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THE BASIS FOR THE MEMBRANE POTENTIAL OF QUIESCENT CELLS OF THE CANINE CORONARY SINUS

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SUMMARY

1. During prolonged periods of quiescence, the membrane potential of cells in the isolated canine coronary sinus, exposed to normal Tyrode solution containing 4 mM-K, declines to about −60 mV. The nature of the resting potential was investigated, in small strips of coronary sinus tissue mounted in a fast-flow system, by recording the membrane potential responses to sudden changes in the extracellular ionic environment.

2. At extracellular K concentrations ($[K]_o$) from 0 to 64 mM the resting potential was little affected by replacing all but 1 mM of external Cl ions with isethionate and methylsulphate ions.

3. At $[K]_o$ levels from 4 to 150 mM the resting potential was reasonably well described by the Goldman–Hodgkin–Katz equation on the assumption that the intracellular K concentration ($[K]_i$) was 155 mM and that the ratio of membrane permeability coefficients for Na and K, P_{Na}/P_K, was 0·07.

4. In the presence of a high concentration of acetylcholine or carbachol ($\geqslant 1$ μM), the resting potentials at $[K]_o$ levels from 1 to 150 mM approximated K equilibrium potentials (E_K) calculated on the assumption that $[K]_i$ was 155 mM.

5. At $[K]_o$ levels $\leqslant 8$ mM replacing most of the external Na with sucrose or Tris caused a substantial hyperpolarization, whereas application of 1–2 μM-tetrodotoxin caused only slight hyperpolarization.

6. A transient hyperpolarization, due to enhanced electrogenic Na extrusion, was recorded on switching back to 4 mM-K following brief exposures to K-free solution; no transient hyperpolarization was recorded in the presence of 5 μM-acetylstrophanthidin. The acetylstrophanthidin itself caused a rapid depolarization of several millivolts.

7. Preliminary conductance measurements made with two micro-electrodes in some smaller preparations indicate that the steady-state current–voltage relationship is N-shaped.

8. We conclude that the low membrane potential of quiescent coronary sinus cells reflects not a low $[K]_i$ but rather a relatively high ratio P_{Na}/P_K, of about 0·07: the Na ions flow into the cells via predominantly TTX-insensitive pathways and are extruded by the electrogenic Na/K exchange pump, which thereby makes a substantial contribution to the resting potential.

INTRODUCTION

While studying triggered activity in cells of the canine coronary sinus, Wit & Cranefield (1977) noted that the resting potential often declined to a steady low level during prolonged periods of quiescence, and sometimes became so low that the cells could not be excited by extracellular stimuli. However, a marked hyperpolarization can be evoked in such cells by application of acetylcholine or noradrenaline (Wit & Cranefield, 1977; Wit, Cranefield & Gadsby, 1980; cf. Boyden, Cranefield & Gadsby 1983). It is necessary to understand the basis of the resting potential of coronary sinus cells before attempting to explain either the effects of noradrenaline or the complex changes in the resting, or maximum diastolic, potential that are seen after, and during, bursts of triggered activity under a variety of conditions (Wit & Cranefield, 1977; Gadsby, Wit & Cranefield, 1979; Wit, Cranefield & Gadsby, 1980, 1981). We have investigated the basis of the resting potential of coronary sinus cells by examining the effects of changes in the extracellular ionic environment. To minimize the eventual changes in intracellular ionic composition expected to result from the changes in extracellular ion concentrations, we wished to keep the exposures to test solutions brief, which meant that the changes in extracellular environment had to be completed rapidly. For that reason, we studied very small strips of coronary sinus tissue mounted in the narrow channel of a modified Hodgkin–Horowicz (1959) fast-flow system. Changes in resting potential could then be recorded in response to steplike changes in the composition of the superfusate, and membrane potential measurements made under several test conditions could, within the space of a few minutes, be bracketed between control measurements.

We present here evidence that the low membrane potential recorded in quiescent cells of the coronary sinus exposed to normal levels of extracellular K concentration ($[K]_o$) is not due to those cells having an abnormally low intracellular K concentration ($[K]_i$) but is due rather, to the ratio of the membrane permeability to Na ions (P_{Na}), to that to K ions (P_K), being somewhat larger than it is, for example, in cardiac Purkinje fibres or working myocardial cells. We find that the Na ions flow into the cells predominantly via tetrodotoxin-insensitive pathways, and appear to be extruded by an electrogenic Na/K exchange pump which makes a substantial contribution to the resting potential. The steady-state current–voltage relationship proves to be sigmoidal or N-shaped and, in the virtual absence of chloride ions, appears to have a region of shallow, or even negative, slope conductance near diastolic membrane potentials (about -60 to -80 mV), so that small changes in membrane current may have relatively large effects on membrane potentials and on the excitability of coronary sinus cells.

METHODS

Hearts were obtained from mongrel dogs anaesthetized with sodium pentobarbitone (30 mg/kg I.V.) and were rinsed and then kept, at room temperature, in a Tyrode solution with the following composition (mM): NaCl, 137; KCl, 4; $NaHCO_3$, 12; dextrose, 5·5; NaH_2PO_4, 1·8; $MgCl_2$, 0·5; $CaCl_2$, 2·7. The coronary sinus was isolated from the heart and was cut open along its length and pinned out flat (see Wit & Cranefield, 1977). Then, using a dissecting microscope at high magnification, small strips of tissue about 2 mm long and less than 1 mm wide were carefully

dissected from the roof or floor of the distal half of the coronary sinus. The strips were suspended between two 100 μm wide insect pins in the narrow channel of a modified Hodgkin–Horowicz (1959) fast-flow system. A continuous flow of superfusate, of 5 ml./min, was maintained throughout all experiments; at that rate, the composition of the superfusate near the centre of the channel could be changed with a half-time of approximately 0·5 sec, by simply manipulating the valve at the entrance to the chamber. For additional details of the flow system see Gadsby & Cranefield (1977, 1982). The superfusing solutions were pre-heated before they entered the chamber and their temperature was monitored with a small thermistor bead positioned close to the preparation. The experiments were carried out at 35·5–37·5 °C but, during any single experiment, the variation in temperature was less than $\pm$0·2 °C.

The coronary sinus cells were initially superfused for 30–60 min with the Tyrode solution described above, to allow complete recovery from the dissection procedure, before starting the experiment proper. Variation of the K concentration of the Cl-containing Tyrode solutions was achieved by substitution of KCl for NaCl, or vice versa. For most experiments, however, after the 30–60 min equilibration period, the superfusate was switched to a virtually Cl-free, modified Tyrode solution in which the major anion was isethionate. That solution contained (mM): Na isethionate, 137 (Koch-Light, Colnbrook, Bucks., England); K methylsulphate, 4 (Hopkin and Williams, Chadwell Heath, Essex, England); $NaHCO_3$, 12; dextrose, 5·5; NaH_2PO_4, 1·8; $MgCl_2$, 0·5; Ca methanesulphonate, 2·7 (made with $CaCO_3$ and methanesulphonic acid; Eastman Kodak Corp., Rochester, NY). The potassium concentration of this low-Cl solution was altered by replacing Na isethionate with K methylsulphate, or vice versa. In some experiments, low-Na superfusates were used in which Na isethionate was replaced by either sucrose or by Tris methanesulphonate (made with Tris base and methanesulphonic acid) in the following proportions: Na isethionate, 137 mM; sucrose, 248 mM; Tris methanesulphonate, 137 mM. In these solutions, K concentration was varied as usual by replacing appropriate amounts of sucrose or Tris methanesulphonate with K methylsulphate or vice versa. All solutions were bubbled with 95 % O_2–5 % CO_2 mixture. In a few experiments, the bicarbonate was replaced with 5 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, Sigma Chemical Co., St. Louis, MO; adjusted to pH 7·3) and these solutions were bubbled with pure oxygen. All of the membrane potential responses to changes in the extracellular environment reported here for bicarbonate-buffered solutions could be obtained also in HEPES-buffered solutions. Small amounts of concentrated stock solutions of the following drugs were added on occasion to the superfusion solutions to produce the uniform concentration indicated in the text: acetylcholine chloride (Sigma), carbamylcholine chloride (carbachol, Sigma), tetrodotoxin (TTX, Sigma), lidocaine hydrochloride (xylocaine, Astra Pharmaceuticals, Worcester, MA), racemic verapamil (gift from Knoll AG, Ludwigshafen, F.R.G.). The cardiac steroid, 3-acetylstrophanthidin (kindly provided by Eli Lilly & Co., Indianapolis, IN) was added from a refrigerated stock solution of 5 mM-acetylstrophanthidin in ethanol. Control experiments showed that 0·1 % by volume ethanol had no effect on the resting membrane potential of coronary sinus cells.

Conventional glass micro-electrodes filled with 3 M-KCl (resistances, 15–40 MΩ; tip potentials less negative than -5 mV) were used both for membrane potential recording and for intracellular current injection. Membrane potential was recorded differentially with respect to a reference half-cell (sintered Ag/AgCl/Pt-black pellet; Annex Research, Santa Ana, CA) connected to the bath via a flowing 3 M-KCl junction; the latter was usually positioned downstream from the preparations, close to the suction tube which removed the continuously flowing superfusate. A similar half-cell connected the bath to an operational amplifier, in ammeter configuration, which held the bath at virtual ground and monitored applied currents. Membrane potentials and applied currents were recorded using a rectilinear pen recorder (frequency response, 3 dB down at 75 Hz; Gilson Medical Electronics, Middleton, WI) and were simultaneously displayed on a Tektronix 5103N storage oscilloscope and photographed on polaroid film. A constant current isolation unit was used to inject 'double ramps' of current, the current rising linearly with time to a maximum and then declining linearly at the same rate. We adopt the usual sign convention for membrane current, viz., that a net outward flow of positive ions across the cell membrane constitutes positive (or outward) current.

Before switching to the low-Cl solution, resting membrane potentials were sampled in the 4 mM-KCl-containing solution, and were routinely found to be about -50 to -60 mV. The resting potentials usually declined slightly in the low-Cl solution and, thereafter, they tended to increase slowly over the course of several hours: in one experiment, for instance, the membrane potential

was monitored continuously during a single impalement and was found to increase gradually by 5 mV over 2 hr, in the presence of a constant extracellular environment. The mechanism of this hyperpolarization is not known, but it seems unlikely that it results from 'healing over' of cells damaged during dissection, since the time course of that process is known to be much faster, i.e. half-time on the order of one minute under comparable conditions (e.g. Ochi & Nishiye, 1973).

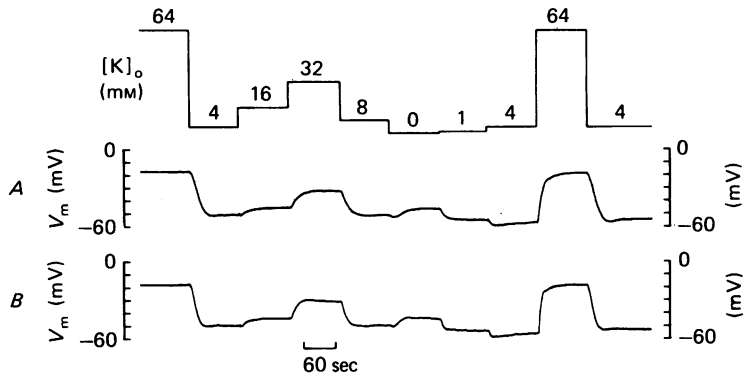

Fig. 1. Changes in resting potential, recorded simultaneously in two coronary sinus preparations, in response to the step changes in $[K]_o$ indicated by the upper line. The two coronary sinus preparations were suspended side-by-side in the centre of the narrow channel of the fast-flow chamber. Note the relative insensitivity of the resting potential to changes in $[K]_o$ below 16 mM. Low-Cl solutions were used throughout this experiment; external Ca concentration, 0·9 mM.

RESULTS

The rapid response of the membrane potential of coronary sinus cells to sudden alteration of the ionic composition of the superfusate under the conditions of our study is demonstrated in Fig. 1, which illustrates results obtained simultaneously in two preparations small enough to be suspended side by side in the flow chamber. The records show changes in resting potential caused by step changes in $[K]_o$ over the range 0–64 mM, in low-Cl solutions. Within about one minute of each change of solution the membrane potential reached a new steady level, and the amplitudes of the changes in potential in the two preparations were similar. The voltage changes were somewhat faster in preparation *B* than in preparation *A*, suggesting that diffusion equilibration occurred more rapidly in the extracellular spaces of preparation *B*.

More importantly, Fig. 1 shows that the membrane potential of quiescent cells of the coronary sinus is relatively insensitive to $[K]_o$ over a considerable range of concentrations, changing little from its steady level at 4 mM-extracellular K of about −55 mV as $[K]_o$ is varied between 1 and 8 mM. The membrane is depolarized by a few millivolts when $[K]_o$ is either lowered to nominally zero or raised to 16 mM, but is more markedly depolarized when $[K]_o$ is raised to 32 or 64 mM.

Fig. 2 summarizes results from a similar experiment, and shows measurements of membrane potential plotted against log $[K]_o$. In this experiment, steady-state resting potentials were determined several times at each of seven levels of $[K]_o$ (from 0 to 64 mM), first in Cl-containing Tyrode solution, and then in low-Cl solution. At least 60–90 sec were allowed for extracellular equilibration before making measurements at each new level of $[K]_o$, and at least 30 min were allowed for cellular equilibration

after changing the extracellular Cl concentration ($[Cl]_o$). Five impalements were required to obtain the sixty-two measurements made in Cl-containing solution and six impalements were needed to complete the sixty-four measurements in virtually Cl-free solution. The results shown in Fig. 2 confirm that the relatively low resting potential of coronary sinus cells is approximately independent of $[K]_o$ over the range 1–8 mM, but that the membrane potential declines progressively as $[K]_o$ is raised from

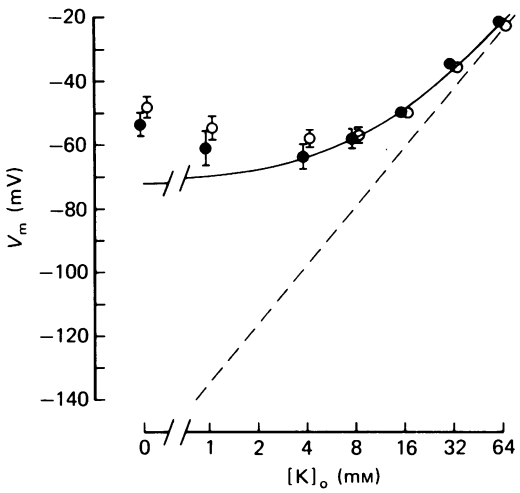

Fig. 2. The relationship between the resting potential and log $[K]_o$ determined in solutions in which the major anion was either chloride (filled circles) or isethionate (open circles). Each point represents the average of three to twenty-three measurements (mean, nine measurements), and the vertical bars indicate ±S.D. where the S.D. is greater than the radius of the circles. The dashed line shows E_K calculated from the Nernst equation assuming $[K]_i$ to be 155 mM and the temperature to be 36·5 °C. The curved line is drawn according to the Goldman–Hodgkin–Katz equation on the additional assumptions that $[K]_o + [Na]_o = 155$ mM, $[Na]_i = 10$ mM, and $\alpha = 0{\cdot}07$.

8 to 64 mM. Moreover, the relationship between the steady resting potential and log $[K]_o$ was practically unaltered by lowering $[Cl]_o$ from 147·4 to 1 mM: the marked reduction in $[Cl]_o$ had no effect on the resting potential at $[K]_o$ levels of 8–64 mM but caused, in this particular experiment, a small depolarization at $[K]_o$ levels of 1–4 mM. Even at low levels of $[K]_o$, however, a change to low-Cl-solutions did not always cause depolarization. In fact, in experiments on twenty preparations that had resting potentials of −50 to −70 mV in 4 mM-K solution, the average depolarization seen a few minutes after switching from normal to virtually Cl-free solution amounted to only 0·5 mV(S.D. ±3·4 mV; i.e. after allowing sufficient time for dissipation of any liquid junction potential in the extracellular space, see, e.g. Spitzer & Walker, 1979). The large standard deviation reflects the fact that on some occasions a small hyperpolarization, rather than a depolarization, was recorded. Although the origin of this variability remains unexplained, the small absolute magnitude of the average potential change recorded in response to such a large change in $[Cl]_o$ suggests that the permeability of the membrane of coronary sinus cells to Cl ions is small relative to the permeability to other ions.

The resting potential can be defined as a level of membrane potential at which the sum of all membrane currents is zero, and at which the membrane conductance has a positive slope. With this in mind, we can attempt to interpret the effects of $[K]_o$ on the resting potential shown in Figs. 1 and 2.

If the surface membrane of coronary sinus cells were to be exclusively permeable to K ions, the resting potential at any level of $[K]_o$ would be the potential at which K current is zero, i.e. the equilibrium potential for K ions (E_K), given by the Nernst equation: $E_K = (RT/F) \ln ([K]_o/[K]_i)$, where R, T and F have their usual meanings. Since the resting potential at 4 mM-external K is about -60 mV, if that resting potential were to approximate E_K then $[K]_i$ would have to be about 40 mM. Fig. 1 shows that 30 sec after $[K]_o$ is suddenly raised from 4 to 64 mM, the steady resting potential is about -20 mV; if E_K under those conditions were also -20 mV then $[K]_i$ would have to be about 150 mM. In other words, were the resting potential always equal to E_K, the potential changes recorded on increasing $[K]_o$ from 4 to 64 mM could result only from an increase in $[K]_i$ from 40 to 150 mM; moreover, that increase would have to occur in less than half a minute. Since that large and rapid a rise in $[K]_i$ is highly improbable we can conclude that the resting potential is not equal to E_K at all levels of $[K]_o$ and, hence, can further conclude that coronary sinus cells are not exclusively permeable to K ions.

If we assume that quiescent coronary sinus cells are permeable not only to K ions but also to Na ions, then, on the basis of further simplifying assumptions, we would expect the steady resting potential, V_r, to vary with $[K]_o$ according to the Goldman (1943), Hodgkin & Katz (1949) equation (see also Mullins & Noda, 1963):

$$V_r = \frac{RT}{F} \ln \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i}, \tag{1}$$

where $[Na]_o$ and $[Na]_i$ are the extracellular and intracellular Na ion concentrations, and α represents the ratio, P_{Na}/P_K, of the membrane permeability coefficients for Na and K ions. When P_K is very high relative to P_{Na}, α will be very small and eqn. (1), predicts that the resting potential will approximate E_K, except at extremely low $[K]_o$ levels, when the term $\alpha[Na]_o$ makes a significant (depolarizing) contribution to the resting potential. But if α is not small, the term $\alpha[Na]_o$ will make an important contribution to the resting potential even at moderate levels of $[K]_o$. This effect is illustrated by the curved line drawn through the resting potentials obtained at moderately high levels of $[K]_o$ in Fig. 2. That curve was drawn according to eqn. (1) on the assumption that $[K]_i$ is 155 mM and that α is 0·07. The dashed line shows the values of E_K predicted by the Nernst equation if $[K]_i$ is 155 mM. Comparison of the two lines reveals that at levels of $[K]_o$ higher than 16 mM, the change in resting potential caused by a change in $[K]_o$ is approximately that predicted by the Nernst equation; that is so because the term $\alpha[Na]_o$ in the numerator of eqn. (1) remains small in comparison to $[K]_o$ so that, at high levels of $[K]_o$, V_r approximates E_K. Under those conditions, the measured values of resting potential suggest that $[K]_i$ is, indeed, about 155 mM. $[K]_i$ may be expected to remain relatively constant when $[K]_o$ is suddenly reduced from 64 to 4 mM, so that the likely explanation for the low (-60 mV) resting potential at 4 mM $[K]_o$ is not that $[K]_i$ is low, but that α is large enough to make the term $\alpha[Na]_o$ assume major importance for $[K]_o$ levels < 16 mM.

In other words, these results suggest that in coronary sinus cells $[K]_i$ is approximately 155 mM and the resting membrane is not exclusively permeable to K ions but is also permeable to some other ions (including, presumably, Na ions).

Effect of acetylcholine

Additional support for these conclusions comes from experiments in which we took advantage of the well-known muscarinic action of acetylcholine (ACh) to cause a

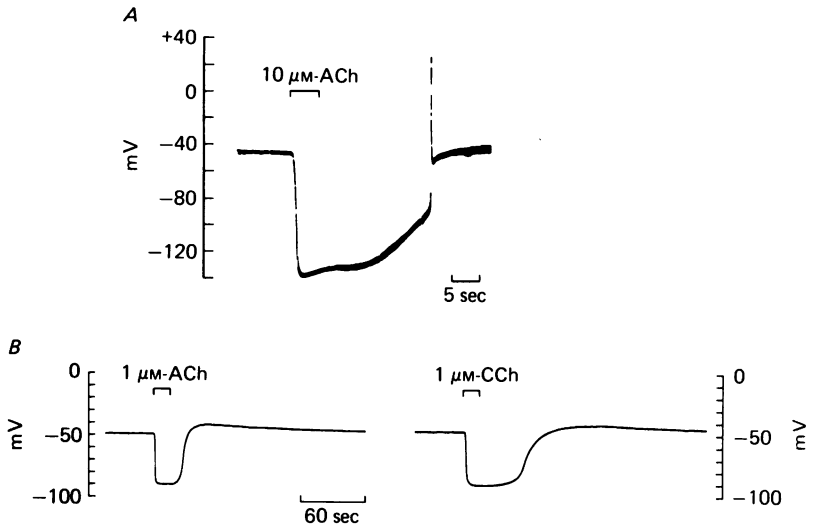

Fig. 3. Changes in membrane potential caused by briefly exposing coronary sinus preparations either to acetylcholine (ACh) or to carbachol (CCh). *A*, response to a 5 sec application of 10 μM-ACh recorded in a preparation superfused with 0 K, low Cl solution. *B* shows effects of 15 sec applications of either 1 μM-ACh or 1 μM-CCh recorded in a preparation exposed to normal, 4 mM-K, Cl-containing solution. Note that all three records, but especially those in *B*, reveal a transient depolarizing overshoot of the steady resting potential after washing out the ACh or CCh (see Discussion).

specific increase in the K ion permeability of atrial cell membranes (for review, see Hutter, 1964). If acetylcholine similarly increases P_K in coronary sinus cells (cf. Wit & Cranefield, 1977; see also Boyden *et al.* 1983), then acetylcholine would be expected to greatly increase the low resting potential seen at low $[K]_o$ values if that low membrane potential reflects a relatively low P_K, but not if it reflects a low $[K]_i$. The records in Fig. 3 show clearly that the muscarinic agonists acetylcholine and carbachol (CCh) cause marked hyperpolarization of quiescent coronary sinus cells exposed to normal or low levels of $[K]_o$. For example, Fig. 3*A* illustrates a hyperpolarization of more than 90 mV caused by a 5 sec application of 10 μM-ACh to a coronary sinus strip exposed to nominally K-free, low-Cl solution; the resting potential was initially −45 mV but increased to −138 mV within 2 sec of exposure to ACh. The subsequent depolarization, caused by quickly washing out the ACh, gave rise at about −75 mV to an action potential with an overshoot of +25 mV. The records shown in Fig. 3*B* were obtained within a few minutes of each other in a preparation superfused with normal Tyrode solution (4 mM-K); they show that

15 sec applications of either 1 μM-ACh (left) or 1 μM-CCh (right) caused rapid hyperpolarization to the same potential, namely −91 mV. The effects of ACh and CCh, determined over a wide range of $[K]_o$ values, are summarized in the graph of Fig. 4. Filled and open symbols are used to distinguish between results obtained in two different coronary sinus strips; each point represents a single measurement, the circles indicating control levels of membrane potential determined just before the increased membrane potentials were recorded in the presence of a high concentration of either

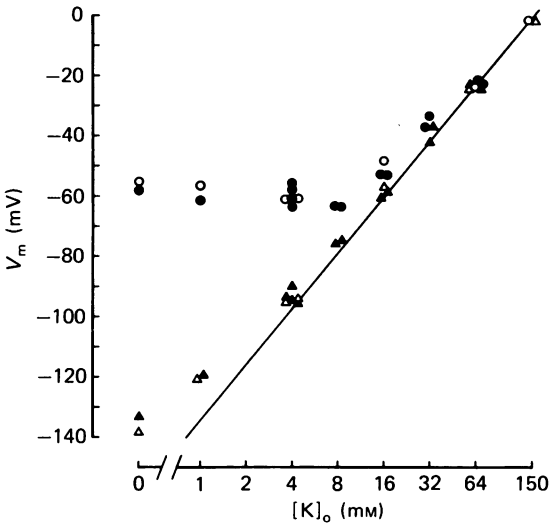

Fig. 4. Effects of ACh and CCh on the resting potentials of coronary sinus preparations exposed to a wide range of extracellular K concentrations. Open symbols summarize results obtained from the preparation of Fig. 3*A* and show resting potentials recorded just before (open circles) and during (open triangles) 5–15 sec applications of 10 μM-ACh. Filled symbols indicate results from a different preparation and show control levels of resting potentials (filled circles) and the maximum levels recorded a few seconds after applying CCh (filled triangles). In this experiment a maximally effective concentration of CCh was applied to the preparation by injecting drops of a 10 mM-CCh solution directly into the superfusate just upstream from the preparation. Low Cl solutions were used throughout both of these experiments. The straight line shows E_K calculated assuming $[K]_i$ to be 155 mM.

ACh (open triangles) or CCh (filled triangles). The straight line indicates E_K calculated on the assumption that $[K]_i$ is 155 mM. The membrane potential at all levels of $[K]_o$ in the presence of either ACh or CCh closely approximated this line, which suggests that under those conditions the membrane is relatively highly permeable to K ions and that $[K]_i$ is approximately 155 mM. The records of Fig. 3 demonstrate that the ACh-induced hyperpolarization occurs within a second or two, much too short a time for $[K]_i$ to change appreciably. These results thus provide strong support for our conclusion that, in quiescent coronary sinus cells at 4 mM-$[K]_o$, $[K]_i$ is normally high and the membrane is permeable not only to K ions but also to some other ion or ions.

The nature of the inward current

The large, positive deviation of the normal resting potential from E_K, illustrated in Figs. 2 and 4, means that an outward current carried by K ions must flow continuously across the resting cell membrane. That K current must be balanced by an inward current of the same magnitude since the net membrane current must be zero at the resting potential. That inward current must be carried by ions with an equilibrium potential which lies *positive* to the resting potential. In solutions virtually

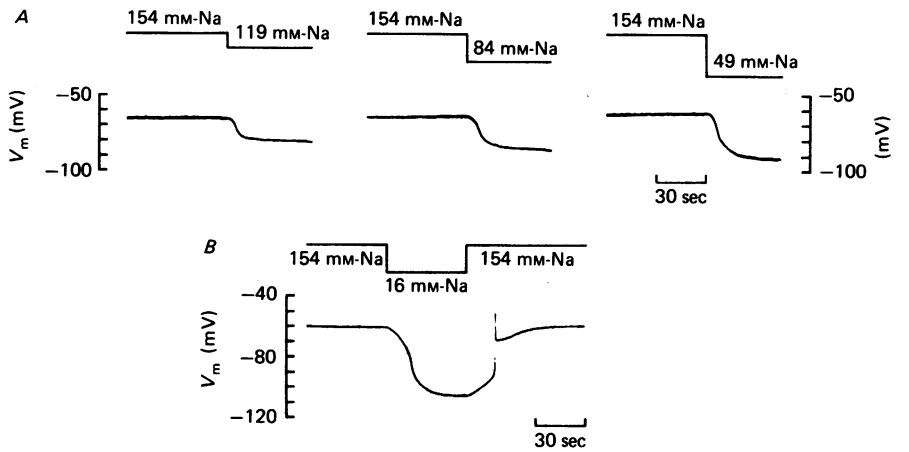

Fig. 5. Effects on the resting potential of coronary sinus preparations of the steplike reductions in extracellular Na concentration represented by the upper lines in *A* and *B*. In both experiments, Na isethionate was replaced by sucrose to give the millimolar levels of $[Na]_o$ indicated; 1 mM-K, low Cl solutions were used throughout; the Ca concentration was 2·7 mM in *A*, and 1 mM in *B*. To correct for the small liquid junction potentials expected to exist in the superfusion fluid between the preparation and the remote reference electrode shortly after switching to the low-Na solutions, the membrane potentials recorded in those low-Na solutions would have to be increased (i.e. made more negative) by up to 1, 3, 5 and 7 mV, respectively, in the 119, 84, 49 and 16 mM-Na solutions (cf. legend to Fig. 6).

free of chloride, the ions most likely to carry inward current are Na and Ca ions. We therefore investigated the effects on the resting potential of coronary sinus cells of suddenly changing the extracellular concentration of Na or Ca ions. If Na ions carry appreciable inward current, then lowering $[Na]_o$ should reduce that depolarizing current and so allow the cells to hyperpolarize towards E_K.

Fig. 5*A* shows that in a preparation exposed to 1 mM-K, a step reduction of $[Na]_o$ to 77%, 55%, or 32% of the control level of 154 mM (sucrose being substituted for Na isethionate on an isosmotic basis) caused a hyperpolarization of corresponding amplitude, and did so within a few seconds. On returning to 154 mM-extracellular Na, the membrane potential quickly returned to its initial resting level (see, e.g. Fig. 5*B*). Fig. 5*B* shows the rapid hyperpolarization recorded in another preparation exposed to 1 mM-K solution, in response to a brief reduction of $[Na]_o$ to 10% of its control level, and the equally rapid return to the control resting potential on restoring $[Na]_o$. In other experiments, resting potentials were measured, over a wide range of

$[K]_o$ levels, just before, and during, reductions of $[Na]_o$ to 14 mM. Average values of those resting potentials, obtained from sixteen preparations, are summarized in the semilogarithmic plot of Fig. 6. The filled circles show resting potentials in 100 % $[Na]_o$; the open circles indicate membrane potentials determined 30–90 sec after Na isethionate was largely replaced either by sucrose (total of 116 measurements), or by Tris methanesulphonate (total of sixty-four measurements). The dashed line shows E_K calculated on the assumption that $[K]_i$ is 155 mM. These results clearly demonstrate that sudden replacement of most of the extracellular Na ions by the larger, and hence presumably less permeant, Tris ions, or by sucrose, causes the resting potential to rapidly increase towards the expected level of E_K. Since the resting potential normally lies closer to E_K at high levels of $[K]_o$, the hyperpolarization seen on lowering $[Na]_o$ is then smaller than that seen when $[K]_o$ is low; thus, the average hyperpolarizations observed were, respectively, only 0·2 and 1·5 mV, at 64 and 32 mM-extracellular K, whereas average hyperpolarizations of 39·8 and 18·9 mV, respectively, were obtained at 1 and 4 mM-external K. The speed with which these potential changes occurred (see, e.g. Fig. 5) suggests that they were predominantly the direct result of changes in the concentration of Na in the extracellular spaces, and were not some indirect effect of subsequent changes in intracellular ion concentrations. We can therefore conclude that Na ions do indeed carry a considerable fraction of the inward current that is responsible for the relatively low resting potential seen at normal or moderately low $[K]_o$ levels.

The effects of TTX and lidocaine

Because the low resting potential seen at normal $[K]_o$ levels usually lies within a voltage range in which some component of steady-state Na current might be expected to flow through non-inactivated excitable Na channels, we investigated the effect on the resting potential of applying TTX (cf. Gadsby & Cranefield, 1977; Attwell, Cohen, Eisner, Ohba & Ojeda, 1979; Colatsky & Gadsby, 1980). In one preparation exposed to 1 mM-K, Cl-containing solution and in two preparations exposed to 4 mM-K low-Cl solutions, 2- to 3-min applications of a sufficient concentration of TTX to block probably at least half of the open Na channels (1–2 μM: Colatsky & Gadsby, 1980; Cohen, Bean, Colatsky & Tsien, 1981), caused an average hyperpolarization of 5 mV (range 2–10 mV). In two additional experiments we tested the effects of lidocaine, another agent known to block excitable Na channels both in axons (for review, see Hille, 1977) and in cardiac cells (e.g. Hondeghem & Katzung, 1977; Lee, Hume, Giles & Brown, 1981; Colatsky, 1982; Carmeliet & Saikawa, 1982): application of 5 μg/ml. lidocaine caused, after 3–5 min, hyperpolarizations of less than 1 mV, and less than 2 mV, respectively, in those two preparations.

The effects of $[Ca]_o$

The effects of varying the extracellular concentration of Ca ions were investigated in two kinds of experiments. In the first kind, $[Ca]_o$ was either raised from 2·7 to 8·1 mM, or lowered from 2·7 to 0·27 mM, by increasing or reducing the amount of Ca methanesulphonate added to the solution without regard for the resulting small changes in tonicity and ionic strength. The resting potential was measured in 2·7 mM-Ca, measured again 2–3 min after raising or lowering $[Ca]_o$, and again after

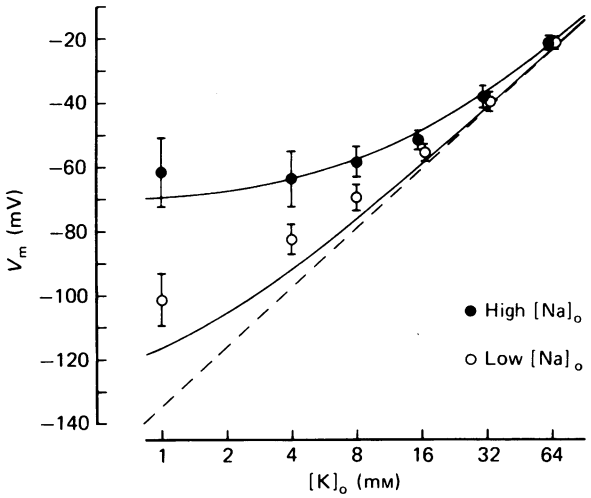

Fig. 6. Effect on the relationship between resting potential and log $[K]_o$ of reducing $[Na]_o$ to 14 mM by replacing Na isethionate with either sucrose or Tris methanesulphonate. Measurements were made on sixteen preparations, but not all preparations were tested at all $[K]_o$ levels: from four to thirteen preparations (yielding twelve to fifty-eight measurements) contribute to the average potentials plotted at each $[K]_o$ value. Direct comparison of the effects of Na replacement by sucrose or by Tris in seven preparations revealed no consistent difference; other experiments indicated that there was no obvious difference between results obtained with HCO_3/CO_2 or with HEPES as pH buffer, and that there was no large difference between results obtained in 2·7 mM-Ca and in 0·9 mM-Ca. In experiments on nine of the preparations, the reference electrode tip was positioned as far away as possible, so that membrane potentials recorded at the end of 30–90 sec exposures to low $[Na]_o$ could be corrected for the small liquid junction potentials existing between the high-Na and low-Na fluids simply by addition of the potential changes measured on repeating the solution changes at the end of the experiment after withdrawing the micro-electrode, breaking it, and positioning the broken tip in the centre of the channel near the preparation; junction potentials recorded in this way for both Na substitutes were similar in size and varied from about 3 mV at 64 mM-extracellular K to about 7 mV at 1 mM-extracellular K. As a check on this procedure, experiments on the other seven preparations were designed to obviate such corrections: in three cases, the flowing 3 M-KCl junction of the reference electrode was positioned just downstream from the preparation and the correct value of the membrane potential could then be measured directly after allowing sufficient time for equilibration of the extracellular space (about 30–90 sec; see e.g. Figs. 1 and 5) and, hence, for dissipation of the liquid junction potential there. For the remaining four preparations, we attempted to obtain a continuously correct recording of the membrane potential by using as a reference a low resistance (low tip potential) micro-electrode inserted into the extracellular space. Because similar results were obtained in all experiments, they have all been combined to give the mean values ± S.D. presented in the graph. The dashed line shows E_K, calculated from the Nernst equation, for a $[K]_i$ of 155 mM. The curve drawn through the points obtained at high $[Na]_o$ is identical to the curve shown in Fig. 2 and was determined from the Goldman–Hodgkin–Katz equation for the conditions: $[K]_o + [Na]_o = 155$ mM, $[K]_i = 155$ mM, $[Na]_i = 10$ mM, $\alpha = 0{\cdot}07$. The curve lying closer to the points obtained at low $[Na]_o$ was calculated from the same equation, but with $[Na]_o$ held constant at 14 mM (see Discussion).

re-exposure to 2·7 mM-Ca, to obtain a bracketed value for the change in membrane potential. In three preparations exposed to 1 mM-K, low-Cl solution, raising $[Ca]_o$ from 2·7 to 8·1 mM caused an average hyperpolarization of 9 mV (S.D. ±5 mV; nine trials), and lowering $[Ca]_o$ from 2·7 to 0·27 mM caused an average depolarization of 3 mV (S.D. ±2 mV; nine trials). In a similar experiment on a preparation exposed to 4 mM-K, low-Cl solution, raising $[Ca]_o$ from 2·7 to 8·1 mM caused an average hyperpolarization of 3 mV (S.D. ±1 mV; eleven trials), whereas lowering $[Ca]_o$ from 2·7 to 0·27 mM caused a 3 mV depolarization (S.D. ±1 mV; eight trials). In the second series of experiments, the effect of increasing $[Ca]_o$ from 2·7 to 8·1 mM was compared with the effect of an equivalent increase in the total extracellular concentration of divalent cations caused by adding either Mn ions or Mg ions to the 2·7 mM-Ca solution. It was found that, at 1 mM-K, Mg was unable to mimic the hyperpolarizing effect of Ca and caused no change in the resting potential, whereas Mn caused a somewhat greater hyperpolarization than Ca. The changes in membrane potential observed in the first series of experiments are in the opposite direction to those expected to result from changes in any steady-state inward current carried by Ca ions, since lowering $[Ca]_o$ causes depolarization and raising $[Ca]_o$ causes hyperpolarization. However, those changes, as well as those caused by adding Mn or Mg, are consistent with the well-known ability of those divalent cations to 'stabilize' muscle cell membranes (see, e.g. Jenden & Reger, 1963), an effect mediated, presumably, by modulation of the surface potential as a result of interactions between the divalent cations and fixed negative charges on the outer surface of the cell membrane (e.g. Frankenhaeuser & Hodgkin, 1957; Hille, 1968): increasing the extracellular divalent cation concentration would make the surface potential more positive and hence alter the electric field within the membrane in the same way that hyperpolarization does. Such a change in electric field might reduce inward current, by causing TTX-sensitive Na channels to close, and so might lead to hyperpolarization.

In three additional experiments, we investigated the effect on the resting potential at 1–4 mM-extracellular K of applying a relatively high concentration (1 μg/ml.) of the Ca channel blocking drug, verapamil. In all three preparations, exposure to verapamil caused the resting potential (−50 to −70 mV) to fall slowly, over a period of about 8 min, by 2·5 to 5 mV. This change in potential, like that seen when $[Ca]_o$ is reduced, is in the opposite direction to the change expected to result from reduction of any steady-state component of Ca channel current (e.g. Gibbons & Fozzard, 1975; Kass, Siegelbaum & Tsien, 1976); of course, the contribution made by any such current might, anyway, be expected to be negligibly small since the threshold voltage for activation of that current is believed to lie near −40 mV, i.e. positive to the range of our control resting potentials (for review, see Reuter, 1973). The mechanism of the slow depolarization caused by verapamil is not known.

These experiments thus lead us to conclude that the inward current responsible for the large deviation of the resting potential from E_K is carried mainly by Na ions, but that only part of that current flows through TTX-sensitive channels.

Role of the Na/K exchange pump

The response of the membrane potential to brief applications of ACh or of high $[K]_o$ was found to remain approximately constant for several hours, suggesting that

the cells of these quiescent coronary sinus preparations maintain a constant level of $[K]_i$ and hence, presumably, a constant level of $[Na]_i$. Our conclusion that a steady current of Na ions flows continuously into the cells therefore requires that there also be a continuous, net Na efflux from the cells of equivalent magnitude. That efflux of Na ions must occur against their electrochemical potential gradient and so represents active transport. The steady outward current of K ions that flows at the resting potential must also be countered by an equivalent active transport of K ions back into the cell. These active movements of Na and K are accomplished by the Na/K exchange pump. If that Na/K pump is electrogenic (see, e.g. Thomas, 1972;

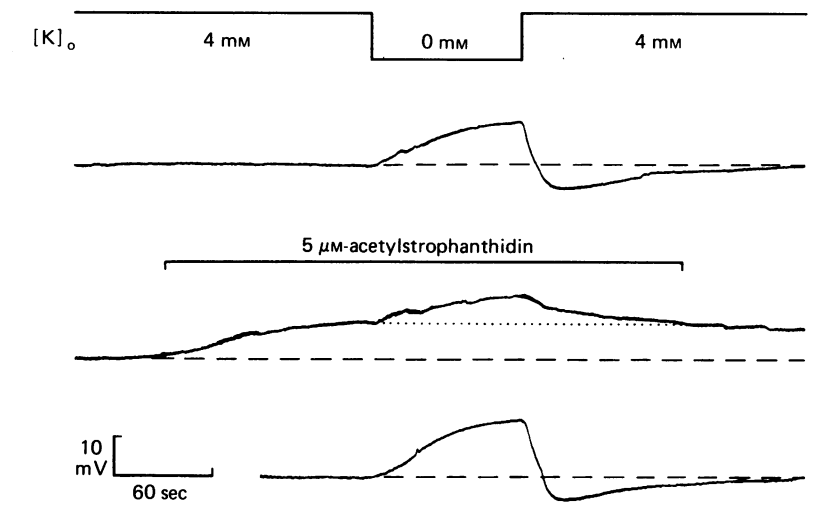

Fig. 7. The transient hyperpolarization following brief (90 sec) exposures to K-free solution, and its abolition by acetylstrophanthidin. The upper line indicates the changes in $[K]_o$ which were repeated for each run. The two control records were obtained 28 min before, and 29 min after, respectively, the test run during which 5 μM-acetylstrophanthidin was applied for the period indicated by the bar. The dashed lines mark the control resting potential at 4 mM-extracellular K, −55 mV, and the dotted line indicates the steady potential level in the presence of acetylstrophanthidin. Low-Cl solutions throughout.

Glitsch, 1979; Gadsby & Cranefield, 1979; cf. Wit *et al.* 1981), some fraction of the net efflux of Na will appear as a steady, hyperpolarizing (outward) membrane current; in that case, sudden inhibition of the Na pump would be expected to result in immediate membrane depolarization. We used the rapidly acting cardiac steroid, acetylstrophanthidin, to specifically inhibit the Na pump (see middle trace of Fig. 7) and found that, in seven experiments on five coronary sinus preparations, a maximally effective concentration, 5–10 μM, caused an average depolarization (±S.D.) of 8±1 mV within 2–3 min, at a $[K]_o$ of 4 mM (cf. Gadsby & Cranefield, 1979). This finding strongly suggests that a steady hyperpolarizing current generated by the Na pump does make a substantial contribution to the resting potential of quiescent coronary sinus cells.

Additional evidence that the Na pump in coronary sinus cells is electrogenic is presented in Fig. 7. The upper and lower records in Fig. 7 show that the depolarization obtained on switching to nominally K-free solution is followed, on switching back

to 4 mM-K solution, by a transient hyperpolarization which reaches a peak within about 20 sec and then decays slowly over the next 2–3 min. The middle record of Fig. 7 shows that 5 μM-acetylstrophanthidin completely abolishes this transient hyperpolarization, indicating that it is somehow generated by activity of the Na pump. A similar transient hyperpolarization has been recorded on readmission of external K, following brief periods of exposure to K-free solution, in sino-atrial node cells (Noma & Irisawa, 1974, 1975), in atrio-ventricular node cells (Kurachi, Noma & Irisawa 1981), in ventricular myocardial cells (Eisner & Lederer, 1979), and in cardiac Purkinje fibres (Ellis, 1977; Gadsby & Cranefield, 1977, 1979; Lee & Fozzard, 1979; Eisner & Lederer, 1980). The explanation for the transient hyperpolarization is as follows: the removal of extracellular K slows the Na pump and, since Na leakage into the cells continues, the diminished rate of Na extrusion allows $[Na]_i$ to rise. The Na pump is known to be stimulated by a rise in $[Na]_i$ (e.g. Glitsch, Pusch & Venetz, 1976; Deitmer & Ellis, 1978; Eisner, Lederer & Vaughan-Jones, 1981) so that, on switching back to 4 mM-external K, the Na pump rate is temporarily enhanced until $[Na]_i$ has been returned to its normal steady-state level. The acetylstrophanthidin-sensitive, transient hyperpolarization thus reflects a temporary increase in the rate of electrogenic Na extrusion. We can rule out as a possible alternative explanation a transient depletion of extracellular K caused by temporarily enhanced activity of an electroneutral Na/K pump, since the records in Fig. 7 show clearly that lowering $[K]_o$ from 4 mM to zero results in monotonic depolarization and not hyperpolarization. We can also rule out other explanations for the hyperpolarization based on changes in passive membrane currents, for example, a reduction in inward Na current or increase in outward K current, secondary to the rise in $[Na]_i$ (and any consequent rise in cellular Ca concentration), because those changes are not expected to be affected by acetylstrophanthidin (cf. Gadsby & Cranefield, 1979).

The steady-state current–voltage relationship of quiescent coronary sinus cells

The rather abrupt changes in resting potential that interrupt both the hyperpolarization caused by lowering $[Na]_o$ and the depolarization caused by raising it again (shown in Fig. 5*B*) as well as those that interrupt the gradual depolarization seen on washing out ACh or CCh (Fig. 3), suggest that the steady-state current–voltage relationship of coronary sinus cells is markedly non-linear and, in fact, is sigmoidal or N-shaped. To test this possibility, we investigated the current–voltage relationship in coronary sinus preparations that were carefully selected for their small size, in order to reduce the extent of cable-like spread of injected current and thereby help maximize uniformity of membrane polarization; these preparations were about 1·5 mm long and about 250 μm in both width and depth. An intracellular micro-electrode positioned near the centre of the preparation was used to inject slowly rising and falling ($\leqslant$ 1·5 nA/sec) double ramps of current, while the resulting changes in membrane potential were recorded via a second micro-electrode inserted nearby (0·1–0·3 mm). Typical records from two of these experiments, one at 4 mM-extracellular K and the other at 1 mM-extracellular K, are presented in Fig. 8. These records share several important features. Both records show the hyperpolarization caused by injection of an inward current whose amplitude increased linearly with time, followed by the subsequent depolarization as the current declined. In both cases, the linear

increase in current caused the membrane potential to increase at first slowly, then rapidly, and then again more slowly; in both cases the change in membrane potential caused by the linear decline in current was interrupted by an abrupt depolarization which gave rise to an action potential. Since the applied currents were changed slowly and linearly, the resulting potential changes (Fig. 8) suggest that the steady-state current–voltage relationship was markedly non-linear under those conditions. Moreover, close inspection of records such as those in Fig. 8 reveals some small hysteresis

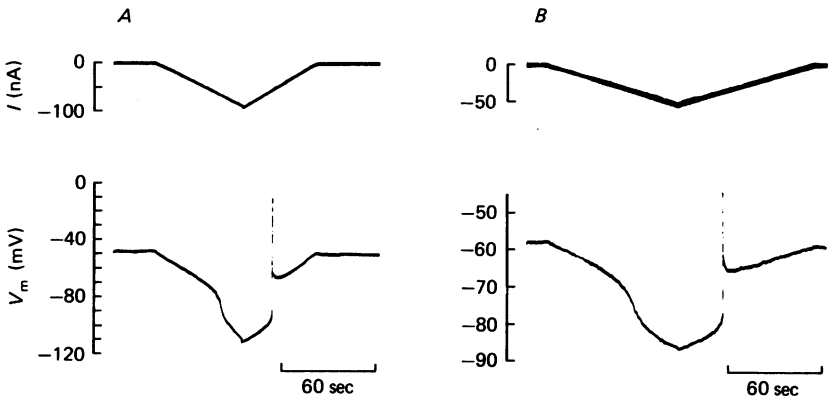

Fig. 8. Changes in membrane potential (lower traces) induced in two small coronary sinus preparations by injection of small, slow ramps of current (upper traces) via a second intracellular micro-electrode. The low Cl solutions contained 1 mM-K in *A* and 4 mM-K in *B*. Note the different voltage and current scales for *A* and *B*. The action potentials elicited during the depolarizing current ramps were imperfectly registered by the pen recorder and so have been retouched by hand.

in the voltage changes caused by the rising and falling limbs of the double ramps of current, the abrupt depolarization during the decline in current occurring at a slightly lower current amplitude than the rapid hyperpolarization during the increase in current. Taken together, the abrupt changes in potential and the hysteresis in the responses to slowly increasing and decreasing currents suggest that the steady-state current–voltage relationship of the resting membrane of coronary sinus cells is N-shaped, like that of other cardiac cells, and includes a region in which the slope conductance is negative (cf., Dudel, Peper, Rudel & Trautwein, 1967; Hall, Hutter & Noble, 1963; McAllister, Noble & Tsien, 1975; Beeler & Reuter, 1977; Gadsby & Cranefield, 1977; Trautwein & McDonald, 1978; Vereecke, Isenberg & Carmeliet, 1980).

DISCUSSION

E_K and $[K]_i$ are both high

The major conclusion we draw from these experiments is that the relatively low resting potential of coronary sinus cells (about −60 mV at 4 mM-extracellular K) cannot be attributed to a correspondingly low level of $[K]_i$, and hence of E_K, because by appropriate experimental interventions, e.g. sudden application of acetylcholine, sudden increase in $[K]_o$, or sudden decrease in $[Na]_o$, the resting potential attains within a few seconds a level that indicates that E_K and $[K]_i$ are high. In fact, the

membrane potentials attained during those interventions indicate that $[K]_i$ is normally about 155 mM in coronary sinus cells (see also Boyden *et al.* 1983), a value similar to that reported for Purkinje fibres (Carmeliet, 1961; Miura, Rosen & Hoffman, 1977; Gadsby & Cranefield, 1977; Wier, 1978; Sheu, Korth, Lathrop & Fozzard, 1980; Wiederholt, Daniesevskis, Hansen, Lickey & Platsch, 1980), for myocardial cells of ventricles (Lee & Fozzard, 1975; Cohen & Fozzard, 1978; Browning & Strauss, 1981; Baumgarten, Cohen & McDonald, 1981) and atria (Singer, Baumgarten & Miller, 1980; Browning, Kerr & Strauss, 1980) and for cells of the sino-atrial node (Browning *et al.* 1980) and atrio-ventricular node (Baumgarten, 1982). Since the resting potential normally lies so far from E_K, the cell membrane clearly cannot be exclusively permeable to K ions. Indeed, the apparent insensitivity of the resting potential to changes in $[K]_o$ between 1 and 8 mM implies that, under these conditions, the ratio of the permeability of the membrane to K ions to its permeability to other ions is not very high; the marked hyperpolarization caused by lowering $[Na]_o$ (Fig. 5, 6) suggests that the 'other ions' to which the membrane is permeable are predominantly Na ions.

The Goldman–Hodgkin–Katz equation (eqn. 1) provides a useful framework for discussing the $[K]_o$ dependence of the resting potential of cells that are permeable to both K ions and Na ions. The smooth curve in Fig. 2, which provides a reasonable fit to measured resting potentials at $[K]_o$ levels $\geqslant 4$ mM, has been drawn according to eqn. 1 assuming that $[K]_i$ is 155 mM and that the permeability ratio, P_{Na}/P_K, has a constant value of 0·07. However, the value of 0·07 for α is almost one order of magnitude greater than that required to fit resting potentials of Purkinje fibres (e.g. Ellis, 1977; Gadsby & Cranefield, 1977; Sheu *et al.* 1980), or ventricular myocardial cells (Walker & Ladle, 1973; Lee & Fozzard, 1975), or frog skeletal muscle fibres (Hodgkin & Horowicz, 1959). We do not know whether the larger α found in coronary sinus cells reflects primarily a larger P_{Na}, or a smaller P_K, or both. All of the resting potentials in Fig. 2 lie within the voltage range in which steady inward current has been demonstrated, in Purkinje fibres, to flow through TTX-sensitive channels (Gadsby & Cranefield, 1977; Coraboeuf, Deroubaix & Coulombe, 1979; Attwell *et al.* 1979; Colatsky & Gadsby, 1980). Our finding that TTX causes a measurable hyperpolarization of coronary sinus cells, presumably by reducing a steady inward current flowing in non-inactivated Na channels, suggests that some contribution to P_{Na} can be expected from those channels. But this cannot explain the much larger α needed to account for resting potentials of coronary sinus cells compared to those of Purkinje fibres at high $[K]_o$ levels (e.g. > 8 mM), since TTX-sensitive current flows in both preparations under those conditions.

A second problem is that the data shown in Fig. 2 cannot be fitted using a constant value of α, since eqn. 1 predicts that if α is constant the resting potential will increase monotonically towards a maximum (negative) level as $[K]_o$ is lowered towards zero. As Fig. 2 shows, however, the resting potential of coronary sinus cells reaches a maximum when $[K]_o$ is about 4 mM and then *declines* (i.e. becomes more positive) as $[K]_o$ is lowered from 4 mM towards 0. A reasonable fit to the data at $[K]_o$ values $\leqslant 4$ mM can be obtained by assuming that α increases from 0·07 to about 0·14 as $[K]_o$ is reduced from 4 mM to zero, i.e. by assuming that P_K falls, or that P_{Na} rises, or both. A fall in P_K might be expected, since the current–voltage relationship for K

currents in resting cardiac or skeletal muscle fibres shows marked inward-going rectification, that is, P_K increases as the electrochemical driving force on K ions (given by the difference between the membrane potential and E_K) is made more inward, and decreases when the driving force becomes more outward (see, e.g. Hodgkin & Horowicz, 1959; Noble, 1965; Haas & Kern, 1966; Vereecke *et al.* 1980). Since the negative shift in E_K that occurs when $[K]_o$ is suddenly reduced from 4 mM to 0 constitutes an increase in the outward driving force on K ions, P_K may be expected to decline. Evidence for such a decline in P_K is seen in the middle record of Fig. 7, where reduction of $[K]_o$ caused a depolarization that cannot be attributed to a reduction in Na/K pump activity because the pump had already been inhibited by a maximal dose of acetylstrophanthidin; that depolarization is presumably attributable to a reduction in outward K current. Of course, any depolarization, however caused, that is associated with an increase in the amplitude of the steady-state, TTX-sensitive, window current will be accompanied by an increase in P_{Na}. In addition, lowering $[K]_o$ from 4 mM to 0 (in the absence of acetylstrophanthidin) causes a reduction in the rate of activity of the Na/K pump. Since the experiment shown in Fig. 7 demonstrates that the Na/K pump in coronary sinus cells is electrogenic, part of the depolarization recorded on lowering $[K]_o$ from 4 mM to 0 can be attributed to a reduction of the steady hyperpolarizing current generated by the pump.

In the context of the Goldman–Hodgkin–Katz equation a reduction in Na/K pump current may be treated as an increase in P_{Na}, and hence in α, for the following reasons (cf. Mullins & Noda, 1963); during operation of the electrogenic Na/K pump, a certain fraction of the Na ions that continuously enter the cells as passive *inward* Na current is continuously extruded as active *outward* Na current. In other words, the *net inward* Na current (represented in eqn. 1) is smaller, by that same fraction, when the pump is operating than when the pump is inhibited. Hence, a reduction in pump current is formally equivalent to an increase in inward Na current, i.e. to an effective increase in P_{Na}, and thus in α, as long as intra- and extracellular Na concentrations can be considered to remain approximately constant.

Thus, the depolarization seen on lowering $[K]_o$ from 4 mM to 0 most probably reflects the following: (*a*) a fall in P_K due to inward-going rectification; (*b*) a reduction in the steady outward current generated by the Na/K pump; (*c*) a voltage-dependent increase in P_{Na}.

Low Na experiments

Although reducing $[Na]_o$ to 14 mM invariably caused a marked hyperpolarization, at $[K]_o$ values $\leqslant$ 8 mM, the membrane potential in low $[Na]_o$ still remained several millivolts positive to the presumed level of E_K (see Fig. 6). This was verified in some experiments by application of ACh in the presence of low $[Na]_o$, which resulted in a further hyperpolarization of a few millivolts (see Fig. 5*B* of Boyden *et al.* 1983). It seems likely that at least some of the remaining deviation of the membrane potential from E_K in low Na solution can be attributed to inward current carried by the remaining extracellular Na ions. Thus, the control resting potentials, in normal $[Na]_o$, illustrated in Fig. 6 can be fitted reasonably well by the Goldman–Hodgkin–Katz equation with identical parameters to those used to fit the similar resting potential data shown in Fig. 2: viz. $[K]_o + [Na]_o = 155$ mM, $[K]_i = 155$ mM, $[Na]_i = 10$ mM, $P_{Na}/P_K = 0{\cdot}07$. Then, on the assumption that only $[Na]_o$ is changed, the same equation can be used to calculate expected resting potentials when

$[Na]_o$ = 14 mM. The equation predicts hyperpolarizations, on lowering $[Na]_o$, of 48 and 29 mV, respectively, at $[K]_o$ levels of 1 mM and 4 mM, whereas the observed hyperpolarizations (Fig. 6) averaged 40 and 19 mV at those K concentrations. The calculation also reveals that, for a P_{Na}/P_K ratio of 0·07, an extracellular Na concentration of 14 mM is sufficient to keep the estimated resting potential positive to E_K by 18 mV at 1 mM-extracellular K and by 6 mV at 4 mM-extracellular K. However, it might be argued that the ratio P_{Na}/P_K is expected to decrease on hyperpolarization secondary to both a reduction in P_{Na} (TTX-sensitive component) and an increase in P_K (via inward-going rectification), for reasons already discussed, so that the discrepancy between observed and calculated resting potentials at low $[Na]_o$ might be somewhat greater than indicated above.

One factor possibly contributing to the discrepancy is that in about half of these experiments the exposures to low Na solutions were only 30 sec in duration. Although the hyperpolarization usually appeared essentially complete after 30 sec of exposure to low $[Na]_o$ (see, e.g. Fig. 5), it is possible that in some cases we have underestimated the steady resting potential in low Na solutions by 2–3 mV. One reason for keeping the low $[Na]_o$ exposures so brief was to minimize possible indirect effects resulting from changes in intracellular Na concentration; another reason was to minimize damage to the preparation caused by occurrence of a contracture (occasionally visible via the dissecting microscope) while the micro-electrode was still in the cell. It seems possible, therefore, that in some instances, a reversible increase in the leakage current flowing at the site of micro-electrode impalement might have been an additional factor contributing to the discrepancy between the observed and calculated resting potentials in low Na solutions. Any remaining discrepancy is presumably attributable to inward current carried across the membrane by other ions, e.g. Ca or Mg ions, via as yet poorly characterized pathways.

The Ca-activated channels known to conduct the transient inward current that underlies the delayed afterdepolarizations seen under various conditions comprise a relatively non-selective pathway for cations across the cardiac cell membrane (Kass, Lederer, Tsien & Weingart, 1978*a*; Kass, Tsien & Weingart, 1978*b*; Vassalle & Mugelli, 1981; Colquhoun, Neher, Reuter & Stevens, 1981). Moreover, delayed afterdepolarizations are readily elicited in canine coronary sinus preparations, at least during exposure to low concentrations of noradrenaline (Wit & Cranefield, 1977; Wit *et al.* 1980, 1981). If, for some reason, these Ca-activated channels were able to carry significant inward current in resting coronary sinus cells, then they might constitute the TTX-insensitive pathway for Na current postulated above to contribute to the low resting potential of those cells; furthermore, such channels might even be expected to pass some inward current in Na-free solutions, since the reversal potential under those conditions seems to be about −35 mV, i.e. significantly positive to E_K (Kass *et al.* 1978*b*). Although these channels are believed to be activated by micromolar concentrations of Ca, because the transient inward current and delayed afterdepolarization are associated with contraction (e.g. Kass *et al.* 1978*a*; cf. Colquhoun *et al.* 1981; Yellen, 1982), it seems possible that they could contribute to the background inward current in coronary sinus cells if it is assumed that either (*a*) the resting intracellular Ca concentration were higher than normal in those cells, or (*b*) the channels were more sensitive to Ca in those than in other cells.

Na/K pump contribution to the resting potential

As already mentioned, the simplest explanation for the rapid depolarization caused by acetylstrophanthidin (Fig. 7) is that it reflects abolition of the steady-state current generated by the electrogenic Na/K pump. We should, however, consider two other possible explanations for that depolarization, based on expected consequences of pump inhibition, whether or not the pump is electrogenic, namely, a fall in $[K]_i$, and a possible accumulation of K ions in extracellular spaces due to the net loss of K from the cells. The effects of these changes in K concentration would be greatest in cells exclusively permeable to K ions (i.e. if α were negligibly small), when the resting potential would be given by E_K and a depolarization (like that shown in Fig. 7) of about 8 mV in less than 3 min could be accounted for if the cells lost about one quarter of their K content in that time. Although the precise effects of changes in $[K]_i$ on the resting potential of coronary sinus cells are unknown, the observed relative insensitivity of the resting potential to *extracellular* K concentration (see, e.g. Fig. 2) would seem to suggest that, by analogy, $[K]_i$ might have to fall by considerably more than one quarter to cause a depolarization of 8 mV. Such a massive, rapid fall in $[K]_i$ seems rather unlikely. The arguments against extracellular K accumulation as an explanation for the acetylstrophanthidin-induced depolarization are somewhat more complicated. Let us start from the extreme assumption that the Na/K pump in coronary sinus cells is not electrogenic so that only passive currents contribute to the resting potentials presented in Figs. 2, 4 and 6. On the basis of those measured potentials, a 3- or 4-fold increase in extracellular K concentration (from a starting level of 4 mM) would be required to account for an 8 mV depolarization. Now, if the acetylstrophanthidin-induced depolarization were due simply to such extracellular K accumulation then, according to Figs. 2, 4 and 6, a subsequent switch to K-free solution would be expected to cause a *biphasic* change in resting potential, with a time course of several seconds as the K concentration just outside the cells fell gradually (slowed by diffusion): thus, as the extracellular K concentration fell from its elevated level (of, e.g. 12–16 mM) back to about 4 mM, the cells would first *hyperpolarize* by 8 mV, and then the further fall to near 0 mM would cause *depolarization*. However, the middle record in Fig. 7 shows unequivocally that the depolarization caused by acetylstrophanthidin is followed, on lowering $[K]_o$ to near zero, by a *monotonic depolarization*; moreover, the subsequent elevation of $[K]_o$ back to 4 mM caused monotonic repolarization. Since, therefore, neither reduction of $[K]_i$ nor extracellular K accumulation seems able to provide a suitable explanation for the observed acetylstrophanthidin-induced depolarization, that depolarization is most reasonably attributed to reduction of steady-state current generated by electrogenic Na/K exchange.

Effects of ACh and CCh

An observation made consistently during this study was that the time courses of the membrane potential changes caused by identical doses of acetylcholine or carbachol were strikingly different (see, e.g. Fig. 3*B*). The most obvious differences were that the drug-induced hyperpolarization persisted for a longer time after beginning the wash-out of the drug, and that the subsequent decline of the membrane

potential was slower, when the agonist was CCh than when it was ACh. On the assumption that these differences reflect a faster decline of agonist concentration in the extracellular space in the case of ACh, a possible explanation is that ACh is hydrolysed by intrinsic, membrane-bound cholinesterases much more rapidly than CCh is. Whatever the underlying mechanism, the slower depolarization on washing out CCh usually occurred without any abrupt potential changes of the kind that often accompanied the depolarization associated with wash-out of ACh (see, e.g. Fig. 3*A*). Since these abrupt potential changes often gave rise to an action potential or even, under appropriate conditions, to a burst of action potentials accompanied by contractions that were often sufficiently vigorous to dislodge micro-electrodes, we used CCh rather than ACh whenever possible (see also Boyden *et al.* 1983).

The records in Fig. 3 illustrate another consistent finding: the decline of the hyperpolarization on washing out the muscarinic agonists (ACh or CCh) is followed by a transient depolarization of the cell membrane with respect to the steady resting potential. This transient depolarization reached a peak amplitude, usually of about 5 mV, within several seconds of the decline of the hyperpolarization and then slowly decayed over the next few minutes. A qualitatively similar temporary depolarization follows the hyperpolarization caused by brief applications of ACh to canine cardiac Purkinje fibres (Gadsby, Wit & Cranefield, 1978). The mechanisms underlying these transient depolarizations are unknown, but either transient depolarization (or both) might be related to the post-vagal, or post-ACh, sinus node tachycardia reported by Loeb & Vassalle (1976: but cf. Gadsby *et al.* 1978; Nishiye, Cranefield & Gadsby, 1982).

Possible implications for arrhythmogenesis

Our results strongly suggest that the low resting potential of quiescent coronary sinus cells exposed to a physiological level of $[K]_o$ (4 mM) is due not to a low $[K]_i$ but to a relatively high value for the permeability ratio P_{Na}/P_K (approximately 0·07). Addition of ACh greatly increases P_K so that it predominates and the resting potential approaches the level of E_K expected for a $[K]_i$ of about 155 mM. Moreover, sudden withdrawal of the ACh can result in membrane depolarization that is rapid enough to give rise to an action potential (see, e.g. Fig. 3*A*). Presumably, the removal of inactivation of fast (TTX-sensitive) Na channels that must occur during the ACh-induced hyperpolarization contributes to this physiological example of 'anode-break excitation' brought about by application and withdrawal of a naturally-occurring transmitter. [Note that this mechanism for post-ACh excitation is distinct from the subsequent, slower post-ACh depolarization, already discussed, that can itself give rise to an action potential or burst of action potentials under appropriate conditions (Nishiye *et al.* 1982).] We are not aware of any other example in the electrophysiological literature of excitation resulting directly from the abrupt cessation of a transmitter-induced hyperpolarization. Conceivably, appropriate fluctuations in the level of vagal activity in the coronary sinus might occur *in vivo* to evoke this kind of excitation and give rise to an extrasystole. Such an extrasystole could, in theory, arise at any time during the cardiac cycle and might therefore be considered potentially arrhythmogenic (see Cranefield, 1983).

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REFERENCES

ATTWELL, D., COHEN, I., EISNER, D., OHBA, M. & OJEDA, C. (1979). The steady-state TTX sensitive ("window") sodium current in cardiac Purkinje fibers. *Pflügers Arch.* **379**, 137–142.

BAUMGARTEN, C. M. (1982). Intracellular potassium and chloride activities in rabbit atrioventricular node. *Biophys. J.* **37**, 243a (abstract).

BAUMGARTEN, C. M., COHEN, C. J. & MCDONALD, T. F. (1981). Heterogeneity of intracellular potassium activity and membrane potential in hypoxic guinea pig ventricle. *Circulation Res.* **49**, 1181–1189.

BEELER, G. W. & REUTER, H. (1977). Reconstruction of the action potential of ventricular myocardial fibres. *J. Physiol.* **368**, 177–210.

BOYDEN, P. A., CRANEFIELD, P. F. & GADSBY, D. C. (1983). Noradrenaline hyperpolarizes cells of the canine coronary sinus by increasing their permeability to potassium ions. *J. Physiol.* **339**, 185–206.

BROWNING, D. J., KERR, C. R. & STRAUSS, H. C. (1980). Electrochemical anatomy of the rabbit right atrium: Potassium. *Circulation* **62**, III-54 (abstract).

BROWNING, D. J. & STRAUSS, H. C. (1981). Effects of stimulation frequency on potassium activity and cell volume in cardiac tissue. *Am. J. Physiol.* **240**, C39–55.

CARMELIET, E. E. (1961). Chloride ions and the membrane potential of Purkinje fibres. *J. Physiol.* **156**, 375–388.

CARMELIET, E. & SAIKAWA, T. (1982). Shortening of the action potential and reduction of pacemaker activity by lidocaine, quinidine, and procainamide in sheep cardiac Purkinje fibers. *Circulation Res.* **50**, 257–272.

COHEN, C. J., BEAN, B. P., COLATSKY, T. J. & TSIEN, R. W. (1981). Tetrodotoxin block of sodium channels in rabbit Purkinje fibers. Interactions between toxin binding and channel gating. *J. gen. Physiol.* **78**, 383–411.

COHEN, C. J. & FOZZARD, H. A. (1979). Intracellular K and Na activities in papillary muscle during inotropic interventions. *Biophys. J.* **25**, 144a (abstract).

COLATSKY, T. J. (1982). Mechanisms of action of lidocaine and quinidine on action potential duration in rabbit cardiac Purkinje fibers. An effect on steady-state sodium currents? *Circulation Res.* **50**, 17–27.

COLATSKY, T. J. & GADSBY, D. C. (1980). Is tetrodotoxin block of background sodium channels in canine cardiac Purkinje fibres voltage-dependent? *J. Physiol.* **306**, 20*P*.

COLQUHOUN, D., NEHER, E., REUTER, H. & STEVENS, C. F. (1981). Inward current channels activated by intracellular Ca in cultured cardiac cells. *Nature, Lond.* **294**, 752–754.

CORABOEUF, E., DEROUBAIX, E. & COULOMBE, A. (1979). Effect of tetrodotoxin on action potentials of the conducting system in dog heart. *Am. J. Physiol.* **236**, H561–567.

CRANEFIELD, P. F. (1983). Triggered Arrhythmias. In *Frontiers of Cardiac Electrophysiology*, ed. ROSENBAUM, M. & ELIZARI, M. The Hague: Martinus Nijhoff.

DEITMER, J. W. & ELLIS, D. (1978). The intracellular sodium activity of cardiac Purkinje fibres during inhibition and reactivation of the Na–K pump. *J. Physiol.* **284**, 241–259.

DUDEL, J., PEPER, K., RUDEL, R. & TRAUTWEIN, W. (1967). The potassium component of membrane current in Purkinje fibres. *Pflügers Arch. ges Physiol.* **296**, 308–327.

EISNER, D. A. & LEDERER, W. J. (1979). The role of the sodium pump in the effects of potassium-depleted solutions on mammalian cardiac muscle. *J. Physiol.* **294**, 279–301.

EISNER, D. A. & LEDERER, W. J. (1980). Characterization of the electrogenic sodium pump in cardiac Purkinje fibres. *J. Physiol.* **303**, 441–474.

EISNER, D. A., LEDERER, W. J. & VAUGHAN-JONES, R. D. (1981). The dependence of sodium pumping and tension on intracellular sodium activity in voltage-clamped sheep Purkinje fibres. *J. Physiol.* **317**, 163–187.

ELLIS, D. (1977). The effects of external cations and ouabain on the intracellular sodium activity of sheep heart Purkinje fibres. *J. Physiol.* **273**, 211–240.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 217.

GADSBY, D. C. & CRANEFIELD, P. F. (1977). Two levels of resting potential in cardiac Purkinje fibers. *J. gen. Physiol.* **70**, 725–746.

GADSBY, D. C. & CRANEFIELD, P. F. (1979). Electrogenic sodium extrusion in cardiac Purkinje fibers. *J. gen. Physiol.* **73**, 819–837.

GADSBY, D. C. & CRANEFIELD, P. F. (1982). Effects of electrogenic sodium extrusion on the membrane potential of cardiac Purkinje fibers. In *Normal and Abnormal Conduction in the Heart*, ed. PAES DE CARVALHO, A., HOFFMAN, B. F. & LIEBERMAN, M. pp. 225–247. New York: Futura.

GADSBY, D. C., WIT, A. L., CRANEFIELD, P. F. (1978). The effects of acetylcholine on the electrical activity of canine cardiac Purkinje fibers. *Circulation Res.* **43**, 29–35.

GADSBY, D. C., WIT, A. L. & CRANEFIELD, P. F. (1979). Overdrive suppression of triggered atrial tachycardia arising in the canine coronary sinus. *Am. J. Cardiol.* **43**, 374 (abstract).

GIBBONS, W. R. & FOZZARD, H. A. (1975). Slow inward current and contraction of sheep cardiac Purkinje fibers. *J. gen. Physiol.* **65**, 367–384.

GLITSCH, H. G. (1979). Characteristics of active Na transport in intact cardiac cells. *Am. J. Physiol.* **236**, H189–199.

GLITSCH, H. G., PUSCH, H. & VENETZ, K. (1976). Effects of Na and K ions on the active Na transport in guinea-pig auricles. *Pflügers Arch.* **365**, 29–36.

GOLDMAN, D. E. (1943). Potential, impedence and rectification in membranes. *J. gen. Physiol.* **27**, 37–60.

HAAS, H. G. & KERN, R. (1966). Potassium fluxes in voltage clamped Purkinje fibers. *Pflügers Arch.* **291**, 69–84.

HALL, A. E., HUTTER, O. F. & NOBLE, D. (1963). Current–voltage relations of Purkinje fibres in sodium-deficient solutions. *J. Physiol.* **166**, 225–240.

HILLE, B. (1968). Charges and potentials at the nerve surface: divalent cations and pH. *J. gen. Physiol.* **51**, 221–236.

HILLE, B. (1977). Local anesthetics: hydrophilic and hydrophobic pathways for the drug receptor reaction. *J. gen. Physiol.* **69**, 497–515.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

HONDEGHEM, L. M. & KATZUNG, B. G. (1977). Time and voltage dependent interactions of antiarrhythmic drugs with cardiac sodium channels. *Biochem. biophys. Acta* **472**, 373–398.

HUTTER, O. F. (1964). The action of the vagus, of acetylcholine and other parasympathomimetic drugs on the heart. In *Second International Pharmacological Meeting*, vol. 5, *Pharmacology of Cardiac Function*, ed. KRAYER, O. & KOVARIKOVA, A., pp. 87–94. Oxford: Pergamon Press.

JENDEN, D. J. & REGER, J. F. (1963). The role of resting potential change in the contractile failure of frog sartorius muscle during calcium deprivation. *J. Physiol.* **169**, 889–901.

KASS, R. S., LEDERER, W. J., TSIEN, R. W. & WEINGART, R. (1978*a*). Role of calcium ions in transient inward currents and aftercontractions induced by strophanthidin in cardiac Purkinje fibres. *J. Physiol.* **281**, 187–208.

KASS, R. S., SIEGELBAUM, S. & TSIEN, R. W. (1976). Incomplete inactivation of the slow inward current in cardiac Purkinje fibres. *J. Physiol.* **263**, 127–128*P*.

KASS, R. S., TSIEN, R. W. & WEINGART, R. (1978*b*). Ionic basis of transient inward current induced by strophanthidin in cardiac Purkinje fibres. *J. Physiol.* **281**, 209–226.

KURACHI, Y., NOMA, A. & IRISAWA, H. (1981). Electrogenic sodium pump in rabbit atrio-ventricular node cell. *Pflügers Arch.* **391**, 127–128.

LEE, C. O. & FOZZARD, H. A. (1975). Activities of potassium and sodium ions in rabbit heart muscle. *J. gen. Physiol.* **65**, 695–708.

LEE, C. O. & FOZZARD, H. A. (1979). Membrane permeability during low potassium depolarization in sheep cardiac Purkinje fibers. *Am. J. Physiol.* **237**, C156–165.

LEE, K. S., HUME, J. R., GILES, W. & BROWN, A. M. (1981). Sodium current depression by lidocaine and quinidine in isolated ventricular cells. *Nature, Lond.* **291**, 325–327.

LOEB, J. M. & VASSALLE, M. (1976). The positive chronotropic action of the vagus nerve on the heart. *Fedn Proc.* **35**, 446 (abstract).

McAllister, R. E., Noble, D. & Tsien, R. W. (1975). Reconstruction of the electrical activity of cardiac Purkinje fibres. *J. Physiol.* **251**, 1–59.

Miura, D. S., Rosen, M. R. & Hoffman, B. F. (1977). The effect of extracellular potassium on the intracellular potassium ion activity and transmembrane potentials of beating canine cardiac Purkinje fibers. *J. gen. Physiol.* **69**, 463–474.

Mullins, L. J. & Noda, K. (1963). The influence of sodium-free solutions on the membrane potential of frog muscle fibers. *J. gen. Physiol.* **47**, 117–132.

Nishiye, H., Cranefield, P. F. & Gadsby, D. C. (1982). Transient depolarization of atrial fibers following exposure to acetylcholine. *Biophys. J.* **37**, 243a (abstract).

Noble, D. (1965). Electrical properties of cardiac muscle attributable to inward-going (anomalous) rectification. *J. cell. comp. Physiol.* **66**, (Suppl. 2) 127–135.

Noma, A. & Irisawa, H. (1974). Electrogenic sodium pump in rabbit sinoatrial node cell. *Pflügers Arch.* **351**, 177–182.

Noma, A. & Irisawa, H. (1975). Contribution of an electrogenic sodium pump to the membrane potential in rabbit sinoatrial node cells. *Pflügers Arch.* **358**, 289–301.

Ochi, R. & Nishiye, H. (1973). Temperature dependence of the healing-over in mammalian cardiac muscle. *Proc. Japan Acad.* **49**, 372–375.

Reuter, H. (1973). Divalent cations as charge carriers in excitable membranes. *Prog. Biophys. molec. Biol.* **26**, 1–43.

Sheu, S. S., Korth, M., Lathrop, D. A. & Fozzard, H. A. (1980). Intra- and extra-cellular K^+ and Na^+ activities and resting potential in sheep cardiac Purkinje strands. *Circulation Res.* **47**, 692–700.

Singer, D. H., Baumgarten, C. M. & Miller, E. D. (1980). Intracellular potassium activity in guinea-pig atrial muscle. *Am. J. Cardiol.* **45**, 425 (abstract).

Spitzer, K. W. & Walker, J. L. (1979). Changes in liquid-junction potential following chloride replacement in cat papillary muscle. *Pflügers Arch.* **382**, 281–284.

Thomas, R. C. (1972). Electrogenic sodium pump in nerve and muscle cells. *Physiol. Rev.* **52**, 563–594.

Trautwein, W. & McDonald, T. F. (1978). Current–voltage relations in ventricular muscle preparations from different species. *Pflügers Arch.* **374**, 79–89.

Vassalle, M. & Mugelli, A. (1981). An oscillatory current in sheep cardiac Purkinje fibers. *Circulation Res.* **48**, 618–631.

Vereecke, J., Isenberg, G. & Carmeliet, E. (1980). K efflux through inward rectifying K channels in voltage clamped Purkinje fibers. *Pflügers Arch.* **384**, 207–217.

Walker, J. L. & Ladle, R. O. (1973). Frog heart intracellular potassium activities measured with potassium microelectrodes. *Am. J. Physiol.* **225**, 263–267.

Wiederholt, M., Daniesevskis, P., Hansen, L. L., Lickey, H. J. & Platsch, K. D. (1980). Effects of extracellular potassium ouabain and prostaglandins on intracellular potassium activity in sheep cardiac Purkinje fibers. *Pflügers Arch.* **388**, 169–175.

Wier, W. G. (1978). Ionic currents and intracellular potassium in hypoxic myocardial cells. *Biophys. J.* **21**, 166a (abstract):

Wit, A. L. & Cranefield, P. F. (1977). Triggered and automatic activity in the canine coronary sinus. *Circulation Res.* **41**, 435–445.

Wit, A. L., Cranefield, P. F. & Gadsby, D. C. (1980). Triggered Activity. In *The Slow Inward Current and Cardiac Arrhythmias*, ed. Zipes, D. P., Bailey, J. C. & Elharrar, V., pp. 437–454. The Hague: Martinus Nijhoff.

Wit, A. L., Cranefield, P. F. & Gadsby, D. C. (1981). Electrogenic sodium extrusion can stop triggered activity in the canine coronary sinus. *Circulation Res.* **49**, 1029–1042.

Yellen, G. (1982). Single Ca^{2+}-activated nonselective cation channels in neuroblastoma. *Nature, Lond.* **296**, 357–359.