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Determination of the rate of K^+ movement through potassium channels in isolated rat heart and liver mitochondria

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ABSTRACT

Both ATP-regulated (mitoK$_{ATP}$) and large conductance calcium-activated (mitoBK$_{Ca}$) potassium channels have been proposed to regulate mitochondrial K^+ influx and matrix volume and to mediate cardiac ischaemic preconditioning (IP). However, the specificity of the pharmacological agents used in these studies and the mechanisms underlying their effects on IP remain controversial. Here we used increasing concentrations of K^+-ionophore (valinomycin) to stimulate respiration by rat liver and heart mitochondria in the presence of the K^+/H^+ exchanger nigericin. This allowed rates of valinomycin-induced K^+ influx to be determined whilst parallel measurements of light scattering (A_{520}) and matrix volume (^3H_2O and [^{14}C]-sucrose) enabled rates of K^+ influx to be correlated with increases in matrix volume. Light scattering readily detected an increase in K^+ influx of <5 nmol K^+ min^{-1} per mg protein corresponding to $<2\%$ mitochondrial matrix volume increase. In agreement with earlier data no light-scattering changes were observed in response to any mitoK$_{ATP}$ channel openers or blockers. However, the mitoBK$_{Ca}$ opener NS1619 (10–50 μM) did decrease light scattering slightly, but this was also seen in K^+-free medium and was accompanied by uncoupling. Contrary to prediction, the mitoBK$_{Ca}$ blocker paxilline (10–50 μM) decreased rather than increased light scattering, and it also slightly uncoupled respiration. Our data argue against the presence of significant activities of either the mitoK$_{ATP}$ or the mitoBK$_{Ca}$ channel in rat liver and heart mitochondria and provide further evidence that preconditioning induced by pharmacological openers of these channels is more likely to involve alternative mechanisms.

1. Introduction

It has been known for many years that the inner membrane of the mitochondria contains an electrogenic mechanism for K^+ entry (K^+ channel) that works in opposition to a K^+/H^+ antiporter to pump K^+ out of the matrix. The relative activities of the two systems are thought to play a key role in the regulation of matrix volume (see [1–3]). However, the identity of these channels and transporters remains uncertain since none has been purified sufficiently to allow sequencing. Nor has genomic analysis revealed any plasma membrane K^+ channel isoform or spliced variant with a mitochondrial targeting sequence [4]. Rather the presence and properties of these K^+ transport mechanisms have been surmised from their functional characterisation in isolated mitochondria or reconstituted proteoliposomes (see [5,6]). Three mechanisms have been proposed for K^+ entry. First, many laboratories have presented evidence for an ATP-regulated K^+ channel (mitoK$_{ATP}$) similar to that found in the plasma membrane but with different sensitivities to openers such as diazoxide, cromakalim, pinacidil and nicorandil and inhibitors including glibencamide and 5-hydroxydecanoate [4,7–10]. The use of these drugs has led to the proposal that this channel plays an important role in ischaemic preconditioning (IP) (see [4,11–14]). However, other studies have questioned the existence of these channels and have implicated non-specific effects of mitoK$_{ATP}$ channel openers and blockers in their ability to mimic and antagonise IP respectively [4,7–10].

A second channel that may mediate electrogenic K^+ entry into the mitochondria is the adenine nucleotide translocase (ANT). This is proposed to occur when mitochondria are depleted of adenine nucleotides [15,16] or when adenine nucleotides have been displaced from the ANT by pyrophosphate (PPi), atractyloside or carboxyatractyloside [10,17,18]. Since micromolar [Ca^{2+}] is a potent inhibitor of mitochondrial pyrophosphatase [19] and causes matrix [PPi] to increase [20], this provides a mechanism to explain the increase in matrix volume seen in isolated mitochondria exposed to micromolar

Abbreviations: 5-HD, 5-hydroxydecanoic acid; BSA, bovine serum albumin; cromakalim, (±)-*trans*-6-cyano-3,4-dihydro-2,2-dimethyl-4-(2-oxopyrrolidin-1-yl)-2H-1-benzopyran-3-ol; diazoxide, 7-chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide; G/M, 5 mM L-glutamate plus 2 mM L-malate; mitoK$_{ATP}$, mitochondrial ATP-regulated potassium channel; mitoBK$_{Ca}$, mitochondrial large conductance calcium-activated potassium channel; NS1619, 1,3-Dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; ROS, reactive oxygen species; R/S, 5 mM succinate plus 200 nM rotenone

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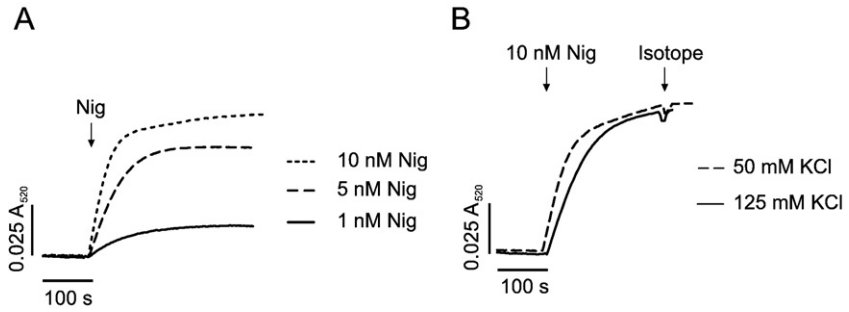

Fig. 1. The effects of nigericin on light scattering and matrix volume of rat liver mitochondria. Liver mitochondria were incubated with continuous stirring at 2 mg protein/ml in the sample and reference cuvette of a split-beam spectrophotometer as described under Materials and methods. The medium contained 50 mM KCl (panel A) or either 125 mM KCl or 50 mM KCl (panel B) and was supplemented with 5 mM succinate plus 200 nM rotenone and 1 μM oligomycin. Where indicated, nigericin was added to the sample cuvette at the concentration shown. In the experiment shown in panel B, 15 μl of buffer containing the required amounts of ^3H_2O and [^{14}C]-sucrose was added to both cuvettes at the end of the run (indicated as isotope). Four 0.8 ml samples were rapidly taken from each cuvette to pellet the mitochondria by centrifugation and to determine the matrix volume as described under Materials and methods. For the experiment shown, which is a representative of three similar experiments on separate mitochondrial preparations, the calculated volumes (±S.E.M. of four replicates) in the absence of nigericin were 1.604±0.0 to 2.02±0.02 μl per mg protein in 125 mM KCl and 50 mM KCl media respectively, whilst following the addition of 10 nM nigericin the volumes decreased to 1.21±0.04 and 1.55±0.01 μl per mg protein respectively.

[Ca^{2+}] and also mitochondria in situ upon addition of hormones that increase intracellular [Ca^{2+}] [18,21]. However, data from O'Rourke's laboratory have suggested the presence of a third mechanism for electrogenic K^+ entry, a large conductance calcium-activated potassium channel (mitoBK$_{Ca}$) that is blocked with charybdotoxin and opened by NS1619. This may provide an alternative Ca^{2+}-regulated mechanism for matrix volume regulation [22]. Pharmacological data has also implicated opening of this channel in IP [14,22–24] although once again the specificity of the pharmacological interventions is open to question [4].

Much of the evidence for the presence of mitoK$_{ATP}$ channels comes from studying the effects of pharmacological channel openers and blockers on both isolated mitochondrial volume, measured by light scattering, and potassium fluxes in detergent-solubilised inner membrane proteins reconstituted into proteoliposomes (see [5,6]). However, we have questioned whether the reconstitution studies may reflect contamination of mitochondria with plasma membranes [25]. Furthermore, data from our own and other laboratories [9,10,25] has failed to provide any evidence for the activity of mitoK$_{ATP}$ in intact heart, liver or brain mitochondria. In this paper we have devised a technique to quantify the potassium movements associated with the small light-scattering changes induced by sub-nanomolar concentrations of valinomycin and use these results to revisit the effects of openers and blockers of mitoK$_{ATP}$ and mitoBK$_{Ca}$ on mitochondrial potassium flux. Our data provide no support for either of these channels mediating significant potassium flux or volume changes in liver and heart mitochondria incubated under physiological conditions.

2. Materials and methods

2.1. Materials

Valinomycin, nigericin, paxilline, cromakalim, diazoxide, 5-hydroxydecanoate (5-HD), bovine serum albumin (BSA≥96% fatty acid free), NS1619 and all substrates used for the oxygen electrode were from Sigma (Gillingham, Dorset, U.K.).

2.2. Isolation of liver and heart mitochondria

Male Wistar rats (250–275 g) were killed by cervical dislocation. The hearts or livers were rapidly removed and placed in isolation buffer A (ISA) (300 mM sucrose, 10 mM Tris–HCl, 2 mM EGTA, pH 7.4) to remove as much blood as possible. Heart tissue was

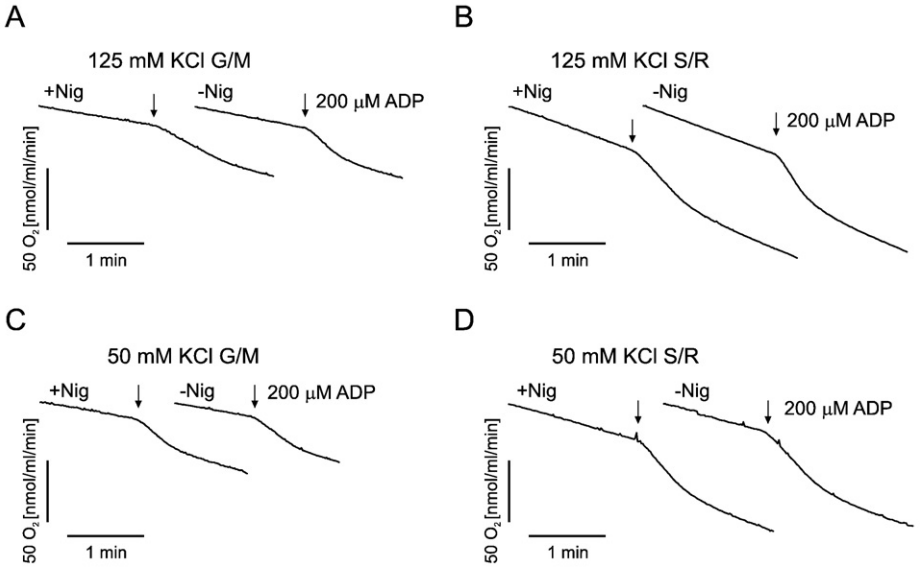

Fig. 2. The effects of nigericin and osmolarity on respiration by rat liver mitochondria. Rat liver mitochondria were incubated in 1 ml buffer containing either 125 mM KCl (panels A and B) or 50 mM KCl (panels C and D) and the respiratory substrate indicated G/M — 5 mM L-glutamate plus 2 mM L-malate; S/R — 5 mM succinate plus 200 nM rotenone. Where indicated (+Nig) nigericin (10 nM) was added before mitochondria. The addition of ADP (200 μM) is marked by arrows. Data shown are typical of at least three separate experiments.

finely cut up with scissors and incubated for 10 min on ice in 5 ml ISA containing 2 mg Nagarse (Bacterial Proteinase P8038 Sigma). For both tissues, homogenisation was performed in ISA containing 0.5% (w/v) BSA (ISB) using a glass Dounce Potter homogeniser and thereafter the preparation of mitochondria was performed by differential centrifugation and Percoll gradient centrifugation as described previously [9,26]. All procedures were performed at 4 °C and mitochondria were kept on ice until use; this was always within 4 h of preparation. The protein concentration of mitochondrial suspensions was determined using the biuret method [27]. In all oxygen electrode and spectrophotometer experiments liver and heart mitochondria were used at 2 and 1 mg protein/ml respectively.

2.3. Experimental buffers

All buffers for oxygen electrode and spectrophotometer experiments contained 20 mM MOPS, 10 mM Tris base, 2 mM phosphate (K- or Na-salt as required), 0.1 mM sodium-EGTA and 1 mg/ml BSA (≥96% fatty acid free) and an osmotic support which was either 125 mM KCl, 50 mM KCl (hypo-osmotic) or 100 mM sucrose (hypo-osmotic potassium free). The pH was adjusted to pH 7.2 with KOH or Tris base (potassium free). The hypo-osmotic media were employed to overcome the decrease in matrix volume caused by nigericin addition and to ensure maximal rates of respiration [28,29]. Other additions were made as required.

2.4. Oxygen electrode studies

The oxygen electrode (Hansatech Instruments Ltd.) was connected to a computer for data recording and all experiments were performed at 30 °C in 1 ml of experimental buffer containing either 5 mM L-glutamate plus 2 mM L-malate (G/M) or 5 mM succinate plus 200 nM rotenone (S/R) as respiratory substrate. Calibration was performed by assuming that air-saturated KCl medium contains 225 nmol O_2 per ml at 30 °C and zero oxygen after dithionite addition.

2.5. Light-scattering and radio-isotope measurements of changes in mitochondrial matrix volume

Light scattering was used to monitor matrix volume changes in real time and was measured as the change in A_{520} as described previously [9]. Mitochondria were added to 7 ml of the required buffer before apportioning 3.5 ml to both the sample and reference cuvettes which were maintained at 30 °C with constant stirring to ensure oxygenation. A_{520} was continuously recorded at 2 data points per second and additions made through an injection port as required. In some experiments absolute determination of the matrix volume of mitochondria was made by measurement of the matrix water content using ^3H_2O and [^{14}C]-sucrose added directly to both reference and sample cuvettes at the end of a run as described previously [9].

3. Results

3.1. Measurement of the rate of electrogenic K^+ entry into the mitochondria using an oxygen electrode

The addition of the K^+/H^+ exchanger, nigericin, to energised mitochondria will cause any K^+ that enters the mitochondria electrogenically through a K^+ channel to leave the matrix again in exchange for a proton. This stoichiometrically converts electrogenic K^+ entry into H^+ entry, leading to a stimulation of respiration as these protons are pumped back out to regenerate the proton motive force. Since the H^+:O ratio is known to be 6 for succinate in the presence of rotenone and 10 for glutamate plus malate this allows the stimulation of the rate of respiration mediated by the opening of a K^+ channel to be converted into the rate of K^+ influx. However, there is a problem associated with this approach in that addition of nigericin to mitochondria causes loss of endogenous K^+ ions and shrinkage of the mitochondrial matrix. This is illustrated in panel A of Fig. 1 where increasing concentrations of nigericin caused a progressive shrinkage of energised liver mitochondria as reflected in an increase in light scattering (A_{520}). This is likely to cause an inhibition of respiration because the flow of electrons from complex I and complex II into complex III is strongly inhibited by a decrease in matrix volume [28,29]. The data of Fig. 2 confirm that this is the case for ADP-stimulated respiration of liver mitochondria in the presence of glutamate plus malate (panel A) or succinate plus rotenone (panel B). In order to overcome this decrease in matrix volume, hypo-osmotic media can be used as illustrated in the data of panels C and D of Fig. 2. In parallel experiments reported in panel B of Fig. 1 we determined matrix volume of rat liver mitochondria using ^3H_2O and [^{14}C]-sucrose in the same mitochondrial samples used to determine the decrease in light scattering induced by 10 nM nigericin. Liver mitochondria were incubated in both sample and reference cuvettes of the spectrophotometer and then 10 nM nigericin added to the sample cuvette. When the light-scattering signal had reached a new equilibrium, ^3H_2O and [^{14}C]-sucrose were added to both sample and reference cuvettes for the determination of matrix volume. The resting matrix volume (μl per mg protein ± S.E.M. of 4 replicates) in the absence of nigericin was determined as 1.60 ± 0.02 in normal medium (iso-osmotic — 125 mM KCl) whilst in hypo-osmotic medium (50 mM KCl) the volume increased to 2.02 ± 0.02. Following 10 nM nigericin addition the volumes decreased to 1.21 ± 0.04 and 1.55 ± 0.01 μl per mg protein in the 125 mM KCl and 50 mM KCl media respectively. Importantly, these data confirm that the presence of nigericin in the 50 mM KCl medium restored the volume to a similar value to that seen in the 125 mM KCl medium in the absence of nigericin. For this reason, subsequent experiments investigating the stimulation of respiration by valinomycin in the presence of nigericin were performed in the 50 mM KCl medium.

In Fig. 3 the effects of adding increasing concentrations of valinomycin in the presence of nigericin on the rate of state 4 respiration are shown for liver (panels A and B) and heart (panel C)

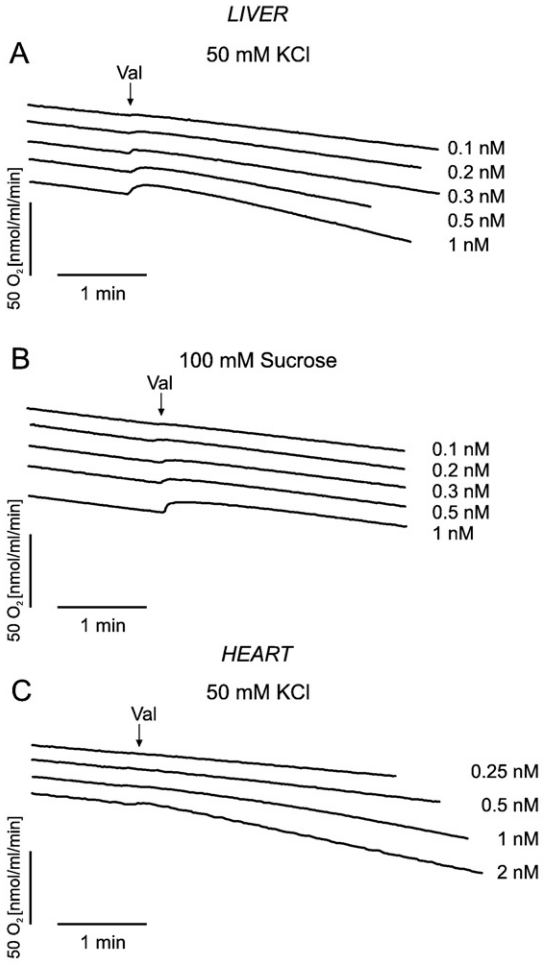

Fig. 3. Changes in the rate of respiration of rat liver and heart mitochondria induced by increasing concentrations of valinomycin. Mitochondria from rat liver (panels A and B) or heart (panel C) were incubated in 1 ml of either 50 mM KCl (panels A and C) or 100 mM sucrose (panel B) respiration buffer containing 1 μM oligomycin and nigericin (10 and 1 nM for liver and heart mitochondria respectively) and 5 mM L-glutamate plus 2 mM L-malate. Rates of respiration were measured by linear regression of the trace before and after addition (at the arrow) of valinomycin at the concentration shown.

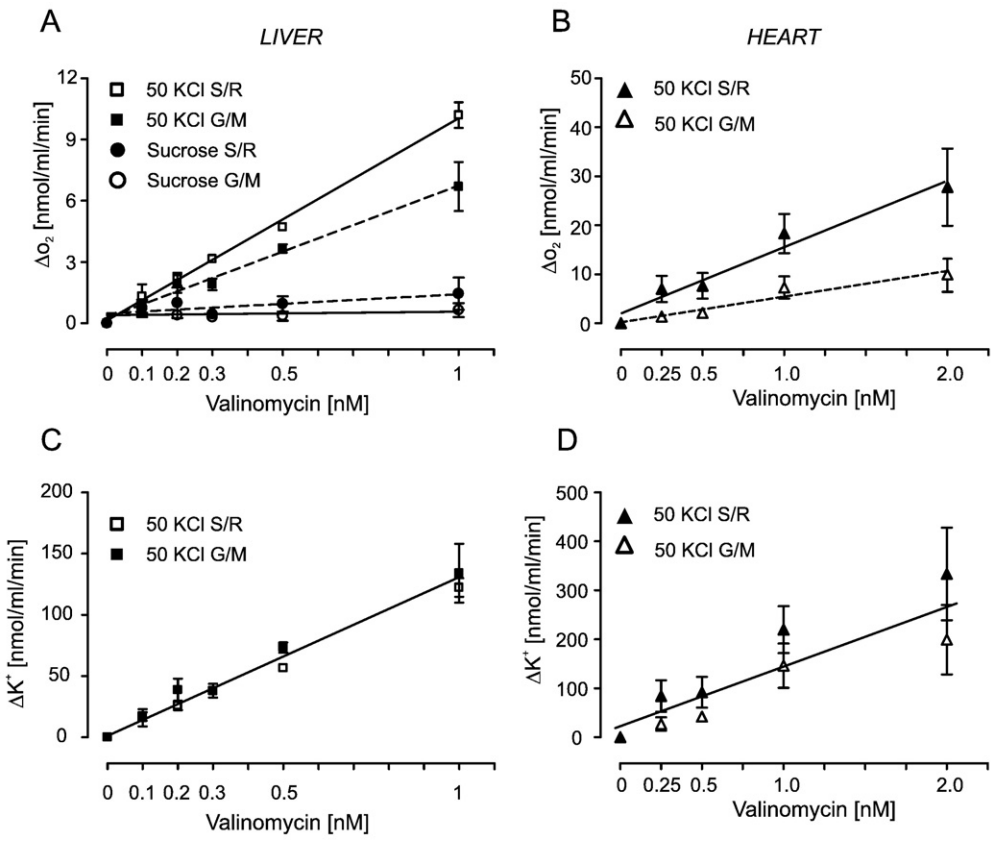

Fig. 4. Quantification of the flux of K^+ into mitochondria induced by increasing concentrations of valinomycin. Rates of state 4 respiration by rat liver (panels A and C) or heart (panels B and D) mitochondria in the presence of 1 μM oligomycin and nigericin at 10 nM (liver) or 1 nM (heart) were determined as described in Fig. 3 both before and after addition of valinomycin at the concentration shown. The respiratory substrate was either 5 mM L-glutamate plus 2 mM L-malate (G/M) or 5 mM succinate plus 200 nM rotenone (S/R) as indicated. In panels C and D the valinomycin-induced increase in respiration was converted into fluxes of K^+ using an H^+:O ratio of 10 or 6 for G/M or S/R respectively as explained in the text. Data are given as means±S.E.M. (error bars) of 3 (liver) or 2 (heart) separate experiments each using a different mitochondrial preparation. In Fig. 6 panel D the data of panel C are plotted against mean data for the changes in A_{520} induced by the same concentrations of valinomycin measured in parallel experiments.

mitochondria. The stimulation of respiration observed in KCl medium (panel A) was not observed in sucrose medium (panel B), confirming that it is potassium entry followed by K^+/H^+ exchange that is leading to the stimulation of respiration. Fig. 4 shows the mean data for the stimulation of respiration mediated by increasing concentrations of valinomycin in liver (panel A) and heart (panel B) mitochondria. Data are given for both glutamate plus malate and succinate plus rotenone as substrate. In panels C and D the valinomycin-induced increase in respiration (nmol min^{-1} per mg protein) has been converted into fluxes of K^+ using an H^+:O ratio of 10 or 6 for G/M or S/R respectively. This brings the data for both substrates onto the same line, providing further confirmation that the stimulation of respiration is providing an accurate estimate of the rate of K^+ entry into the matrix.

3.2. The effects of valinomycin on light scattering and matrix volume

In parallel with measurements of the rate of K^+ entry into mitochondria induced by increasing concentrations of valinomycin we monitored the light-scattering changes induced by valinomycin under identical conditions and determined the increase in matrix volume using ^3H_2O and [^{14}C]-sucrose. These measurements provide an indication of the sensitivity of light scattering as a technique for measuring electrogenic K^+ fluxes. In Fig. 5 panel A we show the increase in light scattering induced by 10 nM nigericin followed by the subsequent decrease in light scattering upon progressive additions of valinomycin. In Panel B we show the effects of the same concentrations of valinomycin in the absence of nigericin. The responses in the absence of nigericin are considerably larger than those observed in its presence which provides good evidence that the nigericin is present at high enough concentrations to facilitate loss of K^+ from the matrix almost as fast as it enters. In panel C we show that in sucrose medium the nigericin gave the anticipated increase in light scattering as K^+ leaves the matrix and mitochondria shrink, but that valinomycin gave no compensating decrease in scattering since no external K^+ was available to enter. Panels D and E show similar data for the effects of increasing concentrations of valinomycin on the light scattering of heart mitochondria in the presence and absence of 1 nM nigericin respectively. We found it necessary to use less nigericin for heart mitochondria than liver mitochondria if we were not to reduce the matrix volume too much and thus inhibit respiration. Since the difference between the change in light scattering seen in the absence and presence of nigericin is not great, it is clear that at 1 nM the nigericin cannot exchange the K^+ entering via valinomycin fast enough to prevent some potassium accumulation and thus swelling. Consequently, for the heart mitochondria experiments the increase in the rate of respiration induced by valinomycin may underestimate the rate of K^+ flux.

In the experiment shown in Fig. 6 panel A we used ^3H_2O and [^{14}C]-sucrose to determine the valinomycin-induced change in matrix volume in the same liver mitochondrial samples used to monitor changes in light scattering. The mitochondria were incubated in 50 mM KCl medium in the presence of 10 nM nigericin prior to adding increasing concentrations of valinomycin to the sample cuvette and then ^3H_2O and [^{14}C]-sucrose added to both cuvettes for absolute matrix volume determination. Panel B confirms that no change in light scattering occurred in response to valinomycin in potassium-free (sucrose) medium. Panel C presents mean data from 3 similar

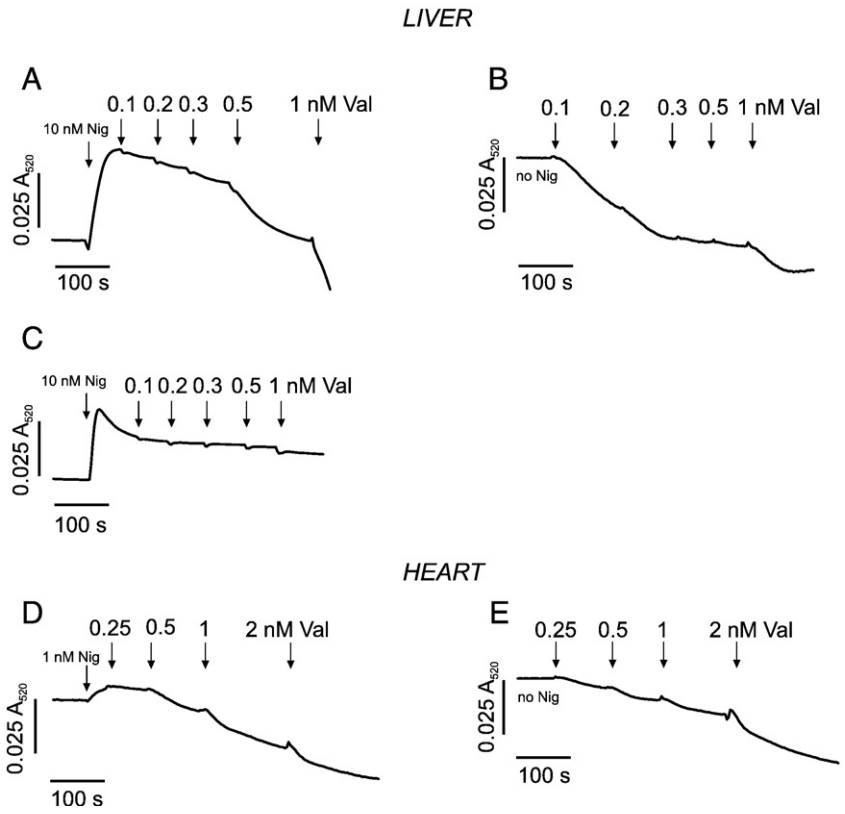

Fig. 5. The effects of nigericin and valinomycin on the light-scattering response of liver and heart mitochondria. Mitochondria from rat liver (panels A–C) or heart (panels D–E) were incubated with continuous stirring in both sample and reference cuvettes of a split-beam spectrophotometer at 2 mg and 1 mg protein per ml respectively as described under Materials and methods. The buffers used were 50 mM KCl medium (panels A, B, D, and E) or 100 mM sucrose medium (panel C) containing 5 mM succinate plus 200 nM rotenone (S/R) and 1 μM oligomycin. Additions were made of nigericin (Nig) and valinomycin (Val) at the times and concentrations indicated.

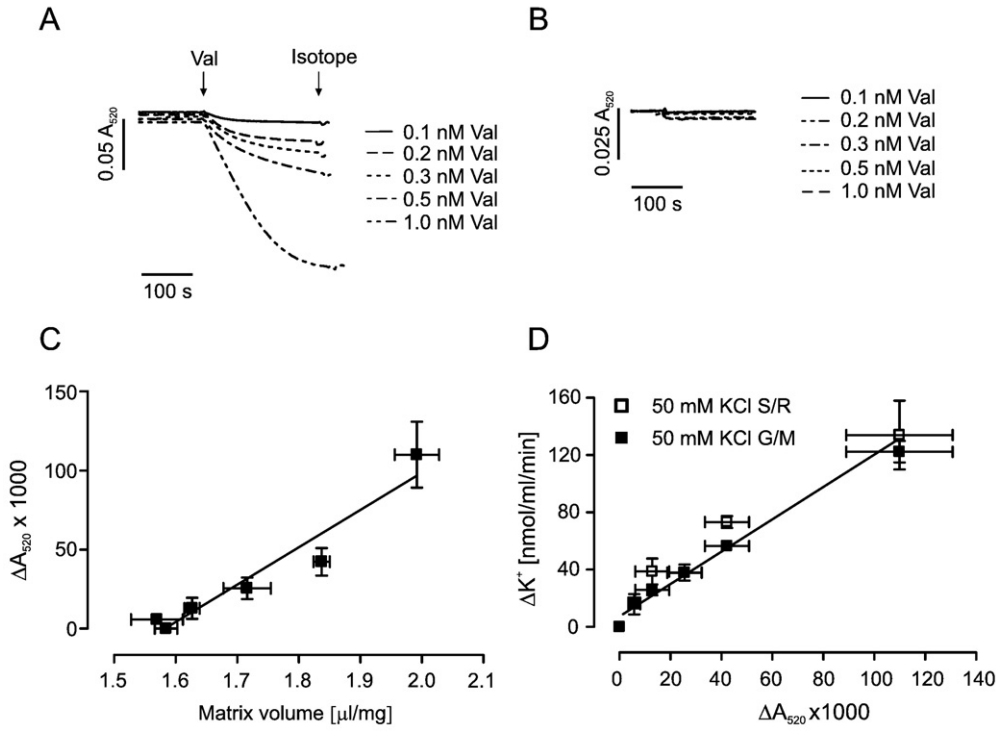

Fig. 6. The relationship between the effects of valinomycin on light scattering, matrix volume and K^+ flux of rat liver mitochondria. Liver mitochondria were incubated in 50 mM KCl medium (panel A) or 100 mM sucrose medium (panel B) containing 10 nM nigericin and 1 μM oligomycin as described for Figs. 1 and 5 and additions of valinomycin (Val) made to the sample cuvette at the times and concentrations indicated. At the end of the run (labelled isotope) ^3H_2O and [^{14}C]-sucrose were added to both cuvettes and matrix volumes measured as described for Fig. 1. Panel C shows the correlation between mean data (±S.E.M. — error bars) for the measured change in light scattering induced by increasing valinomycin concentrations and the matrix volume from 3 separate experiments performed as in panel A. Panel D shows a similar correlation between mean data for the light scattering induced by increasing valinomycin concentrations and the increase in K^+ flux taken from the experiments shown in Fig. 4 panel C.

experiments to those shown in panel A in which the increase in matrix volume induced by valinomycin is plotted against the mean decrease in light scattering induced by valinomycin in the same experiment. In panel D of Fig. 6 we present the correlation between the decrease in light scattering and the calculated rate of K^+ flux into mitochondria presented in panel C of Fig. 4.

3.3. The effects of potassium channel openers and blockers on light scattering and respiration of rat liver and heart mitochondria

Having established the sensitivity of the light scattering and respiration techniques for measuring K^+ flux across the inner mitochondrial membrane we used these techniques to determine changes in K^+ flux induced by agents that have been reported to act as openers or blockers of the putative mitoK$_{ATP}$ and mitoBK$_{Ca}$ channels. The data of panels A and B of Fig. 7 confirm our previous data [9] that neither diazoxide (30 or 50 μM) nor cromakalim (10, 20 or 30 μM), both reported to be mitoK$_{ATP}$ channel openers [5], cause any detectable decrease in light scattering of either liver or heart mitochondria. Nor did 5-hydroxydecanoate (100 or 200 μM), reported to be a mitoK$_{ATP}$ channel blocker [5], cause any detectable increase in light scattering. In panels C–F of Fig. 7 we extend our investigations to NS1619 and paxilline that have been reported to be openers and blockers respectively of the putative mitoBK$_{Ca}$ channel [22]. As might be predicted for a K^+ channel opener, we found that addition of increasing concentrations of NS1619 to either liver (panel C) or heart (panel D) mitochondria caused a decrease in A_{520}. However, we also show that when the experiments were repeated in K^+-free sucrose buffer the same decreases in A_{520} were observed. This implies that NS1619 is exerting a non-specific effect unrelated to the opening of mitoBK$_{Ca}$ channels. The data of Fig. 8 panel A suggest that this non-specific effect may be due to uncoupling of the mitochondria since increasing concentrations of

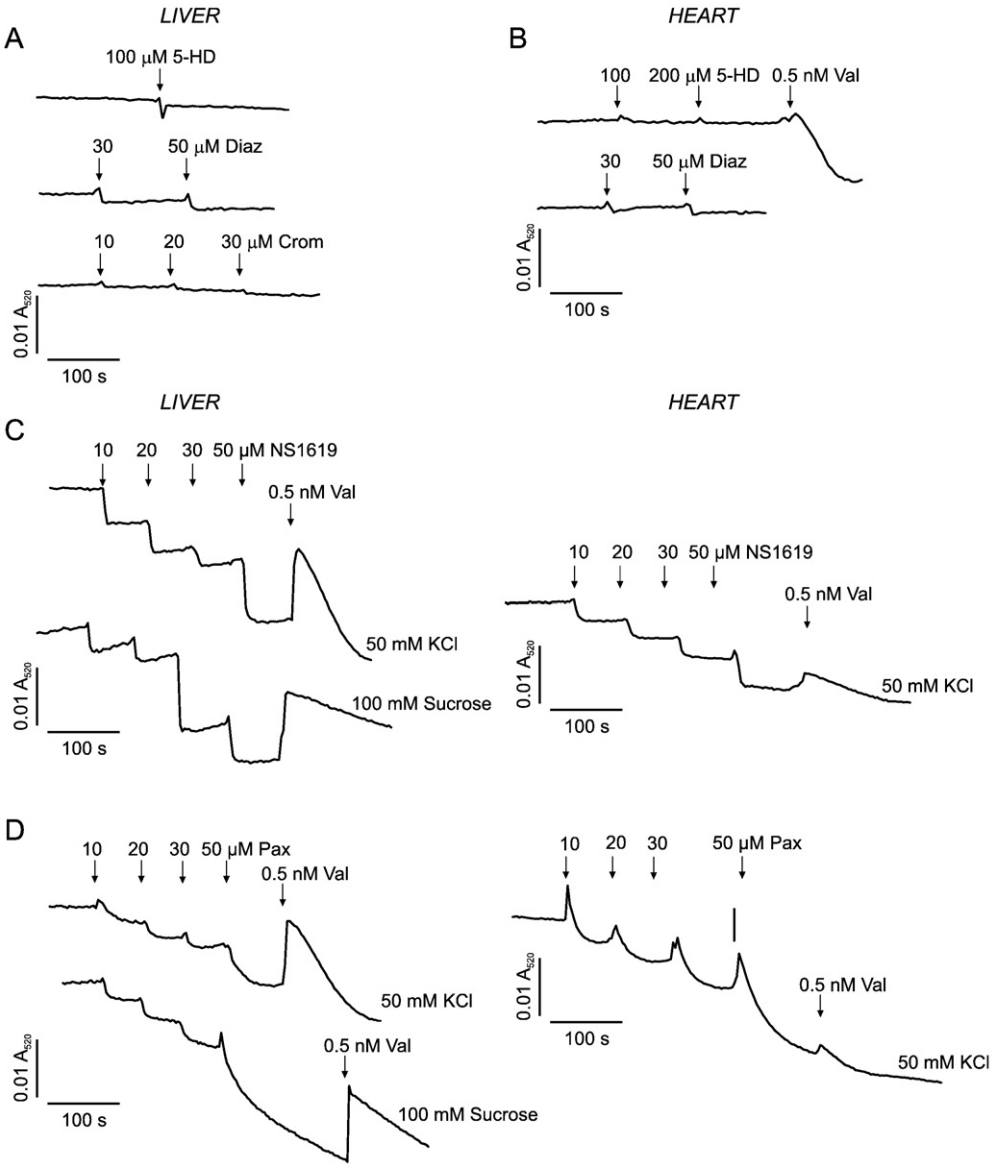

Fig. 7. The effects of potassium channel openers and blockers on light scattering of rat liver and heart mitochondria. The experimental protocol was the same as that described in Fig. 5 using either 50 mM KCl or 100 mM sucrose buffer containing 5 mM L-glutamate plus 2 mM L-malate as respiratory substrate and 1 μM oligomycin. Each panel shows representative data obtained from 2 (heart) or 3 (liver) separate mitochondrial preparations that gave similar results. Panels A and B represent typical traces revealing no effects of K_{ATP} channel openers (diazoxide, cromakalim) or blockers (5-HD) added at the concentrations and times shown whilst panels C and D represents typical traces revealing no effects of BK_{Ca} channel openers (NS1619) or blockers (paxilline). Note that for paxilline and NS1619 the decreases in light scattering observed were also seen in sucrose medium and thus are unlikely to be caused by K^+ movements.

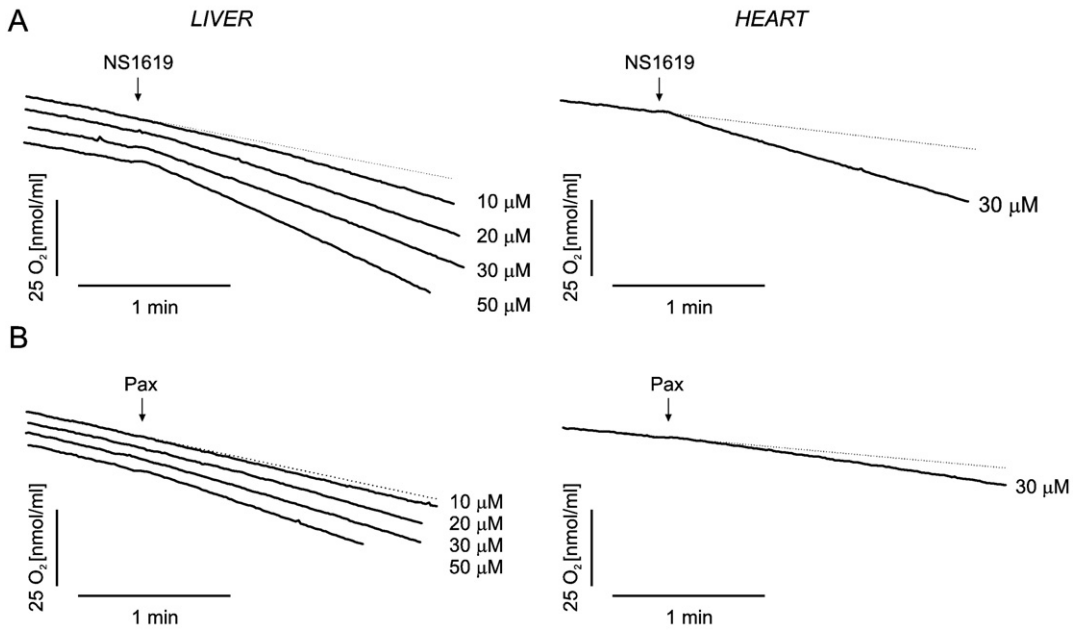

Fig. 8. NS1619 and paxilline stimulate respiration by rat liver and heart mitochondria. Mitochondria were incubated in 1 ml 50 mM KCl respiration buffer containing 5 mM L-glutamate plus 2 mM L-malate and 1 μM oligomycin. Additions of NS1619 or paxilline were made as indicated at the concentrations shown. The data presented are from a single preparation each of rat heart (A) and liver (B) mitochondria.

NS1619 progressively stimulated respiration by liver and heart mitochondria even in the absence of nigericin. Similar non-specific effects were observed with increasing concentrations of paxilline (10–50 μM) which caused a decrease in A_{520} and not an increase as would be expected for a K^+ channel blocker (Fig. 7 panel D). Here too the paxilline-induced decrease in A_{520} was also observed in K^+-free sucrose medium and oxygen electrode experiments suggested that slight uncoupling was occurring (Fig. 8 panel B). In view of the non-specific uncoupling effects of both paxilline and NS1619 on mitochondria, it is not possible to assess whether these agents might also have exerted effects on a mitoBK$_{Ca}$ channel.

4. Discussion

4.1. The sensitivity of light-scattering measurements used to detect modulation of mitochondrial K^+ channel activity

Many laboratories have used light-scattering techniques to detect changes in mitochondrial volume that accompany changes in the permeability of the mitochondrial inner membrane to K^+ (see [3,5,9,10,25]). Using such techniques we [9] and others [10] were unable to provide any evidence for a stimulation of K^+ entry into heart, liver or brain mitochondria by agents such as diazoxide, pinacidil or cromakalim that others have reported to be openers of the putative mitoK$_{ATP}$ channel [5]. However, it could be argued that the changes in K^+ flux are too small to induce detectable changes in light scattering, so in the present paper we have described a technique that allows an approximate quantification of the flux of K^+ that accompanies a detectable change in A_{520}. By incubating mitochondria in the presence of nigericin the entry of K^+ into mitochondria mediated by a K^+ channel is converted into a proton flux that uncouples mitochondria as illustrated for increasing concentrations of valinomycin in Fig. 3. From the resulting stimulation of oxygen consumption the rate of K^+ entry can be calculated as is shown in Fig. 4. The lowest concentration of valinomycin (0.1 nM) that gave a detectable stimulation of respiration by liver mitochondria (2 mg per ml) was calculated to give a K^+ flux of about 19.3±5.7 nmol per min per mg protein. This gave a readily measurable increase in volume in the presence or absence of 10 nM nigericin (Fig. 5 panels A and B respectively) with the effect being at least 5 times greater in the absence of nigericin where the compensatory efflux of K^+ is not stimulated. One caveat in relation to these calculations is that endogenous proton leak increases steeply with membrane potential [30]. Although any depolarisation caused by 0.1 nM valinomycin is likely to be very small, it could lead to a slight underestimate of the proton influx caused by potassium cycling. Thus the absolute values calculated for K^+ flux must be regarded as approximate. Nevertheless, our data lead us to conclude that the light-scattering technique is capable of detecting a stimulation of K^+ influx of less than 5 nmol per min per mg protein corresponding to an increase in matrix volume of <2%.

4.2. Pharmacological agents provide no evidence for detectable activities of mitoK$_{ATP}$ and mitoBK$_{Ca}$ channel in heart or liver mitochondria

Our inability to detect any change in light scattering by either K_{ATP} channel openers such as diazoxide and cromakalim or blockers such as 5-hydroxydecanoate or glibencamide (Fig. 7 and [9]) suggests that the activity of these channels in rat liver and heart mitochondria in vitro is likely to be very low (<5 nmol per min per mg protein) and unable to produce any detectable change in volume or uncoupling. Furthermore our data do not provide any evidence for the presence of a mitoBK$_{Ca}$ channel suggested by others [22]. Thus, although NS1619, which is proposed to be an opener of this channel, induced a decrease in A_{520} (Fig. 7 panel C) and a stimulation of oxygen consumption (Fig. 8 panel A) this is unlikely to be due to K^+ entry because the same decrease in light scattering was seen in K^+-free sucrose medium. Uncoupling by NS1619 has also been described by others [31,32] and it seems likely that the light-scattering effects of NS1619 are the result of such uncoupling rather than the opening of mitoBK$_{Ca}$ channels. Furthermore, the proposed mitoBK$_{Ca}$ channel blocker paxilline produced a decrease in A_{520} rather than the increase that might be expected (Fig. 7 panel D). This effect was also seen in K^+-free sucrose medium and was accompanied by uncoupling (Fig. 8 panel B) implying that paxilline, like NS1619 and some effectors of the putative mitoK$_{ATP}$ channel [33,34] cannot be used as specific pharmacological agents. However, our data do not

allow us to rule out totally the presence of a mitoBK$_{Ca}$ channel because the non-specific effects of NS1619 and paxilline may have masked real effects on this channel activity.

4.3. Mitochondrial K^+ channels are unlikely to mediate preconditioning

Although our data do not allow us to conclude that neither mitoK$_{ATP}$ nor mitoBK$_{Ca}$ channels exist, they do suggest that their activities are likely be too low to affect either matrix volume or membrane potential (via K^+-mediated uncoupling). Yet both an increase in matrix volume and mild uncoupling induced by K^+ channel opening have been proposed to mediate ischaemic preconditioning (see [4,12,14,25]). Two mechanisms have been suggested by which increase in matrix volume might offer protection; either through stimulation of the respiratory chain and enhanced ATP production or by bringing the inner and outer membranes closer together to allow for more efficient ATP export from the mitochondria through contact sites (see [12,25]). However, our inability to detect any changes in volume by K^+ channel openers described here (Fig. 7) and previously [9], and the lack of correlation between cardioprotection and changes in matrix volume determined in situ [8] make either of these possibilities unlikely. Mild uncoupling of mitochondria by K^+ channel openers has been proposed to depolarise the mitochondria sufficiently during reperfusion to reduce ROS production and calcium accumulation and hence prevent the opening of the mitochondrial permeability transition pore. However, as argued by others [35,36] and by our own data presented here, the rate of K^+ entry through K^+ channels would be too low to cause significant uncoupling. Furthermore, direct measurements of mitochondrial membrane potential failed to detect any depolarisation [37,38]. Moreover, there is evidence that diazoxide can enhance ROS production independently of mitoK$_{ATP}$ channel opening, perhaps through its well documented inhibition of the respiratory chain [39].

4.4. Mitochondrial K_{ATP} and BK_{Ca} channel openers may precondition independently of channel opening

There is increasing evidence that the supposed 'specific' mitochondrial K^+ channel openers have other non-specific effects on mitochondrial function, including uncoupling [7,33,34,40] and inhibition of complex I and complex II of the respiratory chain [7,8,36,39,41–42]. These non-specific effects may actually be the mechanism by which preconditioning is exerted rather than through K^+ channel opening. Thus it is known that adding low doses of uncoupler prior to ischaemia can precondition hearts [43,44]. Furthermore, any interference with oxidative phosphorylation during the preischaemic phase seems able to exert a similar protective effect including the addition of a respiratory chain inhibitor [45–48] or a succinate dehydrogenase inhibitor [49]. How this might be translated into a protective effect at reperfusion is not clear, although some respiratory chain inhibitors have been reported to increase ROS production in isolated cardiac myocytes [39,45,50] as have low doses of uncoupler [44]. Since ROS can activate protein kinase C (PKC) which is known to be important in preconditioning (see [25,51]) this may provide a mechanism for the preconditioning effect. Indeed, the protective effects of diazoxide and nicorandil can be overcome by free radical scavengers such as *N*-(2-mercaptopropionyl) glycine [4,39,52,53]. Furthermore, in the case of the established respiratory chain inhibitor, antimycin, cardioprotection is abolished in PKCε knockout mice [50].

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