

# The Pacemaker Current in Cardiac Purkinje Myocytes

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**ABSTRACT** It is generally assumed that in cardiac Purkinje fibers the hyperpolarization activated inward current  $i_h$  underlies the pacemaker potential. Because some findings are at odds with this interpretation, we used the whole cell patch clamp method to study the currents in the voltage range of diastolic depolarization in single canine Purkinje myocytes, a preparation where many confounding limitations can be avoided. In Tyrode solution ( $[K^+]_o = 5.4$  mM), hyperpolarizing steps from  $V_h = -50$  mV resulted in a time-dependent inwardly increasing current in the voltage range of diastolic depolarization. This time-dependent current ( $i_{kdd}$ ) appeared around  $-60$  mV and reversed near  $E_K$ . Small superimposed hyperpolarizing steps (5 mV) applied during the voltage clamp step showed that the slope conductance decreases during the development of this time-dependent current. Decreasing  $[K^+]_o$  from 5.4 to 2.7 mM shifted the reversal potential to a more negative value, near the corresponding  $E_K$ . Increasing  $[K^+]_o$  to 10.8 mM almost abolished  $i_{kdd}$ .  $Cs^+$  (2 mM) markedly reduced or blocked the time-dependent current at potentials positive and negative to  $E_K$ .  $Ba^{2+}$  (4 mM) abolished the time-dependent current in its usual range of potentials and unmasked another time-dependent current (presumably  $i_f$ ) with a threshold of  $\sim -90$  mV ( $>20$  mV negative to that of the time-dependent current in Tyrode solution). During more negative steps,  $i_f$  increased in size and did not reverse. During  $i_h$ , the slope conductance measured with small (8–10 mV) superimposed clamp steps increased. High  $[K^+]_o$  (10.8 mM) markedly increased and  $Cs^+$  (2 mM) blocked  $i_f$ . We conclude that: (a) in the absence of  $Ba^{2+}$ , a time-dependent current does reverse near  $E_K$  and its reversal is unrelated to  $K^+$  depletion; (b) the slope conductance of that time-dependent current decreases in the absence of  $K^+$  depletion at potentials positive to  $E_K$  where inactivation of  $i_{K1}$  is unlikely to occur. (c)  $Ba^{2+}$  blocks this time-dependent current and unmasks another time-dependent current ( $i_f$ ) with a more negative ( $>20$  mV) threshold and no reversal at more negative values; (d)  $Cs^+$  blocks both time-dependent currents recorded in the absence and presence of  $Ba^{2+}$ . The data suggest that in the diastolic range of potentials in Purkinje myocytes there is a voltage- and time-dependent  $K^+$  current ( $i_{kdd}$ ) that can be separated from the hyperpolarization-activated inward current  $i_h$ .

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## INTRODUCTION

For several years, it was believed that the pacemaker potential in cardiac Purkinje fibers was caused by the decline of a potassium specific current that activates during the action potential and deactivates during the subsequent diastole ( $i_{K2}$ ) (Vassalle, 1966; Noble and Tsien, 1968). This interpretation was based on our findings that the time-dependent current reverses near the potassium equilibrium potential ( $E_K$ ) and that the slope conductance decreases as a function of time when the membrane potential is clamped at the maximum diastolic potential (Vassalle, 1966). In addition, the reversal potential for this time-dependent current shifted 61 mV/tenfold change in  $[K^+]_o$  (Noble and Tsien, 1968; Peper and Trautwein, 1969).

Later, it was demonstrated that  $K^+$  depletion in narrow extracellular spaces occurred (probably through the background  $i_{K1}$  channels) during the large hyperpolarization required to attain the reversal potential of the putative  $K^+$  specific pacemaker current. The time-dependent change in this  $K^+$  depletion current during hyperpolarization could result in a spurious reversal of the time-dependent current (Cohen, Daut and Noble, 1976; Baumgarten and Isenberg, 1977; DiFrancesco, Ohba and Ojeda, 1979).

In order to eliminate the confounding effects of  $K^+$  depletion, DiFrancesco (1981*a,b*) used 5–10 mM  $Ba^{2+}$  to block the background  $K^+$  current  $i_{K1}$ . In the presence of  $Ba^{2+}$ , on hyperpolarization a time-dependent current was present which no longer reversed near  $E_K$  and, instead, continued to increase at more negative potentials. The reversal potential of this current (called  $i_f$  by DiFrancesco, 1981*b*) was found in the plateau range of potentials.  $i_f$  was blocked by low concentrations of  $Cs^+$  (1–4 mM) and demonstrated an increasing slope conductance during hyperpolarizing voltage clamp steps. On the basis of these findings, DiFrancesco (1981*a, b*) proposed that in Purkinje fibers the pacemaker current is  $i_f$ , an inward current activated on hyperpolarization and carried by  $Na^+$  and  $K^+$ .

Computer reconstructions provided further support for this interpretation of the pacemaker current (DiFrancesco and Noble, 1985). Thus, it was computed that  $K^+$  depletion could also account for the observed time-dependent decrease in slope conductance on hyperpolarization because of the  $[K^+]_o$  dependence (Sakmann and Trube, 1984) of the background current  $i_{K1}$ . The apparent shift of the reversal potential in different  $[K^+]_o$  could be accounted for by the dependence of  $i_{K1}$  (and thus of  $K^+$  depletion) on  $[K^+]_o$  (DiFrancesco and Noble, 1985).

The  $i_f$  hypothesis is generally accepted and, yet, several findings related to the use of  $Ba^{2+}$  and  $Cs^+$  cast doubts as to its correctness (see Discussion). Therefore, we decided to investigate the pacemaker current in the absence of  $K^+$  depletion (as well as of  $Ba^{2+}$ ) by using one obvious approach, namely, isolated Purkinje myocytes. Because these myocytes have no restricted extracellular spaces (Eisenberg and Cohen, 1983; Callewaert, Carmeliet, and Vereecke 1984; Mathias, Eisenberg, Dwyer, Gintant, and Cohen, 1985), a current reversal could not be due to  $K^+$  depletion.

In the present investigation, we examined the properties of the time-dependent current in the diastolic potential range in isolated canine Purkinje myocytes. We could then adopt procedures intended to identify the ionic nature of the time-dependent current elicited in response to hyperpolarizing voltage steps and study the effects of  $Ba^{2+}$  and  $Cs^+$  on such a current.

As will be demonstrated below, in Tyrode solution (in the absence of  $\text{Ba}^{2+}$ ) there is a time-dependent current in the diastolic depolarization range ( $i_{\text{Kdd}}$ ) which deactivates on hyperpolarization and is blocked by both  $\text{Ba}^{2+}$  and  $\text{Cs}^+$ . In the presence of  $\text{Ba}^{2+}$  (4 mM),  $i_{\text{Kdd}}$  is no longer present and  $i_t$  appears at substantially more negative potentials. These and other results lead us to suggest that the current underlying the pacemaker potential in Purkinje fibers may not be  $i_t$ , but instead a voltage- and time-dependent  $\text{K}^+$  current, as was originally proposed by one of us (Vassalle, 1966) and by others (Noble and Tsien, 1968; Peper and Trautwein, 1969).

#### MATERIALS AND METHODS

Adult dogs of either sex were euthanized by injection of sodium pentobarbital (86 mg/Kg). The hearts were immediately removed and rinsed in warm (40°C), oxygenated (95%  $\text{O}_2$ , 5%  $\text{CO}_2$ ) Tyrode solution of the following composition (in millimoles): NaCl, 140;  $\text{NaHCO}_3$ , 12;  $\text{NaH}_2\text{PO}_4$ , 0.4;  $\text{MgCl}_2$ , 2; KCl, 8;  $\text{CaCl}_2$ , 4; dextrose, 10. Purkinje strands with attached pieces of ventricular muscle were excised, placed in a chamber perfused with warm (40°C) Tyrode solution and stimulated at 0.8 Hz for 30 min. The strands were then dissected free from ventricular muscle and cut into 4–6 mm segments. After an additional 30 min stimulation, the fibers were placed in a test tube, washed three times with calcium-free solution and incubated for 10 min in the same calcium-free solution with the addition of 25 mM taurine, 5 mM beta-hydroxybutyric acid and 5 mM Na pyruvate. The tissue was then dissociated.

The dissociation procedure essentially followed that of Gintant, Datyner, and Cohen (1985), utilizing a mechanical "triturator" that passed the fibers repeatedly through successively smaller bore pipettes. The rate and pattern of agitation were controlled by a Z-80 based microprocessor interfaced to a linear actuator and piston/cylinder (Datyner, Gintant and Cohen, 1985a). Each digestion lasted ~20 min. The digestion solutions contained either type B or type D collagenase (Boehringer Mannheim Corporation, Indianapolis, IN) and bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in the following concentrations of collagenase/albumin (in milligrams/milliliter): 1.4 type B/1.7; 1.2 type D/1.7; 1.0 type D/4.0; and 0.8 type B/4.0. The collagenase/albumin mixture was dissolved in an oxygenated solution containing (in millimolar): KCl, 140;  $\text{KHCO}_3$ , 8;  $\text{KH}_2\text{PO}_4$ , 0.4;  $\text{MgCl}_2$ , 2; dextrose, 10; taurine, 25;  $\beta$ -hydroxybutyric acid, 5; Na pyruvate, 5. This solution was used for the first two digestions. Collagenase and albumin were dissolved in the calcium-free Tyrode solution described above for the last two digestions. An aliquot of 50  $\mu\text{l}$  of 2.5 mM  $\text{CaCl}_2$  was added to the first three digestion solutions of 5-ml vol. Following the third digestion, the solution was centrifuged at 50 *g* for 5 min, the supernatant discarded and the pellet digested with the collagenase/albumin mixture. The final suspension was again centrifuged and the pellet resuspended in calcium-free Tyrode solution.

The single Purkinje cells were placed on a poly-L-lysine (mol wt 55,000) coated glass coverslip at the bottom of a small volume (0.5 ml) perfusion chamber heated to 37°C (held within 0.5°C) (Datyner, Gintant, and Cohen, 1985b). The chamber was located on the stage of an inverted microscope equipped with Hoffman modulation contrast optics (at a magnification of 200). The cells were superfused with Tyrode solution of the following composition (in millimoles): NaCl, 137.7; NaOH 2.3; KCl, 5.4;  $\text{CaCl}_2$ , 1.8;  $\text{MgCl}_2$ , 1; dextrose, 10; Hepes, 5; pH = 7.4. The cells were studied within eight hours following completion of a dissociation procedure.

We employed the whole cell patch clamp technique using an Axopatch 1B amplifier. The pipettes had a resistance of 2–4 M $\Omega$  when filled with the following solution (in millimoles): NaCl, 6.0; K-aspartate, 130.0;  $\text{MgCl}_2$ , 2.0; EGTA, 11.0; Hepes, 10.0;  $\text{Na}_2\text{-ATP}$ , 2.0; Na-GTP, 0.1;  $\text{CaCl}_2$ , 5.0. In some of the initial experiments, 0.2 mM cAMP was added to the pipette solution. The pH was adjusted to 7.2 by adding KOH. The pipette resistance usually increased two- to threefold in the whole cell configuration. The liquid junction potential between the pipette solution and the nor-

mal Tyrode solution was 9 mV (pipette side negative). Because the exchange is never complete due to membrane transport (Mathias, Cohen and Oliva, 1990), no correction was made for this effect. On the assumption of 5–8 M $\Omega$  series resistance and a maximal current of 1 nA in 5.4 mM [K<sup>+</sup>]<sub>o</sub>, the offset due to series resistance was at most 8 mV. This offset is in the opposite direction to the liquid junction offset and again no correction was applied. Data were recorded using the pClamp program (Axon Instruments Inc., Foster City, CA) and analyzed after low pass filtering at 10–15 Hz.

Most recent studies of time-dependent currents at diastolic potentials in Purkinje fibers have employed blockers of potentially overlapping currents, so that the hyperpolarization activated current  $i_h$  could be observed without contamination. We decided to begin our study of the isolated canine Purkinje myocyte in the absence of any of these usual channel blockers (Ba<sup>2+</sup>, Cd<sup>2+</sup>, Mn<sup>2+</sup>, TTX, et cetera). We chose this approach because we were concerned that these blockers may not be specific, and may modify what we were attempting to study, as is in fact shown below for Ba<sup>2+</sup>.

The time-dependent currents flowing during the hyperpolarizing steps were followed by increasing outward tails whose amplitude reflected the degree of change of the time-dependent currents during the steps (e.g., Fig. 1). However, since on purpose we did not generally use blockers of other currents, the tails are likely to be contaminated by other currents flowing on repolarization to the holding potential. This appears to be confirmed by the fact that in some experiments we applied hyperpolarizing steps in the presence of 30  $\mu$ M TTX: the time-dependent current during the step and the direction of the decaying tails were not altered, but the amplitude of the tails was reduced, presumably because an inward TTX-sensitive Na<sup>+</sup> current component activated by the previous hyperpolarization was eliminated.

## RESULTS

The results obtained will be reported in three major sections: in normal Tyrode solution, in the presence of cesium and in the presence of barium.

### NORMAL TYRODE SOLUTION

#### *The Time-dependent Current Reverses Near E<sub>K</sub>*

We first examined the time-dependent diastolic current in Purkinje myocytes superfused in 5.4 mM K<sup>+</sup> Tyrode solution. In Fig. 1, V<sub>h</sub> was –50 mV and, when a 3-s hyperpolarizing voltage clamp step was applied to –55 mV, the initial jump was followed by a relatively unchanging current. However, during the following step to –65 mV, there was a time-dependent increase in a net inward current. The time-dependent increase was larger during the step to –75 mV, but then the time-dependent component changed direction at –85 mV and the direction change was even more conspicuous at –95 mV.

In 21 preparations perfused in 5.4 mM [K<sup>+</sup>]<sub>o</sub> Tyrode solution, the average threshold for diastolic time-dependent current was at  $-61 \pm 5$  mV (mean  $\pm$  SD). Thus, in isolated canine Purkinje myocytes, a slow time-dependent current appears in the potential range of diastolic depolarization. At –75 mV, the amplitude of this current was  $44 \pm 24$  pA ( $n = 16$ ). This current does not continue to increase with hyperpolarization and, instead, it reverses near the predicted equilibrium potential for potassium (in 25 preparations, E<sub>rev</sub> was  $-84 \pm 5$  mV, which is fairly close to an E<sub>K</sub> of –87 mV). This time-dependent current will be referred to as  $i_{Kdd}$ , because it flows in the voltage range of diastolic depolarization and reverses near E<sub>K</sub>. The

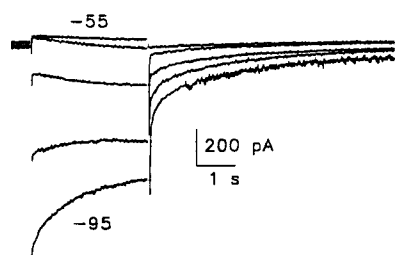


FIGURE 1. Reversal of the time-dependent current in Tyrode solution in an isolated Purkinje myocyte. The holding potential was  $-50$  mV and a 3-s hyperpolarizing step was applied to  $-55$ ,  $-65$ ,  $-75$ ,  $-85$ , and  $-95$  mV. The increase in inward current was minimal at  $-55$  mV and was clearly apparent at  $-65$  and  $-75$  mV. The current reversed at  $-85$  mV. The mean holding current at  $-50$  mV was  $+93$  pA.

presence of a threshold potential indicates that  $i_{Kdd}$  is related to the pacemaker potential, because it begins to change with time within the range of potentials of diastolic depolarization.

*Shift of the Reversal Potential of  $i_{Kdd}$  in Lower  $[K^+]_o$*

If  $i_{Kdd}$  is indeed a  $K^+$  current, its reversal should shift to a more negative value in lower  $[K^+]_o$ . The results of decreasing  $[K^+]_o$  from 5.4 to 2.7 mM are illustrated in Fig. 2. The hyperpolarizing steps were applied from a  $V_h = -50$  mV both in 5.4 mM  $[K^+]_o$  (A) and in 2.7 mM  $[K^+]_o$  (B). It is apparent that the reversal potential shifted from about  $-85$  mV in 5.4 mM  $K^+$  to  $-105$  mV in 2.7 mM  $K^+$  solution.

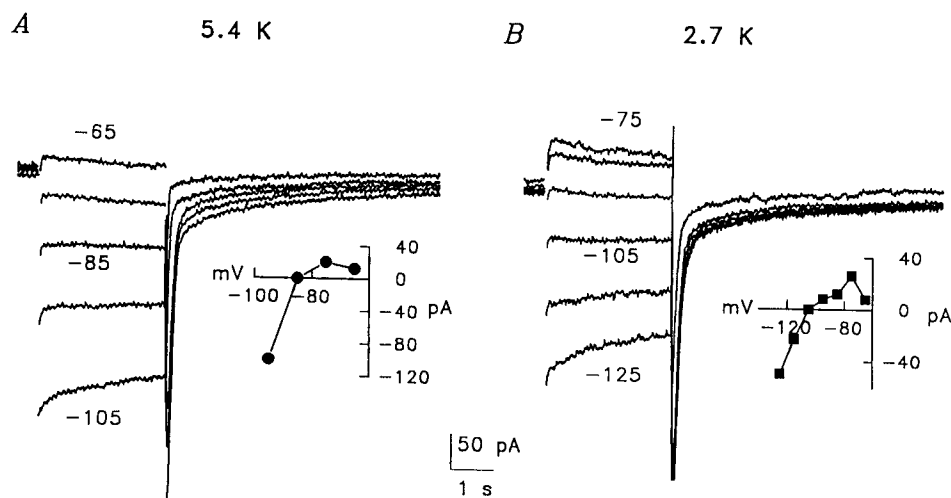


FIGURE 2. The reversal potential of  $i_{Kdd}$  shifts with  $[K^+]_o$ . The current traces were recorded in 5.4 mM  $K^+$  Tyrode solution in A and in 2.7 mM  $K^+$  Tyrode solution in B. The holding potential was  $-50$  mV in both solutions. In 5.4 mM  $K^+$ , the current traces were recorded during 3-s voltage clamp steps to  $-65$ ,  $-75$ ,  $-85$ ,  $-95$ , and  $-105$  mV. In 2.7 mM  $K^+$ , the current traces were recorded during similar steps to  $-75$ ,  $-85$ ,  $-95$ ,  $-105$ ,  $-115$ , and  $-125$  mV. The reversal potential shifted from  $-85$  mV in 5.4 mM  $K^+$  to  $\sim -105$  mV in 2.7 mM  $K^+$ . In B, the current trace for the step to  $-75$  mV was shifted upward by 10 pA to avoid overlap of current traces. The graphs are plots of  $i_{Kdd}$  and indicate that the reversal potential was determined as the intersection with the 0 current axis. The mean holding current at  $-50$  mV was  $+21$  pA (A) and  $-44$  pA (B).

In eight experiments,  $E_{rev}$  shifted from  $-86 \pm 7$  mV in 5.4 mM  $K^+$  to  $-104 \pm 7$  mV in 2.7 mM  $K^+$ , as the predicted  $E_K$  simultaneously shifted from  $-87$  to  $-106$  mV. Thus, changing  $[K^+]_o$  shifts the reversal potential of  $i_{kdd}$  approximately in accordance with the Nernst equilibrium potential for  $K^+$ .

It is apparent that even in the absence of narrow extracellular spaces there is a clear reversal of  $i_{kdd}$  which depends in a predictable way on extracellular  $[K^+]$ . The reversal of this current is unlikely to be due to depletion of extracellular  $[K^+]$  because no restricted spaces exist.

Although we cannot be sure that the inactivation of  $i_{k1}$  did not contribute to the decay of time-dependent current at least at more negative potentials, there should be little inactivation of  $i_{k1}$  at potentials positive to  $E_K$  (Sakmann and Trube, 1984; Tromba and Cohen, 1990). Further, in Purkinje strands the reversal near  $E_K$  may be a complex waveform due to the sum of several currents (Cohen et al., 1976). In general, no such mixed reversals were observed in isolated myocytes in the present experiments (e.g., see Figs. 1, 2, 3, and 7).

#### *Decrease of $i_{kdd}$ in High $[K^+]_o$*

In high  $[K^+]_o$ ,  $i_t$  increases in magnitude (DiFrancesco, 1981a) whereas diastolic depolarization decreases or disappears (Vassalle, 1965). We examined  $i_{kdd}$  in a higher  $[K^+]_o$  (10.8 mM) to find out whether the conductance of our time-dependent current had the same  $K^+$  dependence as that of  $i_t$ .

In Fig. 3, an isolated Purkinje myocyte was exposed to Tyrode solution containing either 5.4 mM  $[K^+]_o$  (A) or 10.8 mM  $[K^+]_o$  (C). Upon elevation of  $[K^+]_o$ ,  $i_{kdd}$  was abolished. Similar results were obtained in three experiments in 10.8 mM  $[K^+]_o$ . The results in B and D will be discussed below.

#### *Change in Slope Conductance during $i_{kdd}$*

In multicellular Purkinje preparations, the slope conductance of the pacemaker current decreases during hyperpolarizing voltage clamp steps positive and negative to  $E_K$  (Vassalle, 1966; Vassalle et al., 1992). These results suggested that the pacemaker current deactivates on hyperpolarization. However (as mentioned above), it has been proposed that at more negative potentials the decrease in slope conductance with time on hyperpolarization might be due to the depletion of  $K^+$  in narrow extracellular spaces (DiFrancesco and Noble, 1985). To determine whether the  $i_{kdd}$  channels are opening or closing during hyperpolarization in the absence of possible  $K^+$  depletion (and of  $Ba^{2+}$ ), we measured in Tyrode solution the changes in slope conductance in response to relatively brief (250 ms) and small (5 mV) hyperpolarizing pulses superimposed on larger (5–65 mV) hyperpolarizing voltage clamp steps.

The results of one such experiment are illustrated in Fig. 4.  $V_h$  was  $-50$  mV, and 3.6-s hyperpolarizing steps were applied to  $-70$ ,  $-80$ , and  $-90$  mV. At each voltage, the small superimposed voltage pulses caused a small step current which progressively decreased in amplitude during the parent pulse.

In 19 preparations, the slope conductance consistently declined during the hyperpolarizing steps. At  $-75$  mV, the mean ratio of the slope conductance mea-

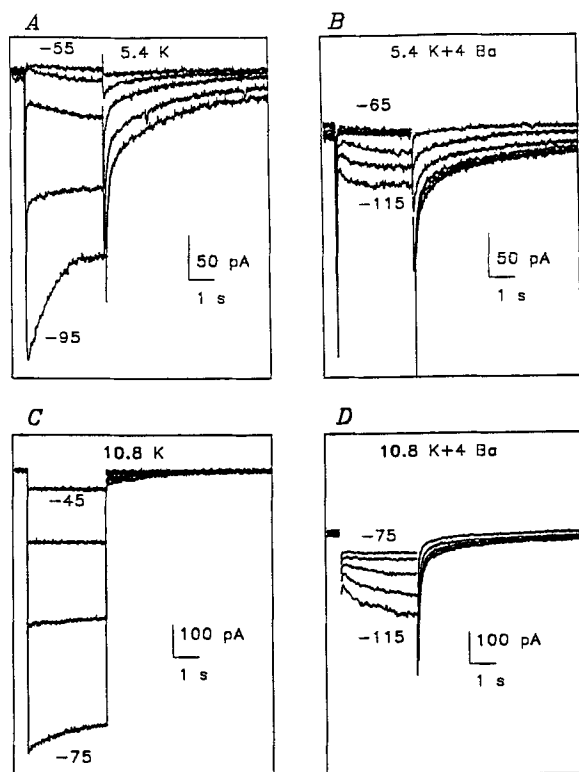


FIGURE 3. High  $[K^+]_o$  decreases the time-dependent diastolic current in Tyrode solution and increases the time-dependent inward current in the presence of  $Ba^{2+}$ . All traces are from the same Purkinje myocyte, which also was exposed to a higher  $[K^+]_o$  (10.8 mM) in the absence (C) and in the presence (D) of  $Ba^{2+}$ . The holding potential was  $-50$  mV in normal and  $-40$  mV in the higher  $[K^+]_o$ . In C (10.8 mM  $K^+$ ), 3-s hyperpolarizing steps were applied to  $-45$ ,  $-55$ ,  $-65$ , and  $-75$  mV. In D (in the presence of 10.8 mM  $K^+$  and 4 mM  $Ba^{2+}$ ), the hyperpolarizing steps were applied to  $-75$ ,  $-85$ ,  $-95$ ,  $-105$ , and  $-115$  mV. The time-dependent diastolic current was smaller in 10.8 mM  $[K^+]_o$  than in 5.4 mM  $[K^+]_o$ . In contrast, the time-dependent inward current in the presence of  $Ba^{2+}$  increased in the higher  $[K^+]_o$  (B and D: note the different calibrations for the current).

In Tyrode solution, the reversal of  $i_{kdd}$  shifted to a less negative value in 10.8 mM  $[K^+]_o$ . In the presence of  $Ba^{2+}$ ,  $i_{kdd}$  was absent and  $i_i$  appeared at a potential of  $-95$  mV and did not reverse at negative potentials (B and D). The mean holding current at  $-50$  mV was  $-20$  pA (A) and  $-160$  pA (B). The mean holding current at  $-40$  mV was  $+88$  pA (C) and  $-120$  pA (D).

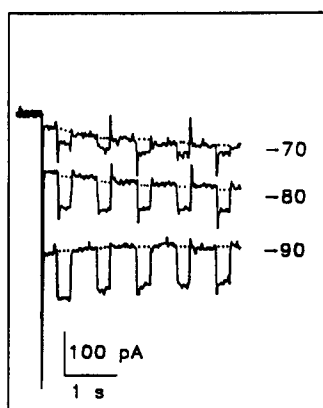


FIGURE 4. Decrease in slope conductance during  $i_{kdd}$  in Tyrode solution. In  $Ba^{2+}$ -free Tyrode solution,  $i_{kdd}$  was elicited upon membrane hyperpolarization from a  $V_h$  of  $-50$  mV to  $-70$ ,  $-80$ , and  $-90$  mV. The 5-mV small pulses superimposed on the parent step show that the membrane conductance decreases during the development of  $i_{kdd}$  at all three potentials. Dashed lines from the  $i_{kdd}$  current traces without slope conductance measurements are superimposed for a better visualization of the slope conductance changes. The mean holding current at  $-50$  mV was  $+23$  pA.

sured at the end of the parent pulse to that measured at the beginning was  $0.75 \pm 0.07$  ( $n = 11$ ), a decrease in conductance similar to that found in multicellular strands (Vassalle, 1966; Vassalle et al., 1992).

These results demonstrate that, in the absence of  $[K^+]_o$  fluctuations in normal Tyrode solution (no  $Ba^{2+}$ ), the slope conductance declines with time on hyperpolarization. This observation is not consistent with the  $i_f$  hypothesis.

#### *Possible Contribution of the Decay of $i_K$ to $i_{Kdd}$*

It might be proposed that  $i_{Kdd}$  is due to deactivation of the delayed rectifier current  $i_K$ . However, if this were the case, no discrete threshold would ever be present on hyperpolarization from a  $V_h$  of  $-50$  mV. Instead, a threshold was present in 57% of cases. To directly assess a possible contribution of  $i_K$  to  $i_{Kdd}$ , two protocols were adopted to gradually activate  $i_K$  to a greater extent and to examine whether on hyperpolarization to a fixed voltage the decay of a larger  $i_K$  would increase  $i_{Kdd}$ .

As seen in Fig. 5 *A*, in the first protocol, the conditioning steps were 1 s in duration and their amplitude was progressively increased by 10 mV to a maximum depolarization of +5 mV. These conditioning steps were followed by a test step to  $-75$  mV. In Fig. 5 *B*, in the second protocol, a depolarizing clamp step to 0 mV was progressively lengthened from 100 ms to 1.7 s in steps of 200 ms (the steps shorter than 700 ms are not shown) and again was followed by a test pulse to  $-75$  mV. Neither protocol altered the amplitude of the subsequent  $i_{Kdd}$ , as shown in Fig. 5 by the traces and by the graphs. In the graphs, the amplitude of  $i_{Kdd}$  has been plotted as a function of the previous depolarization (*A*) and of the duration of the conditioning steps (including the steps  $<700$  ms) (*B*). Similar results were obtained in a total of five experiments (three experiments for the *A* protocol and two for the *B* protocol).

That indeed  $i_K$  was activated during the depolarizing steps is shown in the boxed inset: a depolarizing 3-s step from  $V_h -50$  mV to  $-15$  mV activated the delayed rectifier and on return to  $V_h$  a decaying current tail was present. A similar decay of  $i_K$  on repolarization to a potential positive to the threshold for  $i_{Kdd}$  was found in six experiments.

The absence of a significant change in the time-dependent current after the predepolarizations also argues against significant extracellular  $K^+$  accumulation during the depolarizing steps. The absence of  $K^+$  fluctuations was also supported by two experiments in which a predepolarization of 1 s to  $-20$  mV from a  $V_h$  of  $-50$  mV failed to alter the reversal potential of  $i_{Kdd}$  (not shown).

#### EFFECTS OF CESIUM

In Purkinje fibers, cesium blocks the pacemaker current in Tyrode solution (Isenberg, 1976; DiFrancesco, 1981*a*; Vassalle et al., 1992) as well as  $i_f$  in the presence of  $Ba^{2+}$  (DiFrancesco, 1981*a*). Although  $Cs^+$  in low concentrations (2 mM or less) is considered a specific blocker of  $i_f$ , the possible role of  $i_{Kdd}$  in the pacemaker potential led us to reexamine the effects of  $Cs^+$  on this time-dependent current elicited on hyperpolarization in normal Tyrode solution.



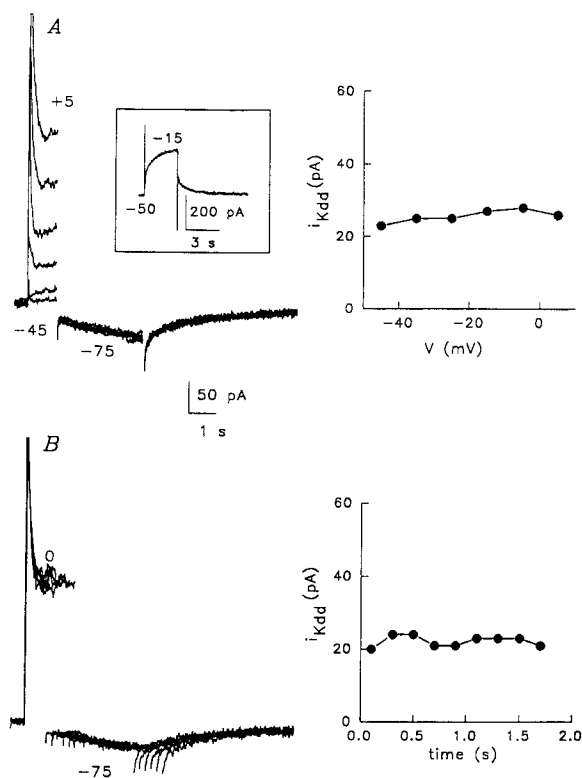


FIGURE 5. Different degrees of activation of the delayed rectifier  $i_K$  do not modify  $i_{Kdd}$  in Tyrode solution. In *A*, the holding potential was  $-50$  mV and 1-s conditioning voltage clamp steps were applied to  $-45$ ,  $-35$ ,  $-25$ ,  $-15$ ,  $-5$ , and  $+5$  mV. Each of the steps were followed by a 3-s test step at  $-75$  mV. In *B*, a 100-ms conditioning step to  $0$  mV was gradually prolonged to  $1.7$  s in 200-ms steps. To avoid overcrowding, the currents flowing during the first three steps have been omitted. The amplitude of  $i_{Kdd}$  is plotted in pA (ordinate) vs the voltage of the conditioning step in mV (abscissa) (*A*, right) or versus the duration of the conditioning step in s (abscissa) (*B*, right). In this panel, the amplitude of  $i_{Kdd}$  after all conditioning steps is shown. In the boxed inset, the activation of the delayed rectifier current during a depolarizing step to  $-15$  mV and the decaying current tail on returning to  $V_h$  are shown. The mean holding current at  $-50$  mV was  $+6$  pA (*A*) and  $+18$  pA (*B*).

#### Cesium Block of $i_{Kdd}$

To find out whether  $\text{Cs}^+$  blocks  $i_{Kdd}$ , we studied the effects of  $2$  mM  $\text{Cs}^+$  in normal Tyrode solution. Fig. 6 shows current traces in response to steps from  $V_h = -50$  mV to  $-75$ ,  $-85$ , and  $-95$  mV before, during and after washout of  $\text{Cs}^+$  containing Tyrode solution. Clearly,  $\text{Cs}^+$  markedly reduced the diastolic current recorded throughout this potential range. In a total of five preparations,  $\text{Cs}^+$  reduced  $i_{Kdd}$  recorded at  $-75$  mV by  $72 \pm 34\%$  (mean  $\pm$  SD).

#### EFFECTS OF BARIUM

##### Barium Blocks $i_{Kdd}$ and Unmasks $i_f$

In the presence of  $5$ – $10$  mM  $\text{Ba}^{2+}$ , a time-dependent current is present which does not reverse at negative potentials ( $i_t$ ) (DiFrancesco, 1981*a,b*). In our previous experiments in isolated canine Purkinje myocytes, there was a time-dependent current ( $i_t$ ) in the presence of  $\text{Ba}^{2+}$ , but only at potentials negative to  $-89$  mV (Yu,

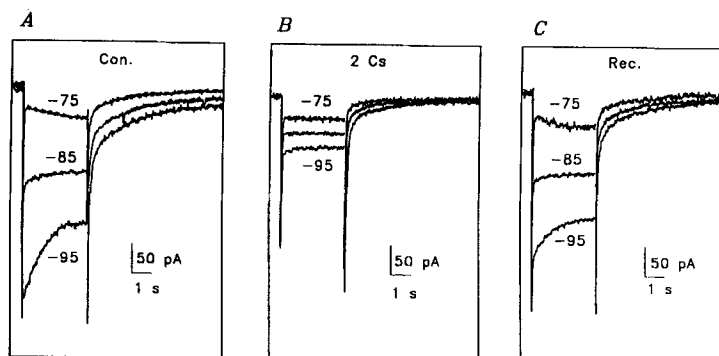


FIGURE 6. Block of  $i_{kdd}$  by cesium. The holding potential was  $-50$  mV and hyperpolarizing steps were applied to  $-75$ ,  $-85$ , and  $-95$  mV in control (A), in the presence of  $2$  mM  $\text{Cs}^+$  (B) and during recovery in Tyrode solution (C). The time-dependent current increased at  $-75$  mV and reversed at  $-85$  and  $-95$  mV.  $\text{Cs}^+$  dramatically reduced  $i_{kdd}$ , which appeared again during the washout of  $\text{Cs}^+$ . A shows some of the data already presented in A of fig. 3. The mean holding current at  $-50$  mV was  $+8$  pA (A),  $-34$  pA (B), and  $-23$  pA (C).

Chang, and Cohen, 1993). Since the present results suggest that in the absence of  $\text{Ba}^{2+}$  a current ( $i_{kdd}$ ) exists at more positive potentials (threshold  $-61$  mV), the possibility arose that the different thresholds for the two time-dependent currents might result from the fact that  $\text{Ba}^{2+}$  abolishes  $i_{kdd}$  and unmasks  $i_t$ . For this reason, we compared the threshold for time-dependent currents in the absence and presence of  $\text{Ba}^{2+}$  in the same canine Purkinje myocytes.

In Fig. 3, A and B, in each panel the holding potential was  $-50$  mV. In A (Con.), in the absence of  $\text{Ba}^{2+}$ ,  $i_{kdd}$  increased with time during the steps to  $-65$  and  $-75$  mV and reversed at more negative potentials. Instead, in the presence of  $4$  mM  $\text{Ba}^{2+}$  (4 Ba, B), no time-dependent current was observed positive to  $-95$  mV, and the current did not reverse at potentials as negative as  $-115$ . Thus,  $i_{kdd}$  was suppressed in the presence of  $\text{Ba}^{2+}$  and the time-dependent current  $i_t$  with a more negative threshold and no reversal at potentials more negative than  $E_K$  appeared. The changes induced by  $\text{Ba}^{2+}$  were reversible, since on washout of  $\text{Ba}^{2+}$  the current patterns returned towards control (data not shown).

In nine experiments, in Tyrode solution (no  $\text{Ba}^{2+}$ ), the average threshold for  $i_{kdd}$  was  $-62 \pm 5$  mV (mean  $\pm$  SD). In the presence of  $4$  mM  $\text{Ba}^{2+}$ , in the same Purkinje myocytes, the threshold for the time-dependent current was  $-88 \pm 10$  mV (mean  $\pm$  SD). Negative to  $E_K$ , in the absence of  $\text{Ba}^{2+}$   $i_{kdd}$  reversed whereas in the presence of  $\text{Ba}^{2+}$   $i_t$  did not reverse. These results suggest that barium either blocked  $i_{kdd}$  and unmasked  $i_t$ , or caused a dramatic shift in the activation range for  $i_{kdd}$  which is inconsistent with previous data (DiFrancesco, Porciatti, and Cohen, 1991).

Because  $i_t$  is a time-dependent increasing inward current, it might contaminate our measure of the reversal of  $i_{kdd}$ . However, in  $5.4$  mM  $\text{K}^+$ , the threshold for  $i_t$  is negative to the reversal of  $i_{kdd}$ . To determine the threshold for the activation of  $i_t$  in lower  $[\text{K}^+]_o$  (where the reversal potential is more negative), hyperpolarizing steps were applied in the absence and presence of  $\text{Ba}^{2+}$  in  $2.7$  mM  $\text{K}^+$  (Fig. 7). In Tyrode

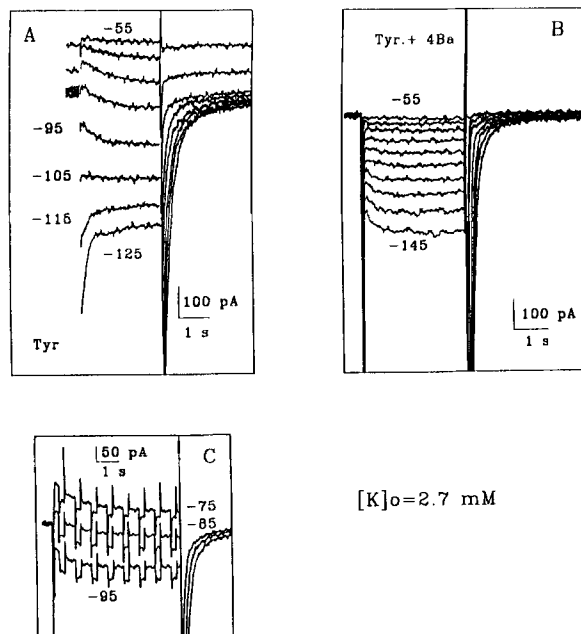


FIGURE 7. Time-dependent currents in lower  $[K^+]_o$ . All panels were recorded from a Purkinje myocyte superfused with 2.7 mM  $[K^+]_o$ . The holding potential was  $-50$  mV and hyperpolarizing pulses were applied to  $-55$ ,  $-65$ ,  $-75$ ,  $-85$ ,  $-95$ ,  $-105$ ,  $-115$ , and  $-125$  mV in the absence (A) and to  $-55$ ,  $-65$ ,  $-75$ ,  $-85$ ,  $-95$ ,  $-105$ ,  $-115$ ,  $-125$ ,  $-135$ , and  $-145$  mV in the presence of 4 mM  $Ba^{2+}$  (B). In C, the changes in slope conductance during  $i_{Kdd}$  were measured by applying small hyperpolarizing steps superimposed on the parent pulse in Tyrode solution (no  $Ba^{2+}$ ). In A, the  $-55$  mV trace was shifted upward by 150 pA and the  $-65$  mV trace by 60 pA to avoid overlap of the current traces. The mean holding current at  $-50$  mV was +137 pA (A), +38 pA (B) and +152 pA (C).

solution (no  $Ba^{2+}$ ), the reversal potential of  $i_{Kdd}$  was  $-105$  mV (A); in the presence of  $Ba^{2+}$ ,  $i_{Kdd}$  was blocked and the threshold for  $i_f$  activation was  $-95$  mV. Note that at  $-95$  mV,  $i_f$  in the presence of  $Ba^{2+}$  (B) had a smaller amplitude and different kinetics than  $i_{Kdd}$  in the Tyrode solution (A). Also in 2.7 mM  $[K^+]_o$  (no  $Ba^{2+}$ ), the slope conductance decreased during the decay of  $i_{Kdd}$  (C).

In six experiments in 2.7 mM  $[K^+]_o$ , in the absence of  $Ba^{2+}$ , the reversal of  $i_{Kdd}$  was  $-106 \pm 10$  mV, whereas in the presence of  $Ba^{2+}$  the threshold for  $i_f$  activation was at  $-95 \pm 6$  mV. Thus, in the lower  $K^+$  a small contribution of  $i_f$  might have shifted the  $i_{Kdd}$  reversal a few mV more negative. This assumes that the  $i_f$  activation threshold in the  $Ba^{2+}$  containing solution is identical to that in normal Tyrode solution and would raise the question of how a clean reversal like that observed in Fig. 7 could be observed.

#### Time-dependent Current and Slope Conductance in the Presence of $Ba^{2+}$

If  $Ba^{2+}$  eliminated  $i_{Kdd}$  (by blocking a decaying potassium conductance) while unmasking the hyperpolarization activated  $i_f$ , the slope conductance should increase during the time-dependent current in the presence of  $Ba^{2+}$  (DiFrancesco, 1981a) (in contrast to the above demonstrated decrease in slope conductance during  $i_{Kdd}$  in  $Ba^{2+}$ -free Tyrode solution).

In Fig. 8, hyperpolarizing voltage clamp steps were applied from a  $V_h$  of  $-50$  mV. In Tyrode solution (top), during a hyperpolarizing voltage clamp step to  $-75$  mV,

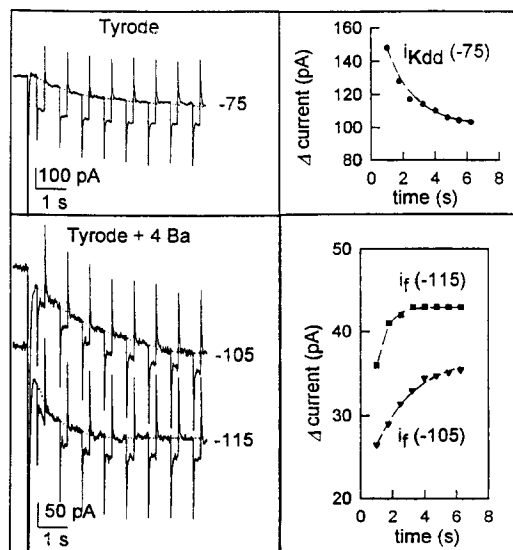


FIGURE 8. Increase in slope conductance during the time-dependent current in the presence of  $Ba^{2+}$ . The myocyte was first perfused in Tyrode solution in the absence of  $Ba^{2+}$  (top). The decrease in amplitude of the current steps in response to the 5-mV pulses superimposed on the 6.4 s test step to  $-75$  mV show that the slope conductance decreases during  $i_{Kdd}$  in the absence of  $Ba^{2+}$ . In the presence of 4 mM  $Ba^{2+}$  (bottom), the increase in amplitude of the current steps in response to the 8-mV pulses superimposed on the test steps to  $-105$  and  $-115$  mV show that the slope conductance increases during the activation of  $i_f$ . The dashed lines, showing the same  $i_{Kdd}$  and  $i_f$  traces without slope conductance measurements, have been added for a better visualization of the

slope conductance changes. The current trace for the step to  $-105$  mV was shifted upward by 130 pA to avoid current overlap. The graphs show the changes in the amplitude of the small current pulses during  $i_{Kdd}$  (top graph, ●) in Tyrode solution and during  $i_f$  at  $-105$  (▼) and  $-115$  (■) mV in the presence of  $Ba^{2+}$  (bottom). In the graphs, the time constants of the currents changes (dashed lines) have been superimposed on the changes in slope conductance (symbols). The time constants of the time-dependent current and slope conductance changes were as follows. For  $\tau[-75(i_{Kdd})]$ : onset current (s) 1.757, slope conductance (s) 1.803. The corresponding numbers for  $[-105(i_f)]$  were 2.436 and 2.070; and for  $[-115(i_f)]$  0.615 and 0.664, respectively. The mean holding current at  $-50$  mV in Tyrode was +181 pA and in Tyrode + 4Ba was  $-58$  pA.

the amplitude of the small superimposed step currents decreased, the ratio between the last and the first step current being 0.7. In the presence of  $Ba^{2+}$  (bottom), the hyperpolarizing steps were applied from the same  $V_h$  to  $-105$  and  $-115$  mV. The amplitude of the superimposed step currents increased during the slowly increasing inward current, the ratio between the last and the first step current at  $-115$  mV being 1.2. The time course of the change in slope conductance should mimic the time course of the current change if only a single conductance with a fixed reversal generates the time-dependent current. The comparison of the time course of the slope conductance changes (symbols) and of the membrane current changes (dashed lines) clearly shows that the time constants were very well matched.

The fact that the slope conductance decreases during  $i_{Kdd}$  in the absence of  $Ba^{2+}$  whereas it increases during  $i_f$  in the presence of  $Ba^{2+}$  supports the notion that  $Ba^{2+}$  blocks one conductance within the diastolic potential range, while revealing another which activates at much more negative potentials. Similar results were observed in a total of three experiments in Tyrode solution with  $Ba^{2+}$ .

That the time-dependent currents in the absence and presence of  $Ba^{2+}$  are different is supported also by the findings illustrated in Fig. 3. As already discussed, in

the absence of  $\text{Ba}^{2+}$ , increasing  $[\text{K}^+]_o$  from 5.4 to 10.8 mM shifted the reversal potential and markedly *reduced* the amplitude of  $i_{\text{Kdd}}$  (A and C). Instead, in the presence of  $\text{Ba}^{2+}$  (B and D), increasing  $[\text{K}^+]_o$  *increased* the amplitude of hyperpolarization activated current (note the different current calibrations in B and D) and did not change its voltage range or direction (as would be expected for  $i_t$ , DiFrancesco, 1981a).

*Cs<sup>+</sup> Effects on the Time-dependent Current in the Presence of Ba<sup>2+</sup>*

Low concentrations of  $\text{Cs}^+$  block  $i_t$  in Purkinje fibers (DiFrancesco, 1981a) and in sinus node myocytes (DiFrancesco, Ferroni, Mazzanti, and Tromba, 1986). The results illustrated in Fig. 9 show that 2 mM  $\text{Cs}^+$  blocked the time-dependent current

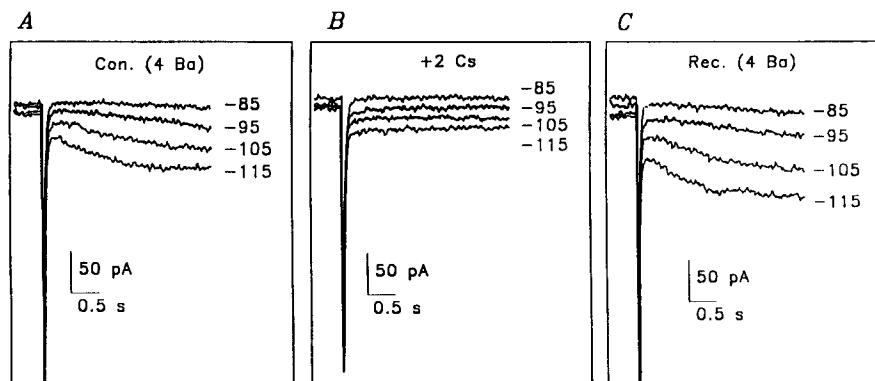


FIGURE 9. Block of  $i_t$  by  $\text{Cs}^+$  in the presence of  $\text{Ba}^{2+}$ . All traces were recorded in the presence of 4 mM  $\text{Ba}^{2+}$ . The holding potential was  $-50$  mV and hyperpolarizing voltage clamp steps were applied to  $-85$ ,  $-95$ ,  $-105$ , and  $-115$  mV. In A,  $i_t$  appeared at  $-95$  mV and increased at more negative voltages. In B, adding 2 mM  $\text{Cs}^+$  to the  $\text{Ba}^{2+}$  solution completely blocked  $i_t$  at all potentials. The block was reversible, as during the washout of  $\text{Cs}^+$   $i_t$  reappeared (C). The mean holding current at  $-50$  mV was  $-177$  pA (A),  $-173$  pA (B), and  $-177$  pA (C).

in the presence of  $\text{Ba}^{2+}$  also in our Purkinje myocytes. Similar results were obtained in a total of three experiments. Thus,  $\text{Cs}^+$  (which has been used as a fingerprint for the  $i_t$  current) blocks a diastolic current associated with a decreasing membrane conductance in the absence of  $\text{Ba}^{2+}$  as well as time-dependent current associated with an increasing membrane conductance in the presence of  $\text{Ba}^{2+}$ .

#### DISCUSSION

The initial investigations of the pacemaker current in Purkinje fibers indicated that diastolic depolarization was caused by the decay of a  $\text{K}^+$  current (Vassalle, 1966; Noble and Tsien, 1968; Peper and Trautwein, 1969). This interpretation was challenged by the experiments of DiFrancesco (1981a,b) and the role of a decaying  $\text{K}^+$

current ( $i_{k2}$ ) as the pacemaker current was replaced by  $i_i$ . Our results suggest that the pacemaker current was not initially misinterpreted, but rather that to avoid certain technical limitations (e.g.,  $K^+$  depletion) experimental procedures were adopted (e.g., block of  $i_{k1}$  by  $Ba^{2+}$ ) that lead to the study of a different time-dependent current. To clarify the factors involved in this controversy, it is necessary to first consider the findings that led to the two different interpretations of the pacemaker current in Purkinje fibers and then examine how our results shed light on this controversial matter.

#### *The Evidence for and Against the $K^+$ Pacemaker Current*

The pacemaker current was believed to be a  $K^+$  current on the basis that it was associated with a decrease in slope conductance (Vassalle, 1966), that it reversed near the potassium equilibrium potential (Vassalle, 1966; Noble and Tsien, 1968; Peper and Trautwein, 1969), and that its reversal potential shifted as a function of  $[K^+]_o$  (Noble and Tsien, 1968; Peper and Trautwein, 1969).

The relationship between the action potential and the pacemaker current was believed to be as follows (Vassalle, 1966). The pacemaker current would be partially activated at the resting potential. During the action potential the pacemaker current would activate and during phase 3 repolarization the increased membrane conductance induced by the pacemaker channel would allow the potential to approach  $E_K$ . However, the pacemaker channel would slowly deactivate at negative potentials and diastolic depolarization would ensue. If  $[K^+]_o$  is lowered, the action potential is followed by a larger undershoot and a larger diastolic depolarization as the activation of  $i_{kdd}$  during the action potential would allow the membrane to approach the more negative  $E_K$  (Vassalle, 1965).

This series of events appeared straightforward. However, there were findings that could not be easily explained on the basis of a simple  $K^+$  selective pacemaker current. The reversal potential was found to be somewhat more negative than the theoretical value (Cohen et al., 1976; Peper and Trautwein, 1969). In addition, there was evidence that in ungulate Purkinje fibers (the preparation most often studied) there were fluctuations of  $[K^+]$  in narrow clefts between cells (Cohen et al., 1976; Baumgarten and Isenberg, 1977). Further, the reversal potential was not fixed but shifted to less negative potentials if the hyperpolarizing clamp step was preceded by a depolarizing step, consistent with fluctuations in  $[K^+]$  in the narrow clefts during each voltage clamp step (DiFrancesco et al., 1979).

These findings prompted the suggestion that the reversal of the time-dependent current might not reflect the ionic selectivity of the pacemaker current. Instead, large hyperpolarizing voltage steps would deplete cleft  $[K^+]$  and cause a time-dependent decline in the background  $K^+$  current  $i_{k1}$ . The inward current that decreases as a function of time would then not be the reversed  $i_{kdd}$ , but instead it would be the declining  $i_{k1}$  due to  $K^+$  depletion. The depletion would be largest at potentials negative to  $E_K$ , because the driving force for  $K^+$  becomes inward directed and  $i_{k1}$  conductance increases: according to this hypothesis, the reversal of the time-dependent current negative to  $E_K$  would be spurious.

*The Evidence for and Against the  $i_f$  Pacemaker Current*

The possible role of  $K^+$  depletion in determining a spurious reversal potential was investigated by blocking  $i_{K1}$  with  $Ba^{2+}$  (5–10 mM) (DiFrancesco, 1981*a,b*). It was reasoned that the block of  $i_{K1}$  (by preventing the  $K^+$  depletion) would abolish the depletion current through the  $i_{K1}$  channel. If the reversal were due to  $K^+$  depletion, it should no longer occur in the presence of  $Ba^{2+}$ . Indeed, in the presence of  $Ba^{2+}$ , a time-dependent current ( $i_f$ ) was present that was associated with an increasing slope conductance and did not reverse at negative potentials. It was concluded that the evidence supporting the  $K^+$  current was spurious and that in fact the pacemaker current was  $i_f$  (DiFrancesco, 1981*a,b*).

The actions of  $Cs^+$  on the time-dependent current in Tyrode solution appeared to further support the  $i_f$  hypothesis. The elimination of an outward pacemaker current was expected to cause a larger inward jump on hyperpolarization. Because  $Cs^+$  abolished the pacemaker current, but did not increase the initial jump during a hyperpolarizing step, it was concluded that  $Cs^+$  was a specific blocker of the inward pacemaker current  $i_f$  (DiFrancesco, 1981*a*).

Computer simulations by DiFrancesco and Noble (1985) lent additional support to the  $i_f$  hypothesis by accounting for some of the findings which had been presumed to support  $i_{K1}$ . Thus, the dependence of the apparent reversal on  $[K^+]_o$  could be accounted for by a similar dependence of  $K^+$  depletion on  $[K^+]_o$ . Also, the decrease in slope conductance (in the absence of  $Ba^{2+}$ ) was explained by the progressive decline of cleft  $[K^+]$  which reduces  $i_{K1}$  conductance. However, in this connection, it should be pointed out that we found that the slope conductance decreases during the pacemaker current at potentials *positive* to  $E_K$  (Vassalle, 1966; Vassalle et al., 1992), where several different tests (with  $Ba^{2+}$ ,  $Cs^+$ , and high  $[K^+]_o$ ) failed to detect  $K^+$  depletion at those potentials (Vassalle et al., 1992).

The  $i_f$  hypothesis has gained wide acceptance. However, some findings emerged that could not be readily fitted by the explanation offered. We found that the depletion current was not large enough to be responsible for the pseudo-reversal near  $E_K$  (Cohen and Falk, 1980). Further,  $Ba^{2+}$  reduced or eliminated the time-dependent current at the most positive diastolic potentials (Cohen, Falk, and Mulrine, 1983). Also, in canine Purkinje strands the extracellular spaces are larger than in the ungulate tissue (Eisenberg and Cohen, 1983) and yet the pacemaker current still reversed near the expected  $E_K$  (Cohen et al., 1983).

The interpretation of many of the experiments supporting the  $i_f$  hypothesis relies on specific actions of  $Ba^{2+}$  and  $Cs^+$ : a specific block of the background potassium current  $i_{K1}$  by  $Ba^{2+}$  and a specific block of  $i_f$  by  $Cs^+$ . It is necessary to consider how specific the actions of these agents are.

 *$Ba^{2+}$  Blocks Currents Other Than  $i_{K1}$* 

$Ba^{2+}$  blocks  $i_{K1}$  in Purkinje fibers (DiFrancesco, 1981*a*, 1982), and so it was employed to eliminate the  $K^+$  depletion on hyperpolarization. However,  $Ba^{2+}$  also blocks other  $K^+$  currents such as the delayed rectifier  $i_K$  (Gintant et al., 1985), the  $K^+$  current induced by acetylcholine (Carmeliet and Mubagwa, 1986) and the one dependent on ATP (Takei and Noma, 1984). Therefore, if a time- and voltage-

dependent  $K^+$  current exists in the diastolic potential range as we suggest, it is not surprising that the application of 5–10 mM  $Ba^{2+}$  to study the pacemaker current would eliminate  $i_{Kdd}$ .

#### *Effects of Cesium on $a_{Na}^i$ , Membrane Potential, and Membrane Currents*

Because  $Cs^+$  did not increase the instantaneous current on hyperpolarization, it was concluded that  $Cs^+$  blocks not an outward current but the inward  $i_f$  (DiFrancesco, 1981a). Because of this finding,  $Cs^+$  has been employed as a fingerprint for  $i_f$ .

However, the above rationale appears uncertain: the steady state holding current changes in an inward direction when  $Cs^+$  is applied (Isenberg, 1976; Vassalle et al., 1992). This would occur if  $Cs^+$  blocks an outward  $K^+$  pacemaker current. In  $Cs^+$  containing Tyrode solution, the residual outward pacemaker current would be smaller, because the maximal conductance is reduced. Therefore, on voltage clamp hyperpolarization the instantaneous decrease in outward current would be less and the instantaneous current jump would be smaller (Vassalle et al., 1992).

$Cs^+$  decreases  $a_{Na}^i$  and it has been proposed that it does so by blocking  $i_f$  (Glitsch, Pusch, and Verdonck, 1986; Chae, Wang, Gong, and Lee, 1990). However,  $Cs^+$  stimulates the  $Na^+/K^+$  pump activity in Purkinje fibers (Eisner and Lederer, 1980) and therefore it could decrease  $a_{Na}^i$  by that mechanism, as several of our experiments indicate (Iacono and Vassalle, 1990). Thus,  $Cs^+$  decreases  $a_{Na}^i$  also in the absence of  $i_f$ , e.g., in the absence of diastole or in zero  $[K^+]_o$  (where  $i_f$  is absent, DiFrancesco, 1982), or in quiescent myocardial fibers.  $Cs^+$  does not decrease  $a_{Na}^i$  in the presence of toxic doses of strophanthidin (Iacono and Vassalle, 1990), which do not affect (Lederer and Tsien, 1976) the pacemaker current but block the  $Na^+/K^+$  pump. In quiescent Purkinje fibers,  $Cs^+$  causes a *transient* hyperpolarization, which might be attributable to a block of  $i_f$ . However, this transient hyperpolarization also occurs when  $i_f$  is absent or deactivated, but not when the  $Na^+/K^+$  pump is almost maximally stimulated or is blocked (Sternlicht and Vassalle, 1992).

In guinea pig ventricular myocytes,  $Ba^{2+}$  induces a diastolic depolarization and a  $K^+$ -selective pacemaker current (see Valenzuela and Vassalle, 1991) in a voltage range where there is no  $i_f$ .  $Cs^+$  (4 mM) abolishes the  $Ba^{2+}$ -induced pacemaker potential and current (Shen and Vassalle, 1994), providing another example that  $Cs^+$  is not a specific blocker of  $i_f$ .

#### *Contribution by Other Currents to $i_{Kdd}$*

During the action potential,  $i_K$  is activated at the plateau and its decay could conceivably contribute to the pacemaker potential. Our results show that  $i_{Kdd}$  is little affected by different degrees of  $i_K$  activation up to 1.7 s in duration and therefore the decay of  $i_K$  seems to contribute little to diastolic depolarization in Purkinje fibers. We have not tested whether the full activation of  $i_K$  ( $i_{Kr}$  and  $i_{Ks}$ ) would distort the measurements of  $i_{Kdd}$ , but none of our protocols used to analyze the time-dependent currents on hyperpolarization would give rise to the complete activation of  $i_{Ks}$ . Therefore, it would appear that neither component of  $i_K$  is responsible or distorts our measurement of  $i_{Kdd}$  with the protocols we employed.



The background  $i_{K1}$  can undergo time-dependent inactivation (e.g., Tromba and Cohen, 1990). However, the inactivation of  $i_{K1}$  occurs at potentials negative to  $E_K$ , i.e., outside the range of the pacemaker potential (Sakmann and Trube, 1984; Tromba and Cohen, 1990). Therefore, the time-dependent current positive to  $E_K$  should not be due to a time-dependent inactivation of  $i_{K1}$ . Although we cannot be sure that inactivation of  $i_{K1}$  does not contribute to the reversed  $i_{Kdd}$ , it is worth pointing out that the time constant of decay of  $i_{Kdd}$  is similar at potentials immediately positive and immediately negative to  $E_{rev}$ . In this regard, it should be pointed out that the decrease in conductance during  $i_{Kdd}$  occurred at potentials both positive and negative to  $E_K$ , showing that we are not dealing with  $i_f$  artificially reversed by the inactivation of  $i_{K1}$ . The absence of mixed reversals supports this conclusion. It should be added that even if the time-dependent current positive to  $E_K$  were to be due to the inactivation of  $i_{K1}$ , it would still be a  $K^+$  time-dependent pacemaker current. However, such an inactivation of  $i_{K1}$  at potential positive to  $E_K$  is contrary to actual findings (Sakmann and Trube, 1984; Tromba and Cohen, 1990) and would raise the question as to why  $i_{K1}$  inactivation should occur in Purkinje but not in myocardial fibers.

One might inquire as to whether the overlapping of  $i_f$  should shift the reversal potential of  $i_{Kdd}$  to more negative values. The answer seems to be that the threshold for  $i_f$  is negative to  $E_K$  in both 5.4 and 10.8 mM  $[K^+]_o$ , and therefore  $i_f$  would not interfere with the reversal potential of  $i_{Kdd}$ . In 2.7 mM  $[K^+]_o$ , we found that the average threshold for  $i_f$  (-95 mV) was fairly close to the reversal potential of  $i_{Kdd}$  (-106 mV). Therefore, in some experiments  $i_f$  could have shifted the reversal potential in 2.7 mM  $K^+$ . However, this suggestion assumes that the threshold for  $i_f$  is the same in the absence and presence of  $Ba^{2+}$ . We have no way to prove or disprove this point since  $i_f$  was not apparent in Tyrode solution in the absence of  $Ba^{2+}$ . If  $i_f$  was contaminating  $i_{Kdd}$  in Tyrode solution, the kinetics of  $i_f$  and  $i_{Kdd}$  would have to be perfectly matched since mixed reversals were not observed (see Fig. 3, A and B, and Fig. 7).

### Conclusions

Our results show that at negative potentials two time-dependent currents are present in Purkinje myocytes that have several distinguishing characteristics.

Thus, the time-dependent current that occurs in the range of diastolic depolarization ( $i_{Kdd}$ ) appears at a less negative threshold, reverses near  $E_K$ , its reversal depends on  $[K^+]_o$ , undergoes a decrease in slope conductance at potentials positive and negative to  $E_K$ , decreases in high  $[K^+]_o$ , is blocked by  $Cs^+$  and disappears in the presence of  $Ba^{2+}$ . These findings are consistent with a voltage- and time-dependent decay of a  $K^+$  current.

The other time-dependent current that appears in the presence of  $Ba^{2+}$  ( $i_f$ ) has an average threshold near or negative to the predicted  $E_K$  in 5.4 and 2.7 mM  $[K^+]_o$ , does not reverse at more negative potentials, increases in high  $[K^+]_o$ , undergoes an increase in slope conductance on hyperpolarization and is blocked by  $Cs^+$  but not by  $Ba^{2+}$ . Most of these characteristics are consistent with the  $i_f$  (or  $i_h$ ) found also in cardiac (Earm, Shimoni, and Spindler, 1983; Yu et al., 1993) and noncardiac (e.g., see Schlichter, Bader, and Bernheim, 1991) tissues that do not have a pacemaker current.

The block of both  $i_{Kdd}$  and  $i_f$  by  $\text{Cs}^+$  shows that  $\text{Cs}^+$  is not a selective blocker of  $i_f$  and the block of  $i_{Kdd}$  but not of  $i_f$  by  $\text{Ba}^{2+}$  shows that  $\text{Ba}^{2+}$  is not a selective blocker of  $i_{K1}$ . Thus, for both  $\text{Ba}^{2+}$  and  $\text{Cs}^+$ , assumptions about their selectivity led to potentially erroneous conclusions concerning the role of  $i_f$  in Purkinje pacemaker activity.

Our experiments argue for the existence of a time- and voltage-dependent decaying  $\text{K}^+$  conductance which is important for diastolic depolarization in Purkinje myocytes. The absolute magnitude of the measured current at  $-75$  mV (44 pA) could generate a change in the pacemaker depolarization rate of 160 mV/s in a Purkinje myocyte of average capacitance 280 pF (Cohen, Datyner, Gintant, Mulrine, and Pennefather, 1987). This current has been missed by some of the previous investigators, either because they employed  $\text{Ba}^{2+}$  in their bathing solution which blocks this current, or did not attempt to investigate the conductance changes in the diastolic range of potentials. We also employed a pipette solution with a [Ca] buffered to the physiologic range (see Methods), which differs from most previous patch clamp investigations.

Finally, it is not surprising that a function as important as pacemaker activity has a number of potentially redundant mechanisms. Although a decaying  $\text{K}^+$  current may be important in the normal pacemaker potential,  $i_f$  could play a role in preventing excessive hyperpolarization. For example, following periods of rapid activity,  $i_f$  could antagonize the  $\text{Na}^+/\text{K}^+$  pump current, activated to a larger degree (see Vassalle, 1987) because of the increased Na load.

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