Measurements of mitochondrial K⁺ fluxes in whole rat hearts using ⁸⁷Rb-NMR

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Gruwel, Marco L. H., Bozena Kuzio, Roxanne Deslauriers, and Valerie V. Kupriyanov. Measurements of mitochondrial K⁺ fluxes in whole rat hearts using ⁸⁷Rb-NMR. Am. J. Physiol. 276 (Cell Physiol. 45): C193–C200, 1999.—The rubidium efflux from hypothermic rat hearts perfused by the Langendorff method at 20°C was studied. At this temperature ⁸⁷Rb-NMR efflux experiments showed the existence of two ⁸⁷Rb pools: cytoplasmic and mitochondrial. Rat heart mitochondria showed a very slow exchange of mitochondrial Rb⁺ for cytoplasmic K⁺. After washout of cytosolic Rb⁺, mitochondria kept a stable Rb⁺ level for >30 min. Rb⁺ efflux from mitochondria was stimulated with 0.1 mM 2,4-dinitrophenol (DNP), by sarcolemmal permeabilization and concomitant cellular energy depletion by saponin (0.01 mg/ml for 4 min) in the presence of a perfusate mimicking intracellular conditions, or by ATP-sensitive K⁺ (KATP) channel openers. DNP, a mitochondrial uncoupler, caused the onset of mitochondrial Rb⁺ exchange; however, the washout was not complete (80 vs. 56% in control). Energy deprivation by saponin, which permeabilizes the sarcolemma, resulted in a rapid and complete Rb⁺ efflux. The mitochondrial Rb⁺ efflux rate constant (k) decreased in the presence of glibenclamide, a KATP channel inhibitor (5 μM; k = 0.204 ± 0.065 min⁻¹; n = 8), or in the presence of ATP plus phosphocreatine (1.0 and 5.0 mM, respectively; k = 0.134 ± 0.021 min⁻¹; n = 4) in the saponin experiments (saponin only; k = 0.321 ± 0.079 min⁻¹; n = 3), indicating the inhibition of mitochondrial KATP channels. Thus hypothermia in combination with ⁸⁷Rb-NMR allowed the probing of the mitochondrial K⁺ pool in whole hearts without mitochondrial isolation.

rubidium ion permeability; hypothermia; mitochondria; ATP-sensitive potassium channels; energy depletion; nuclear magnetic resonance

TO ACHIEVE A HIGH TRANSPLANTATION success rate, organs are often preserved under conditions that significantly limit metabolism or decrease their metabolic rates. One of the standard preservation procedures involves hypothermia (25), whereas other techniques rely on reducing the ion gradients across the cell membrane (13). Since the discovery of sarcolemmal ATP-sensitive K⁺ (KATP) channels by Noma (23), research has focused on the regulation of K⁺ fluxes by means of these channels (9, 10, 12). Modulation of KATP channel activity could be beneficial during organ transplantation or ischemic events. With the discovery of mitochondrial KATP channels in the inner mitochondrial membrane, regulation of K⁺ fluxes across the mitochondrial membrane could be manipulated without the use of ionophores (15). Although K⁺ channels and their activity in both the sarcolemma and the inner mitochondrial membrane have been studied, not much is known about the regulation of K⁺ fluxes between the cytoplasm and the mitochondrial matrix, especially in intact tissues. The study of transmitchondrial K⁺ fluxes relies on the separation of these fluxes from those occurring across the sarcolemma. The separate detection of these fluxes has not been achieved.

Recently we observed a K⁺ efflux anomaly in rat hearts cooled to 20°C by using ⁸⁷Rb as a K⁺ congener (11). Our ⁸⁷Rb-NMR measurements showed incomplete Rb⁺ efflux with a dramatically (5-fold) increased rate constant at 20°C compared with that for efflux observed at 36°C (11). The Rb⁺ plateau reached after this initial rapid washout remained stable for >30 min. This observation led to the hypothesis that at 20°C residual Rb⁺ is of mitochondrial origin. Additional evidence for this phenomenon was obtained from ³¹P-NMR spectra. These measurements revealed the appearance of Pi resonance in a compartment with a pH higher than that of the cytoplasm. This additional resonance could originate from an alkaline mitochondrial pool.

Kinetic studies of ⁴²K⁺ efflux from hearts have failed to disclose an ionic fraction that might reflect the loss of K⁺ from a large mitochondrial compartment with a relatively low permeability (20). However, Altschuld et al. (2) reported that a relatively constant amount of mitochondrial K⁺ was produced when isolated rat heart cells were washed with cold medium. Conditions that produce low levels of ATP, e.g., uncoupling of the mitochondria, also promoted a loss of mitochondrial K⁺. The authors concluded that mitochondrial K⁺ is not in equilibrium with that in the cytosol, even after long periods of incubation.

This work presents data from studies of hypothermic rat hearts perfused by the Langendorff method at 20°C. K⁺ transport was monitored by using its biological congener, ⁸⁷Rb (1, 18). In addition to the study of Rb⁺ fluxes, intracellular H⁺ concentration ([H⁺]) was measured by ³¹P-NMR, which also provided information on the energy status of the heart during the experiments. The measurements indicate the existence of at least two intracellular Rb⁺ pools at 20°C. One of these pools was attributed to mitochondrial Rb⁺. This pool showed efflux kinetics much slower than that of the cytosol. Rb⁺ efflux from the mitochondrial pool was stimulated by a variety of methods to exchange Rb⁺ for K⁺ in the cytosol, including cellular energy depletion and a method involving KATP channel openers.
MATERIALS AND METHODS

Experiments performed in this study were carried out in accordance with the Guide for the Care and Use of Experimental Animals, published by the Canadian Council on Animal Care (2nd ed., Ottawa, ON, Canada, 1993).

Solutions. $^{87}$Rb- and $^{31}$P-NMR experiments were performed on hearts perfused with a phosphate-free Krebs-Henseleit (KH)-K buffer containing (in mM) 118 NaCl, 25 NaHCO$_3$, 4.7 KCl, 1.75 CaCl$_2$, 1.2 MgSO$_4$, 0.5 EDTA (Sigma, St. Louis, MO), and 11 glucose. The perfusate was equilibrated with a mixture of O$_2$ and CO$_2$ gas, the flow ratio of which was manually adjusted to maintain a buffer pH of 7.4 at all temperatures. Perfusion pH was continuously monitored throughout the experiments with a pH meter. For $^{87}$Rb-NMR loading studies the perfusate contained (in mM) 3.76 KCl and 2.14 RbCl (KH-Rb) (Sigma) instead of 4.7 mM KCl, resulting in a 36% substitution of K$^+$ with Rb$^+$. Extracellular K$^+$ replacement by 20, 36, or 100% Rb$^+$ resulted in only a slight decrease in heart rate (−10 and −20% for 36 and 100% Rb$^+$ substitution, respectively) and no changes in the systolic and diastolic pressures or in the $^{31}$P-NMR spectrum (17–19). In addition, in vivo, in the chronic case, experiments showed that substitution of 30–40% of the intracellular K$^+$ with Rb$^+$ is not toxic (22). As the present work focuses on the properties of a second Rb$^+$ pool (possibly mitochondrial), changes in intracellular water volume in response to Rb$^+$ loading and washout were not measured. Switching from Rb$^+$-loading with KH-Rb to Rb$^+$-washout with a medium containing no Rb$^+$ results in a change in total K$^+$ plus [Rb$^+$] from 5.9 to 4.7 mM. This results in a return of the slightly depolarized membrane potential during Rb$^+$ loading to that characteristic of control conditions. Changes in membrane potential are fast, and a new equilibrium will be established during the first few minutes of the washout. Because of the nonvanishing exchange time of perfusates in the extracardiac space (see below), the equilibration time of the membrane potential is easily absorbed by the protocol. In a recent publication (19) describing the opposite experiment, an increased (from 5.9 to 21 mM) extracellular K$^+$ was shown not to change the Rb$^+$ washout kinetics, compared with that of the control. Extracellular K$^+$ is thus not critical for Rb$^+$ efflux kinetics. Rb$^+$ efflux studies were performed with several perfusates. The control efflux was studied with Rb$^+$-free KH, whereas two other media were used after the induction of Rb$^+$ efflux by saponin (Sigma). After saponin treatment, the perfusion medium was switched to a solution mimicking an intracellular compartment of metabolites. Two different media were used for this purpose. The first intracellular medium (ICM1) contained (in mM) 25 NaHCO$_3$, 25 KCl, 100 C$_2$H$_5$O$_2$K (potassium gluconate, Sigma), 20 taurine (Sigma), 2 C$_7$H$_7$O$_3$Na (sodium pyruvate, Sigma), 1.2 MgSO$_4$, and 1.0 EGTA (Sigma). The second medium (ICM2) was the same as ICM1, except that it contained 2.2 mM MgSO$_4$, 5 mM disodium phosphocreatine (PCr), and 1.0 mM ATP (the last 2 from Sigma). All reagents were purchased from BDH (Toronto, ON, Canada) unless mentioned otherwise. Saponin, glibenclamide, bumetanide, and 2,4-dinitrophenol (DNP; all from Sigma) were added to the perfusion buffer as aqueous solutions through separate infusion lines. The concentrations in the stock solutions were 0.6 mg/ml for saponin, 0.6 mM for both glibenclamide and bumetanide, and 6 mM for DNP. The final concentrations in the perfusate for these four agents were 0.01 mg/ml, 5 µM, 10 µM, and 0.1 mM, respectively.

Heart perfusion. Male Sprague-Dawley rats with an average body weight of 350 ± 28 g (n = 34) and an average heart wet weight of 1.56 ± 0.24 g were used. The rats were anesthetized with pentobarbital (120 mg/kg body wt ip). After anesthesia, the heart was quickly removed and retrogradely perfused via the aorta at a constant flow of 10–12 ml · min$^{-1}$ · g wet wt$^{-1}$. After placement of a left ventricular apical drain, a water-filled balloon was inserted through the mitral valve into the left ventricle. The balloon was connected to a Statham P23Db pressure transducer and a Digi-Med model 210 heart performance analyzer (Micro-Med, Louisville, KY), which allowed monitoring of the heart rate (HR) and left ventricular pressure (LVP). Functional parameters such as HR, LVP, end-diastolic pressure (EDP; set to 5–10 mmHg), and systolic pressure were recorded continuously. An ultrasonic flowmeter (Transonic Systems, Ithaca, NY) in the aortic inflow line allowed the coronary flow to be monitored. Perfusion pressure was measured continuously through the catheter connecting the aortic line and the pressure transducer.

NMR spectroscopy. All NMR experiments were performed on a Bruker 360 AM spectrometer. $^{87}$Rb spectra were acquired at 117.8 MHz by using a 20-mm broad band dedicated probe from Morris Instruments (Gloucester, ON, Canada). $^{31}$P spectra were obtained with a dedicated Bruker 20-mm 13C/31P probe tuned to 145.8 MHz. The signal of $^{23}$Na in the heart and perfusate was used to shim the field. By using a spectral width of 18 kHz, a recycle time of 10 ms, and a pulse length of 55 µs (90° pulse length), $^{87}$Rb-NMR spectra were acquired in 2-min blocks containing 256 data points zero-filled to 512 points. $^{87}$Rb spin lattice relaxation times (T1) in rat hearts typically are 2–3 ms (1). With a recycle time of 10 ms the $^{87}$Rb resonance was not saturated. As a standard, a capillary containing 10 µl of a solution composed of 1 M RbCl plus 5 M KI was included in the setup. I$^{-}$ served as a shift reagent to shift the $^{87}$Rb resonance of the standard away from that of the heart (1). To minimize the signal from extracellular $^{60}$Rb during signal acquisition, the hearts were perfused in the "dry mode." In this approach, the superfusion line placed at the bottom of the NMR tube was used as a suction line to remove fluid surrounding the heart. As a result, the contribution from the extracellular component was reduced to ~16% of the total equilibrium $^{87}$Rb signal after loading at 36°C. $^{31}$P spectra were obtained by using a spectral width of 10 kHz and a 24-µs pulse length (60° flip angle), and 4,096 data points per scan were collected. The acquisition of 360 scans with a recycle time of 2.0 s per scan took 12 min. In some experiments, after saponin infusion, $^{31}$P-NMR spectra were collected continuously every 3.25 min in 96 scans, with a recycle delay of 2.0 s. During the experiments the heart was monitored continuously by means of a copper-constantan thermocouple (Omega Engineering, Stamford, CT) placed in the right ventricle. At the bore of the magnet the thermocouple was connected to a low-pass filter to minimize the noise picked up by the wires outside of the magnet.

Both magnetic resonance probes could be connected to a Bruker variable-temperature control unit supplied with dried air. For temperatures above room temperature, the temperature in the probe was regulated with dried air and a heater built into the probe. To reach temperatures below room temperature, the air flow to the probe was precooled with a chiller. The temperatures of the perfusion solutions were thermostated with two Lauda RM6/super (Brinkmann Instruments, New York, NY) circulating baths. After equilibration the temperature in the heart remained stable within ±0.2°C.

Experimental protocols. After stabilization of mechanical function (~10 min) the hearts were subjected to the following protocols. Rb$^+$ accumulation and washout were measured by
using the two protocols shown in Fig. 1. Protocol A started
with a 60-min Rb\textsuperscript{+} accumulation at 36°C. After Rb\textsuperscript{+} loading
the temperature was changed to 20°C, and this was followed
by a 30-min equilibration period during which the hearts
were still perfused with KH-Rb. After this equilibration period \textsuperscript{87}Rb-NMR spectra were obtained for a 10-min period.
Subsequently, the perfusate was switched to a (Rb\textsuperscript{+}-free)
KH-K buffer, and Rb\textsuperscript{+} efflux was monitored during the next
40 min. A second protocol (schematics are shown in Fig. 1B)
began with hearts preequilibrated to 20°C. This protocol
facilitated the measurement of Rb\textsuperscript{+} influx and efflux at 20°C.
The perfusion time with KH-Rb at 20°C was extended to 90
min to allow equilibration of the hearts with Rb\textsuperscript{+} at this
temperature.

At 36°C, \textsuperscript{31}P-NMR spectra were acquired for 12 min after
stabilization of mechanical functions. After this acquisition
the temperature was lowered to 20°C, and after a 40-min
equilibration period the next spectrum was recorded. In some
experiments \textsuperscript{31}P-NMR spectra were acquired during the final
washout, initiated by saponin infusion. These spectra were
continuously collected every 3.25 min.

Measurement of Rb\textsuperscript{+} fluxes kinetics. To measure Rb\textsuperscript{+}
uptake, the perfusate was switched from KH to KH-Rb and
the experimental apparatus was changed to the “dry-mode,”
which reduced the contribution of Rb\textsuperscript{+} present in the bath
to the observed NMR signal. With a coronary flow of 15 ml/min,
the perfusate reached the heart in 3 min, after which NMR
data acquisition was started. The duration of KH-Rb perfu-
sion was determined by the protocol (Fig. 1). To measure Rb\textsuperscript{+}
efflux, perfusion was switched to the KH solution. After a 3-min delay (flow of 12–15 ml/min, depending on tempera-
ture) \textsuperscript{87}Rb-NMR spectra were acquired during the final
washout, initiated by saponin infusion. These spectra were
continuously collected every 3.25 min.

RESULTS

Rb\textsuperscript{+} compartmentation caused by hypothermia. Cooling
the hearts from 36 to 20°C resulted in a change in Rb\textsuperscript{+}
efflux kinetics. In a control experiment (Fig. 2A), washout of Rb\textsuperscript{+} with Rb\textsuperscript{+}-free KH-K was incomplete

The Rb\textsuperscript{+} washout curves obtained from the experiments
were fitted to a three-parameter expression

\[ I(t) = C - B \cdot [1 - \exp(-k \cdot t)] \]  

(1)

This equation can be obtained by assuming that Rb\textsuperscript{+} trans-
port across the sarcolemma involves two passive terms, each
equal to the product of a rate constant and the intra- or
extracellular [Rb\textsuperscript{+}], and an active term. For the passive terms
only one of the rate constants will occur in the equation (efflux
rate constant), whereas the active term describing Na\textsuperscript{+}/K\textsuperscript{+}
pump activity is absorbed in parameter B. The only assump-
tion used in the derivation of Eq. 1 is a constant Na\textsuperscript{+}/K\textsuperscript{+}
pump activity. A similar expression for the Rb\textsuperscript{+} uptake kinetics
can be obtained, and it can be shown (1) that for Rb\textsuperscript{+} uptake
the obtained rate constant is that describing efflux. The three-
parameter fit was used to take into account any baseline
offset (parameter C). Nonlinear regression of the experimen-
tal data to the three-parameter expression was performed by
using the Marquardt-Levenberg algorithm. Parameters C and B
in Eq. 1 represent signal amplitudes, and k represents the
rate constant for ion efflux. C represents the initial
amount of intracellular Rb\textsuperscript{+} present in the heart at the start
of the efflux experiments. Parameter B describes the total
amount of Rb\textsuperscript{+} expelled from the heart during the efflux
experiments. The first two data points in each study were
excluded from the analysis as these points mainly reflect
equilibration of the extracellular space. During this time both
the intra- and extracellular ion signals were time dependent.

After these initial 4 min of perfusion, the extracellular bath
had equilibrated to its final value and remained stable. The
equilibration of the extracellular reservoir was rapid, as
shown previously (18).

Statistical analysis. Data are represented as means \pm SD.
The number of experiments averaged is indicated by n. To
compare data between different groups, Student’s t-test was
performed and P < 0.05 was taken as a significant difference
between the groups.
after the heart was loaded with Rb\(^+\) according to protocol B. Only 44 ± 2% of the Rb\(^+\) present after loading could be washed out, leaving a Rb\(^+\) plateau (11). This part of the experiment is referred to as the first phase of the washout. The remaining cellular pool of Rb\(^+\) (the 2nd pool) remained stable for >30 min of perfusion with Rb\(^-\)-free KH. The second Rb\(^+\) pool and its size were independent of the loading temperature (36°C for protocol A and 20°C for protocol B). To investigate the properties of this second Rb\(^+\) pool, the effects of agents that increase sarcolemmal permeability were tested. After washout of the first Rb\(^+\) pool, the washout of the second Rb\(^+\) pool could be initiated through a 4-min infusion of 0.01 mg/ml saponin. Saponin is known to permeabilize the sarcolemma (10). Upon the infusion of saponin, the perfusion medium was changed to a medium mimicking intracellular conditions (ICM1). This led to the disappearance of the second Rb\(^+\) pool and resulted in a complete washout of Rb\(^+\) (Fig. 2B).

Infusion of 0.1 mM DNP, a mitochondrial uncoupler that prevents mitochondrial ATP synthesis, resulted in a faster and more significant washout of Rb\(^+\) at 20°C (Fig. 2C) compared with the control (80 vs. 44%), indicating a role for the mitochondrion as a possible Rb\(^+\) trap at this temperature. Infusion of 0.1 mM DNP, after the Rb\(^+\) plateau was reached, also resulted in the onset of an additional Rb\(^+\) efflux in the second phase (not shown). However, Rb\(^+\) washout was not complete, and residual Rb\(^+\) accounted for ~20% of the total pool size or 35% of the second Rb\(^+\) pool. In subsequent experiments diazoxide (20–60 µM), a mitochondrial K\(_{\text{ATP}}\) channel opener (7), was used, whereas in other experiments an attempt was made to increase the mitochondrial K\(^+\) permeability by using nigericin (10–20 µM). Each agent was applied after the Rb\(^+\) plateau was reached in the first phase of the efflux experiments. In both cases the onset of a slow Rb\(^+\) efflux from the heart was observed (k < 0.01 min\(^{-1}\); not shown). As indicated above, the second Rb\(^+\) pool could effectively be removed by the infusion of saponin. Figure 3 summarizes the effects of saponin on the washout of the second Rb\(^+\) pool by using ICM1 or ICM2 as the perfusate. Saponin creates large holes in the cholesterol-rich sarcolemma and allows the exchange of metabolites between the cytoplasm and the perfusion medium. To control the ionic and metabolic compositions of the extramitochondrial space, perfusion was switched to ICM1 upon saponin infusion. Perfusion with ICM1 resulted in a rapid disappearance of the second Rb\(^+\) pool, with a rate constant of 0.321 ± 0.079 min\(^{-1}\)
(n = 3). This efflux rate constant could be reduced by 40% by an additional infusion of 5 μM glibenclamide, resulting in an efflux rate constant of 0.204 ± 0.065 min⁻¹ (n = 8). This value is significantly different from the rate constant obtained for perfusion with ICM1 plus saponin but without glibenclamide infusion (P = 0.03). In the saponin experiments with ICM2 perfusion, a Rb⁺ efflux rate constant of 0.134 ± 0.021 min⁻¹ (n = 4; P = 5.5 × 10⁻³ compared with ICM1 plus saponin; 60% inhibition) was obtained. The addition of PCr and ATP to ICM1 (=ICM2) resulted in an efflux rate constant lower than that observed for ICM1 plus saponin plus glibenclamide; however, the difference was not significant (P = 0.066).

Changes in phosphates and cytosolic pH during hypothermia and saponin treatment. 31P-NMR spectra were acquired at 36 and 20°C. The spectra did not show any large changes in high-energy phosphates during the transition from 36 to 20°C. The cytosolic pH was estimated from the chemical shift of the Pi resonance relative to that of PCr (11). At 36 and at 20°C, pH values of 7.05 ± 0.04 and 7.21 ± 0.05, respectively, were measured. Figure 4 shows the time dependence of the high-energy phosphate signal intensities after switching to ICM1 perfusate and saponin infusion (n = 3). After saponin infusion the Pi resonance vanished rapidly while the resonance amplitudes of ATP and the PCr decayed more slowly. Figure 4 shows that both γ- and β-ATP diminish significantly in amplitude but do not vanish during the 30-min observation period. During this time a large portion of the second Rb⁺ pool was washed out (see Fig. 1). The data on the decay of the PCr and γ-ATP signal amplitudes (Fig. 4) were fitted to a single-exponential function as given by Eq. 1. For PCr and γ-ATP, rate constants of 0.164 ± 0.018 and 0.128 ± 0.027 min⁻¹, respectively, were obtained. Although P, was washed out, both PCr and ATP signals remained after 30 min of ICM1 perfusion and had relative amplitudes of 34 and 31%, respectively, of their initial values. Figure 4, inset, shows the relative decay of the total integrated 31P-NMR signal intensity as a function of time. This integral was set at 100% before saponin infusion. During the course of the experiment the total integral decayed to ~40% of the initial amplitude with a rate constant of 0.223 ± 0.082 min⁻¹. Throughout the experiment the ratio of the PCr signal to the ATP signal remained ≥1.

Heart function. At 36°C, before Rb⁺ loading, the hearts showed an average pressure-rate product (PRP) of 27.4 ± 2.8 mHg · beats · min⁻¹ (n = 18). In agreement with previous observations (18), Rb⁺ loading slightly decreased (~10%) the HR and did not affect systolic pressure and EDP. After equilibration to 20°C, PRP was 6.4 ± 1.5 mHg · beats · min⁻¹ (n = 5) as a consequence of the decrease in HR from 245 ± 30 beats/min at 36°C to 52 ± 16 beats/min, whereas left ventricular developed pressure remained constant, from 112 ± 10 to 120 ± 27 mmHg, due to an increase in systolic pressure. As the hearts were cooled from 36 to 20°C, EDP increased by a factor of three to four from 10.2 ± 1.7 to 36.8 ± 5.6 mmHg (n = 8). A simultaneous increase in systolic pressure from 106 ± 7 to 147 ± 5 mmHg was observed, in agreement with previous observations (20). The parallel increase in EDP and systolic pressure upon cooling the hearts to 20°C may reflect an increase in intracellular [Ca²⁺] or sensitivity of the myofibrils to Ca²⁺. Saponin treatment resulted in a complete loss of heart function due to permeabilization of the myocyte sarcolemma. After saponin infusion, perfusion pressure increased from 129 ± 6 to 165 ± 25 mmHg (n = 5), whereas EDP further increased to 45 ± 12 mmHg (n = 5), indicating vasoconstriction. After saponin treatment the hearts appeared white, probably because of a loss of myoglobin.

DISCUSSION

87Rb-NMR has been shown to be a useful tool in the study of K⁺ fluxes in rat hearts and has been shown to accurately reflect K⁺ movement across the sarcolemma with 100% visibility (3, 18). Sarcomemmal Rb⁺ rate constants obtained during normothermic experiments are in excellent agreement with rate constants previously published (18). Neither 87Rb visibility data nor rate constants have been published for mitochondria; however, measurements of cardiomyocytes (cytoplasm plus organelles) indicate that 87Rb is also visible in mitochondria (3, 18). A recent study of the temperature dependence of monovalent cation fluxes in rat hearts showed incomplete washout of Rb⁺ from hearts equilibrated at 20°C (Fig. 2A) (11). These results were interpreted in terms of a large difference in Rb⁺ permeability between the sarcolemma and the inner mitochondrial membrane. Other cell compartments are less able to furnish an explanation for these observations because they have a negligibly small volume fraction and

![Fig. 4. Typical 31P-NMR signal amplitudes of Pi, PCr, and γ- and β-ATP during final washout in 2nd phase with ICM1 and saponin infusion. Values are averages of values obtained in 3 separate experiments. Within 5 min of saponin infusion the Pi resonance amplitude vanished while the amplitudes of the high-energy phosphates steadily declined. For readability, points are connected by broken lines. Solid lines represent fits of data to a single-exponential function (see Eq. 1). However, the ratio of the PCr signal to the ATP signal remained ≥1 throughout the experiment. Inset: decay of total 31P-NMR signal integral as function of time. Before saponin infusion this integral was set to 100% in each of the 3 experiments.](http://ajpcell.physiology.org/Downloadedfrom)
do not maintain a large membrane potential or pH gradient. Thus, at 20°C, the sarcolemmal permeability was assumed to be much larger than that observed for the inner mitochondrial membrane. It was also shown that at this temperature the size of the mitochondrial Rb\(^+\) pool was comparable to that of the cytoplasmic pool, 56 and 44% of the total observed Rb\(^+\), respectively. Mitochondria are known to swell upon hypothermia and occupy ~30% of the total cell volume in rat ventricular myocytes and hepatocytes at 37°C (5, 14, 16). In addition, mitochondria are known to have a high K\(^+\) content, corresponding to a \([K^+] = 180\) mM (7). NMR measures the number of nuclei present and not their direct concentration. Mitochondria thus represent the perfect compartment to contain this nonremovable Rb\(^+\) pool observed in the 20°C Rb\(^+\) efflux experiments. Research of Altschuld et al. (2) has shown that the number of K\(^+\) in rat heart mitochondria remains relatively constant when cellular K\(^+\) is depleted by washing the cells with cold perfusate. This observation is in agreement with our observations.

To study the pool of Rb\(^+\) that remains in myocytes at 20°C, the experiments described in this work were performed. Two different approaches are discussed. In one approach mitochondrial ion metabolism was manipulated directly with DNP or a K\(_{ATP}\) channel opener. In the second approach the myocardial sarcolemma was permeabilized with saponin, which led to cytoplasmic energy deprivation. This depletion of PCr and ATP was expected to stimulate the mitochondrial K\(_{ATP}\) channels and consequently enable Rb\(^+\) efflux in the second phase of the washout experiments.

The experiment shown in Fig. 2C displays Rb\(^+\) efflux stimulated by 0.1 mM DNP at 20°C; the results indicate a specific role for mitochondria in this process. DNP is known to prevent ATP synthesis through uncoupling of the protonmotive force, resulting in a collapse of the H\(^+\) gradient. Arresting mitochondrial ATP production should result in a decrease in [ATP] in both the matrix and the cytoplasm and could result in an opening of mitochondrial K\(_{ATP}\) channels. These channels are known to have ATP regulatory sites facing the cytosol (7). The K\(^+\)/H\(^+\) antiporter could also increase its activity due to the uncoupling of the protonmotive force. These two effects could allow previously trapped mitochondrial \(^{87}\)Rb to exchange with cytosolic K\(^+\) and then with extracellular K\(^+\) during the efflux experiment. This is schematically represented by Fig. 5, A and B. Direct stimulation of K\(_{ATP}\) Channels, without the disruption of the protonmotive force, by using the opener diazoxide resulted in only a slow release of Rb\(^+\) from the mitochondrial pool. However, in experiments with reconstituted channels, diazoxide has recently been reported to be ~3 orders of magnitude more effective in opening mitochondrial K\(_{ATP}\) channels than in opening sarcolemmal channels (7). Our measurements at 20°C did not show a large effect of 20–60 \(\mu\)M diazoxide on Rb\(^+\) efflux. Similar observations were made by Szewczyk et al. (26), who also reported diazoxide to be less effective in opening rat liver mitochondrial K\(_{ATP}\) channels. A similar mild stimulation was observed with the antibiotic nigericin, which promotes K\(^+\)/H\(^+\) exchange across the inner mitochondrial membrane (24). At 20°C, however, nigericin might not be distributed effectively across the inner mitochondrial membrane. Our experimental results are consistent with those of Gamble (6), who first showed that the level of K\(^+\) exchange in nonrespiring mitochondria is low and that exchange can be stimulated by respiration. Stimulation of respiration results in increased activity of the protonmotive force and the

![Fig. 5. Schematic representation after 1st phase of Rb\(^+\) washout of a myocyte equilibrated at 20°C. The most important pathways for the mitochondrial (Mito) K\(^+\) cycle as described by Garlid (7) are indicated. Transport proteins are indicated by shaded circles embedded in a membrane. Mitochondrial K\(^+\) leak is shown as a thin arrow. Stimulation of a pathway is indicated by a thick arrow. A: control conditions. B: results of DNP infusion. DNP uncouples the protonmotive force from the mitochondrial ATP production, stimulating the K\(^+\)/H\(^+\) antiporter (exchanger). In turn, K\(^+\) uptake from cytosol accelerates through K\(^+\) leak and possibly through opening of mitochondrial ATP-sensitive K\(_{ATP}\) channels as ATP levels decrease. C: saponin treatment results in large sarcolemmal holes. Because of a loss of cytoplasmic ATP, mitochondrial K\(_{ATP}\) channels are stimulated while respiration increases. As a result, the K\(^+\)/H\(^+\) antiporter also increases in activity.](http://ajpcell.physiology.org/issue)
K⁺/H⁺ antiporter. The net result of this increase in activity is a decrease in the average residence time of mitochondrial K⁺. However, a reduction in respiration alone cannot explain all our observations because at 10°C no ⁸⁷Rb plateau was observed during the washout (11).

Another way of opening K<sub>ATP</sub> channels is through depletion of cellular ATP by saponin, schematically shown in Fig. 5C. Saponin forms large holes in cholesterol-rich membranes through the formation of large molecular complexes with cholesterol (8). The sizes of these holes, which can be as large as 80 Å in diameter, allows the exchange of many metabolites, such as ions and high-energy phosphates, between the compartments separated by the membrane. A 4-min infusion with a low saponin concentration of 0.01 mg/ml after reaching the Rb<sup>+</sup> plateau already resulted in the onset of an additional Rb<sup>+</sup> efflux in the second phase. That saponin indeed caused large holes in the sarcolemma was supported by the color of the hearts after the final washout, which was white, the hearts likely having lost most of their myoglobin. From Fig. 4 it is clear that saponin treatment results in a rapid loss of all cell phosphates as revealed by the disappearance of P<sub>i</sub> in addition to the decay in ATP and PCr levels. The inner mitochondrial membrane does not contain any cholesterol; this membrane therefore presumably remains intact during the experiment, allowing the mitochondria to maintain the synthesis of ATP under appropriate conditions. To maintain intact mitochondria, the perfusate used for Rb<sup>+</sup> efflux studies was switched to ICM1 upon saponin infusion. This ensured that the mitochondria were submerged in a medium mimicking the cytosol and energized by 2 mM pyruvate present in ICM1.

The washout of PCr and ATP (Fig. 4) occurred at similar rates, indicating enzymatic coupling between the two high-energy phosphates in addition to sustained mitochondrial ATP production after saponin permeabilization of the sarcolemma. There was a residual amount of PCr and ATP, and the ³¹P-NMR spectrum integral contains contributions of two pools. The first pool comprises contributions from the cytoplasm and mitochondria in intact cells that were not affected by saponin. The second pool comprises mitochondrial ATP in permeabilized cells. It is uncertain if this last pool is visible by NMR. If mitochondrial ATP were not visible by NMR, the percentage of permeabilized cells would be equal to the depletion in the total phosphate signal intensity (60%; Fig. 4, inset). However, if mitochondrial ATP were visible by NMR, permeabilization by saponin would exceed 60% because a fraction of the total phosphate signal is mitochondrial and not removed by saponin treatment. In reality we have a spectrum of cells with different degrees of permeabilization and different degrees of phosphate loss. It is not possible that ~40% of the cells remain normal and nonpermeabilized, because saponin was successful in the complete removal of residual Rb<sup>+</sup>. In other words, varying degrees of high-energy phosphate loss must have taken place in the entire cell population, causing complete Rb<sup>+</sup> washout from the mitochondria.

Figure 2B shows that saponin treatment of the second Rb<sup>+</sup> pool results in complete washout of this mitochondrial Rb<sup>+</sup> in the second phase. Part of this efflux is apparently regulated by mitochondrial K<sub>ATP</sub> channels because 5 µM glibenclamide reduced the efflux rate constant by ~40% (Fig. 3). Other evidence for ATP-regulated Rb<sup>+</sup> efflux was obtained from perfusion experiments with ICM2, which partially restored cytoplasmic ATP levels (Fig. 3). When ICM2 was used, the observed Rb<sup>+</sup> efflux rate constant was significantly smaller (by ~60%) than that obtained with ICM as the perfusate. However, perfusion with ICM1 plus 5 µM glibenclamide and ICM2 did not result in significantly different Rb<sup>+</sup> efflux rate constants. These experiments show that a considerable part of the final Rb<sup>+</sup> washout in the second phase is regulated by mitochondrial K<sub>ATP</sub> channels. Inasmuch as the sarcolemmal permeability was significantly increased by saponin treatment, these K<sub>ATP</sub> channels must reside in the inner mitochondrial membrane. The remainder of the Rb<sup>+</sup> efflux could be provided through the K⁺/H⁺ exchanger pathway (7).

A possible explanation for the observed Rb<sup>+</sup> plateau in efflux experiments carried out at 20°C may be related to the thermodynamics of lipid bilayers at this temperature. The sarcolemma contains a large cholesterol fraction, whereas the inner mitochondrial membrane shows no presence of cholesterol. Cholesterol is known to smoothen or remove phase transitions in the bilayer system (4). McMurchie et al. (21) observed a discontinuity in the Arrhenius behavior of the state 3 and 4 rates of respiration in isolated rabbit heart mitochondria at ~20°C. This discontinuity was explained in terms of a membrane phase transition. Such a phase transition could reduce the chemical exchange of ions between the cytoplasm and the mitochondrion, resulting in an alkalinization of the latter. It is likely that the observation of an incomplete Rb<sup>+</sup> efflux at 20°C is related to the observations of McMurchie et al. (21). An additional ⁸⁷Rb-NMR experiment showed that this Rb<sup>+</sup> plateau could also be removed by either cooling or warming the heart (11).

Conclusion. Using ⁸⁷Rb as a biological K⁺ congener, we monitored mitochondrial K⁺ efflux in the perfused rat heart at 20°C without the superposition of transsarcolemmal fluxes. Myocardial hypothermia at 20°C caused mitochondria to retain previously accumulated Rb<sup>+</sup> for ~30 min. This phenomenon allowed the complete exchange of cytosolic Rb<sup>+</sup>, leaving the mitochondrial Rb<sup>+</sup> pool unchanged. Rb<sup>+</sup> efflux from this pool could be stimulated by cellular energy depletion or direct manipulation of mitochondrial ion metabolism. The addition of ATP and PCr to the perfusate or the addition of glibenclamide reduced the Rb<sup>+</sup> efflux rate constant relative to that resulting from perfusion without ATP and PCr or glibenclamide. This reduction in rate constant indicates the inhibition of mitochondrial K<sub>ATP</sub> channels.

These experiments allow a more detailed study of mitochondrial K⁺ fluxes. By using hypothermia, mito-
-chondria can be prepared in a Rb\(^+\)-loaded state, whereas the cytoplasmic pool can be exchanged for K\(^+\). Fast temperature switching would then allow the study of mitochondrial K\(^+\) fluxes in intact cell systems under physiological conditions.

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