density in four LP cells, three HP cells, and seven
ventricular cells. In all three cell types, the activation threshold of \( I_{\text{to}} \) was around −30 mV. However, at all membrane potentials positive to −30 mV, the \( I_{\text{to}} \) density of LP cells was higher than that of HP or ventricular cells.

4-AP (2 mM) not only inhibited a transient current but also a part of the current at the end of the voltage steps from −93 mV to +47 mV (Fig. 4A). The data for this late 4-AP-sensitive current (\( I_{\text{4AP,late}} \)) in five LP cells, four HP cells, and eight ventricular cells are summarized in Table 3. The nature of this 4-AP-sensitive current is uncertain, but it could represent 1) a part of \( I_{\text{to}} \) that is still not inactivated, 2) the rapid delayed rectifier current (\( I_{\text{Kr}} \)) (15), 3) the ultrarapid delayed rectifier (\( I_{\text{Kur}} \)) (45), or 4) any combination of these possibilities. Apart from the question of the nature of this current, the differences in density are not significant. Therefore, we think that \( I_{\text{4AP,late}} \) does not contribute to the observed differences in action potential configuration.

Taking these findings together, we conclude that \( I_{\text{to}} \) density of LP cells is higher compared with that of either HP or ventricular cells. A prominent \( I_{\text{to}} \) combined with a small \( I_{\text{Ca,L}} \) in LP cells explains the short and low plateau phase of their action potential configuration. Furthermore, the significantly higher \( I_{\text{Ca,L}} \) density in ventricular cells compared with that in HP cells corroborates the notion that these two cell populations are different.

Delayed rectifier current and inward rectifier current. Finally, we examined the contributions of \( I_{\text{K}} \) and \( I_{\text{Ks}} \) to the three principal types of action potential configuration that we observed in this study. These currents were evoked by 500-ms depolarizing and hyperpolarizing voltage-clamp steps from a holding potential of −53 mV. The cells were exposed to 2 mM 4-AP to minimize the interference of \( I_{\text{to}} \) with the determination of \( I_{\text{K}} \) densities. The mean current during the final 20 ms of a 500-ms depolarizing voltage step was taken as a measurement of \( I_{\text{K}} \). We are aware that this definition of \( I_{\text{K}} \) may not reflect complete activation of \( I_{\text{K}} \) if the slow component of \( I_{\text{K}} \) (\( I_{\text{Ks}} \)) is present. However, the particular time interval is longer than the observed action potentials (Table 2); therefore, we expect no contribution of more slowly activating currents in the three principal types of action potential configuration. We did not observe a time-dependent increase in current during hyperpolarizing voltage steps. We thus conclude that the hyperpolarization-activated current (\( I_{\text{h}} \)) is not dominantly present in our preparation or under our recording conditions. We therefore could take the mean current in the last 20 ms of a 500-ms hyperpolarizing voltage step as a measurement of \( I_{\text{K1}} \).

Figure 6A shows typical membrane currents at −113 and +37 mV recorded in an LP cell, an HP cell, and a ventricular cell. Figure 6B shows the I-V relationships of mean current in the last 20 ms of 500-ms hyperpolarizing and depolarizing steps as a function of the applied membrane potential in five LP cells, six HP cells, and six ventricular cells. We attributed currents observed during these final 20 ms of 500-ms voltage step positive to −30 mV to \( I_{\text{K}} \). Normalized \( I_{\text{K}} \) densities observed at +37 mV in the three cell types are summarized in Table 3. We did not observe substantial differences in \( I_{\text{K}} \) densities between the three cell types at this membrane potential or in the membrane potential interval between −30 and +40 mV (Fig. 6B).

### Table 3. Differences in current densities in LP, HP, and ventricular cells of sheep

<table>
<thead>
<tr>
<th></th>
<th>LP Cells</th>
<th>HP Cells</th>
<th>Ventricular Cells</th>
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<tbody>
<tr>
<td>( I_{\text{to}} ), PA/pF</td>
<td>7.6 ± 2.1</td>
<td>1.2 ± 0.4*</td>
<td>1.0 ± 0.4†‡</td>
</tr>
<tr>
<td>( I_{\text{4AP,late}} ), PA/pF</td>
<td>1.4 ± 0.4</td>
<td>0.6 ± 0.4</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>( I_{\text{Ca,L}} ), PA/pF</td>
<td>−0.8 ± 0.3</td>
<td>−2.3 ± 0.6*</td>
<td>−5.4 ± 0.5†‡</td>
</tr>
<tr>
<td>( I_{\text{K}} ), PA/pF</td>
<td>3.0 ± 1.0</td>
<td>3.2 ± 0.9</td>
<td>1.9 ± 0.6</td>
</tr>
<tr>
<td>( I_{\text{Kr}} ), FS/pF</td>
<td>82 ± 27</td>
<td>85 ± 22</td>
<td>161 ± 17†‡</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; \( n = \) no. of cells. \( I_{\text{to}} \), transient outward \( K^+ \) current measured at +47 mV as 4-aminopyridine (4-AP)-sensitive transient current; \( I_{\text{4AP,late}} \), 4-AP-sensitive current measured at end of voltage steps to +47 mV; \( I_{\text{Ca,L}} \), L-type \( Ca^{2+} \)-current measured at 0 mV; \( I_{\text{K}} \), delayed rectifier current measured at +37 mV; \( I_{\text{Kr}} \), inward rectifier current slope conductance measured at −80 mV. *\( P < 0.05 \), HP cells vs. LP cells. †\( P < 0.05 \), ventricular cells vs. LP cells. ‡\( P < 0.05 \), ventricular cells vs. HP cells.
We attributed currents observed during the final 20 ms of 500-ms voltage step negative to −40 mV to $I_{K1}$. Typical of $I_{K1}$, all three I-V relationships showed inward rectification at potentials positive to −70 mV and had a reversal potential of approximately −80 mV, which is close to $E_K$. In ventricular cells we observed a prominent negative chord conductance in the membrane potential interval between −70 and −30 mV. Negative chord conductance had a maximum value of $-58 \pm 7$ fS/pF ($n = 6$, normalized for $C_m$) in the membrane potential interval between −40 and −30 mV. In HP cells, however, this value was significantly less negative ($-26 \pm 12$ fS/pF; $n = 6$), whereas in LP cells this value was even positive ($1.6 \pm 14$ fS/pF; $n = 5$). The mean slope conductances at −80 mV normalized for $C_m$ for the three cell types are summarized in Table 3. The normalized slope conductance of $I_{K1}$ in ventricular cells was significantly higher than that in both types of Purkinje cells. Also, at membrane potentials negative to −80 mV, the normalized density of $I_{K1}$ in ventricular cells was significantly higher.

We conclude that there are no clear differences in $I_K$ densities, as defined in our experiments, between LP, HP, and ventricular cells. We also conclude that ventricular cells possess a larger $I_{K1}$ than Purkinje cells. This lends further credit to the notion that HP cells and ventricular cells are different.

**DISCUSSION**

In this paper we employed patch-clamp methodology to investigate the electrophysiological properties of single Purkinje cells of the sheep. We found two distinct types of action potential configuration. The LP cell action potential was characterized by a prominent phase 1 repolarization and a relatively negative plateau phase. The HP cell action potential showed little phase 1 repolarization and a relatively positive plateau phase. HP cell action potential resembled that of ventricular cells, a finding that forced us to include these cells in the study. In our current-clamp experiments we observed fundamental differences in frequency dependency of the action potential configuration of LP, HP, and ventricular cells. Voltage-clamp experiments revealed substantial differences in the membrane currents that shape the action potential of the three cell types. We conclude that LP, HP, and ventricular cells each have a unique action potential configuration.

**Comparison of Electrophysiological Properties of LP and HP Cells**

Our results indicate that the two types of action potential configuration in single isolated Purkinje cells are caused by significant differences in the density of $I_{K1}$ and $I_{Ca,L}$. However, we cannot exclude the possibility that other ionic currents such as the fast TTX-sensitive

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**Fig. 5.** Calcium current ($I_{Ca,L}$) in single Purkinje and ventricular cells of the sheep. $I_{Ca,L}$ was measured in presence of 2 mM 4-AP. A: examples of membrane currents of an LP cell (left), an HP cell (middle), and a ventricular cell (right) in response to 500-ms test potentials to −3 mV and +17 mV. Holding potential was −53 mV. B: I-V relationship of $I_{Ca,L}$ density of LP, HP, and ventricular cells. Values are means ± SE; $n = no. of cells$. *P < 0.05, HP cells vs. LP cells. †P < 0.05, ventricular cells vs. LP cells. ‡P < 0.05, ventricular cells vs. HP cells.
Na current ($I_{Na}$), T-type Ca$^{2+}$ current ($I_{Ca,T}$), the Ca$^{2+}$-activated Cl$^{-}$ current ($I_{to2}$), or the delayed rectifier currents ($I_{Kr}$ and $I_{Ks}$) also play a role. Nevertheless, we have some indications that the role of these currents is limited.

It has been shown that a sustained component of $I_{Na}$ may determine the height and duration of the plateau phase in Purkinje strands of the sheep (1), dog (8), and rabbit (7). In our study, we did not measure $I_{Na}$ directly. However, $dV/dt_{max}$ of the action potential is a convenient index for $I_{Na}$ (35). We found that $dV/dt_{max}$ of action potentials in LP cells was higher than that of action potentials in HP cells. This suggests a larger $I_{Na}$ in LP cells and conceivably a larger sustained $I_{Na}$. The latter would result in an increased height of the plateau phase, yet in action potentials of LP cells we observed the lowest and shortest plateau phase.

Apart from $I_{Ca,L}$, the well-known $I_{Ca,T}$ has been described in Purkinje cells. $I_{Ca,T}$ activates at more negative membrane potentials and decays more rapidly than $I_{Ca,L}$ (20, 40). Because differences in action potential configuration persist long after $I_{Ca,T}$ presumably has inactivated, it is not very likely that variations in $I_{Ca,T}$ density contribute to the observed differences between LP and HP cell action potentials.

In Purkinje cells of the rabbit a prominent $I_{to2}$ is present (31, 37). This current plays an important role in phase 1 repolarization. In contrast to these observations, we found in the sheep Purkinje cells that $I_{to2}$ activity is so small that it presumably contributes little to the shape of the action potential, let alone explaining differences between LP and HP cell action potentials.

In Purkinje cells of the rabbit, the delayed rectifier current ($I_{Kr}$) consists of two components, $I_{Ks}$ and $I_{Kr}$ (9). In our experiments, $I_{Kr}$ is measured in the presence of 4-AP, which might affect $I_{Kr}$ (15). In our experiments, however, both 4-AP-sensitive steady-state current ($I_{Kr,late}$) and 4-AP-insensitive steady-state current during potentials positive to $-30$ mV were not significantly different between HP and LP cells. This indicates that outward currents during the plateau of the action potential do not contribute to the observed differences between LP and HP cell action potentials.

Taking these findings together, we conclude that LP and HP cells constitute two different cell populations and that differences in their action potential configuration are best explained by the differences in density of $I_{to2}$ and $I_{Ca,L}$.

Fig. 6. Inward rectifier current ($I_{K1}$) and delayed rectifier current ($I_{K}$) in single Purkinje and ventricular cells of the sheep. $I_{K1}$ and $I_{K}$ were measured in presence of 2 mM 4-AP. A: examples of membrane currents of an LP cell (left), an HP cell (middle), and a ventricular cell (right) in response to 500-ms test potentials to $-113$ and $+37$ mV. Holding potential was $-53$ mV. $I_{K1}$ was defined as current at end of hyperpolarizing steps; $I_{K}$ was defined as current at end of depolarizing steps. B: $I-V$ relationship of $I_{K1}$ and $I_{K}$ density of LP, HP, and ventricular cells. Values are means ± SE; n = no. of cells. †P < 0.05, ventricular cells vs. LP cells. ‡P < 0.05, ventricular cells vs. HP cells.

Phase 4 Depolarization in Purkinje Strands and Single Purkinje Cells

We never observed phase 4 depolarization in our single cell experiments. Also, in isolated Purkinje cells of the dog (3, 5, 17, 32, 36), cow (5), and rabbit (9, 23, 33) a diastolic depolarization was not systematically observed. However, in our microelectrode experiments with intact Purkinje cells we were able to record a slow phase 4 depolarization. Robinson et al. (32) suggested that the absence of phase 4 depolarization in isolated...
Purkinje cells may be a result of the absence of cell-cell interactions, the absence of extracellular defects, and/or the enzymatic isolation. Recently, it was also shown (4) that external proteolysis can abolish the hyperpolarization activated current (I). This current is thought to be important for the pacemaker potential in Purkinje strands (30). We looked for signs of I by lowering the external K concentration. It has been demonstrated (5) in single Purkinje cells that this maneuver results in the occurrence of pacemaker activity, probably because it decreases the conductance of I more than that of I channels (5, 11). However, we did not observe such a phenomenon in our single sheep Purkinje cell preparation, which agrees with results obtained in single rabbit Purkinje cells (9).

We conclude that our isolation method and measuring conditions yield single Purkinje cells that are not spontaneously active, presumably because they lack a clearly active I.

Comparison of Electrophysiological Properties of HP Cells and Ventricular Cells

At first glance the action potential configuration of HP cells and ventricular cells looks similar. Closer investigation reveals a number of striking differences between HP cell and ventricular cell electrophysiology. Of the action potential parameters, both APD20 and APA of HP cell action potential proved smaller than those of ventricular cells. There were also substantial differences in the frequency dependency of action potential duration. Furthermore, I and I were significantly smaller in HP cells than in ventricular cells. Finally, HP cell dimensions and membrane capacitance are significantly smaller than those of ventricular cells.

Taking these findings together, we conclude that HP cells and ventricular cells constitute two different cell populations.

Comparison of Electrophysiological Properties of Purkinje Cells and Ventricular Cells

We demonstrated that the density of I in ventricular cells was higher than that in Purkinje cells, a finding that agrees with a previous study in the dog (40). I plays an important role in excitation-contraction coupling (14). The differences in density of I between ventricular and Purkinje cells, therefore, are in agreement with their physiological function, namely, contraction and conduction, respectively. Another typical parameter for conductive tissue, dV/dt max, is higher in Purkinje than in ventricular cells (Table 2). This is also similar to what has been found in the dog (29). We found that the density of I negative to 80 mV was higher in ventricular than in Purkinje cells. We also found that I was not significantly different between ventricular and Purkinje cells. Both observations were also made in rabbit Purkinje and ventricular cells (9).

Functional Role of Two Types of Purkinje Action Potentials

Sheep Purkinje cells share the propensity of having two distinct types of action potential configuration with the rabbit (A. O. Verkerk, unpublished observations), dog (13), and baboon (10). Hauswirth et al. (19) observed progressive changes in these types of action potential configuration after the impalement of intact sheep Purkinje strands. They noticed what we now would call transition from HP cell to LP cell action potential morphology. Moreover, a model study of Purkinje cells by McAlister et al. (27) revealed HP cell action potentials, LP cell action potentials, and transition forms. These authors found that the action potential configuration could be modulated, depending on what Ca current density was chosen. In our hands, the density of I also differed between LP and HP cells. However, HP cells never changed into LP cells or vice versa. Furthermore, we never observed changes of I density within one cell. It therefore seems unlikely that in our experiments the two distinct types of action potential configuration are introduced by an altered state of I. The functional role of two Purkinje action potential configurations, however, is not at all clear. We hypothesize two possible roles.

HP cell action potential may be considered intermediate between LP cell and ventricular cell action potential. At the junction between Purkinje strands and ventricular muscle (Purkinje-ventricular junction), a zone is found that is populated with cells called transitional cells (26, 39). These transitional cells have several electrophysiological parameters intermediate between those of Purkinje strands and the ventricle (25). However, the transitional cell zone at the Purkinje-ventricular junction is extremely short (39). It therefore seems unlikely that all our HP cells, which constituted ~50% of all Purkinje cells, were derived from this zone.

In the conduction system trajectory, beginning at the atrioventricular node and ending at the Purkinje-ventricular junction, variations in action potential duration have been described. It has been shown that action potential duration increases from the bundle of His to the distal end of the Purkinje strands (21, 22, 28). This increase continues until a region of maximum action potential duration is reached, a few millimeters before the Purkinje-ventricular junction. Between this region and the actual Purkinje-ventricular junction, action potential duration decreases again (21, 22, 28). We cannot exclude the possibility that cells with HP action potentials originate from the area of maximum action potential duration and LP cells from an area closer to the bundle of His. The significantly higher dV/dt max in LP cells points to this suggestion. However, further studies are required to clarify this issue.

In conclusion, acutely isolated Purkinje cells of the sheep exhibit two types of action potential configuration. The action potential shape may be related to the position of the Purkinje cell in the intact strand.
REFERENCES


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