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Single Anion Channels Reconstituted from Cardiac Mitoplasts

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Abstract. Ion channels from sheep cardiac mitoplast (inverted inner mitochondrial membrane vesicle) preparations were incorporated into voltage-clamped planar lipid bilayers. The appearance of anion rather than cation channels could be promoted by exposing the bilayers to osmotic gradients formed by Cl^- salts of large, relatively impermeant, cations at a pH of 8.8. Two distinct activities were identified. These comprised a multi-substate anion channel of intermediate conductance (~60 pS in 300 *vs.* 50 mM choline Cl, ~100 pS in symmetric 150 mM KCl), and a lower-conductance anion channel (~25 or ~50 pS in similar conditions), which only displayed two well-defined substates, at ~25 and ~50% of the fully open state. The larger channels were not simple multiples of the lower-conductance channels, but both discriminated poorly, and to a similar extent, between anions and cations ($P_{Cl^-}/P_{choline^+}$ ~12, P_{Cl^-}/P_{K^+} ~ 8). The lower-conductance channel was only minimally selective between different anions ($P_{NO_3^-}(1.0) = P_{Cl^-} > P_{Br^-} > P_{I^-} > P_{SCN^-}(0.8)$), and its conductance failed to saturate even in high (>1.0 M) activities of KCl. The channels were not obviously voltage dependent, and they were unaffected by 0.5 mM SITS, H_2O_2, propranolol, quinine or amitriptyline, or by 2 mM ATP, or by variations in pH (5.5–8.8). Ca^{2+} and Mg^{2+} did not alter single channel activity, but did modify single current amplitudes in the lower-conductance channel. This effect, together with voltage-dependent substate behavior, is described in the following paper.

Key words: Anion channel — Mitochondrion — Planar bilayer — Substate

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Introduction

Direct electrophysiological studies have revealed a number of distinct ion channels in the inner, as well as the outer, mitochondrial membrane (Mannella & Tedeschi, 1992; Sorgato & Moran, 1993). Putative inner membrane channels include a voltage-dependent channel with a conductance ~100 pS (in 150 mM KCl), first observed in patches of inner mitochondrial membrane vesicles isolated from the livers of cuprizone-fed mice (Sorgato, Keller & Stühmer, 1987), but which has as yet no known function; non-selective multiconductance channel activity of ~5 pS to ~1 nS (Kinnally, Tedeschi & Campo, 1989 and Petronilli, Szabò & Zoratti, 1989), at least some of which may correspond to the "Ca-dependent pore" of Crompton, Costi and Hayat (1987); low-conductance (~20 pS) pH-sensitive channels, described by Antonenko, Kinnally and Tedeschi (1991), which may be related to the pH-dependent anion pathway of Garlid and Beavis (1986); low-conductance channels in contact sites and inner membranes (Moran et al., 1990); and finally Inoue et al. (1991) have demonstrated a low-conductance, ATP-inactivated channel.

All these channels were initially identified by patch clamping native, inner mitochondrial membranes. Planar bilayer reconstitution, on the other hand, can reveal ion channels which are of very low abundance, or which remain anatomically inaccessible to patch-clamp microelectrodes. The addition of highly purified sheep cardiac mitoplast membrane vesicles to bilayers bathed by chloride salts of large, relatively impermeant cations, at a pH of 8.8 (not dissimilar to mitochondrial matrix pH), minimized the appearance of complicated, mainly cationic, channel activity, and allowed us to observe multi-substate anion channels. These fell into two clear groups, the first characterized by multiple sublevels

and a conductance of ~60 pS in a 300 : 50 mM gradient of choline Cl, and low-conductance channels (~25 pS in equivalent conditions), which only had two well-defined substates.

The conductance, selectivity and substate behavior of these apparently novel anion channels, and the effectiveness of a range of inhibitors, are described in this report. Two striking properties of the low-conductance channels, namely their Ca^{2+}-dependent rectification and their multisubunit type of substate behavior, are analyzed in detail in the succeeding paper (Hayman & Ashley, 1993).

Materials and Methods

Preparation of Sheep Heart Mitochondria

Sheep hearts freshly obtained from a local abattoir were immersed in ice-cold cardioplegia solution (mM: 100 NaCl, 30 Na lactate, 20 KCl, 16 $MgCl_2$, 2 $CaCl_2$, 2 EGTA) and transported to the laboratory within 15–30 min. Subsequent steps were carried out immediately, either at 4°C or on ice. After removing fat, epi- and endocardium, left ventricular and septal muscles were minced and suspended at 50% (w/v) in isolation medium (0.25 M sucrose, 1 mM $CaCl_2$, 5 mM KH_2PO_4, 0.1% BSA, 5 mM MOPS-KOH, pH 7.4) containing 7 mg collagenase per 100 ml (Toth, Ferguson-Miller & Suelter, 1986). Following digestion for 40 min, 2 mM EGTA was added to inactivate the protease and the medium was drained through cheesecloth. The partially disrupted tissue was resuspended at 15% (w/v) in isolation medium containing 2 mM EGTA and homogenized in two stages, using first a loose-fitting Teflon/glass homogenizer (100–300 rpm, 6 up-and-down strokes), and then a tight-fitting Teflon/glass homogenizer (800 rpm, 12 up-and-down strokes). Mitochondria were isolated by differential centrifugation as follows. Cell debris was first removed by centrifugation at 1,500 × g_{max} for 10 min, retaining the supernatant and washing and discarding the pellet. The (combined) supernatants were centrifuged at 10,000 × g_{max} for 20 min to give a mitochondrial fraction which was washed once and repelleted. Measurements of mitochondrial respiration and P : O ratios showed the preparations to be metabolically competent and well coupled (*results not shown*).

Isolation of Mitoplast Membrane Vesicles

Inverted inner membrane vesicles were obtained by hyposmotic lysis and sonication of mitochondria which had been exposed to digitonin (Williams & Pedersen, 1986), a modification of the original method of Chan, Greenwalt and Pedersen (1970). Briefly, freshly isolated mitochondria were suspended in ice-cold isolation medium (100 mg/ml) and stirred on ice for 20 min with 12 mg/ml digitonin. The mixture was then diluted with three volumes of isolation medium and centrifuged for 10 min at 10,000 × g_{max} at 4°C. The pellet was washed in twice the original volume of isolation medium and finally resuspended in distilled, deionized water (100 mg original protein/ml) and sonicated using a probe sonicator. At this point samples (on ice) were subjected either to "mild" sonication (Dawe Soniprobe, 5 mm, 6 × 5 sec bursts at half-maximal power) or "vigorous" sonication (Ultrasonics Rapidis 150, 9 mm, 6 × 5 sec bursts at maximal power). Inverted inner membrane vesicles (mitoplast membrane vesicles) were obtained by a two-stage centrifugation procedure as described by Williams and Pedersen (1986). The vesicles were suspended in 0.4 M sucrose (~5 mg protein/ml) and aliquots were frozen in liquid N_2 and stored at −70°C. Protein content was measured by using a modification of the Lowry method (Markwell et al., 1981). These vesicles, hereafter referred to as "mitoplast membrane vesicles" (Schnaitman & Greenwalt, 1968; Chan et al., 1970; Williams & Pedersen, 1986), were characterized (in the original liver preparation) by their high ATPase activity (~6 μmol/min/mg protein in washed membranes, Williams & Pedersen, 1986).

Planar Bilayer Recording

Inner mitochondrial membrane ion channels were incorporated into planar lipid bilayers formed from palmitoyl-oleoyl phosphatidylthanolamine (POPE) and palmitoyl-oleoyl phosphatidylserine (POPS), having dispersed the lipids in decane at a concentration of 30 mg total lipid per ml (in a 1 : 1 w/w ratio unless otherwise noted). The lipid dispersions were cast across a 300–400 μm hole in a polystyrene partition separating two solution-filled chambers, each containing 600 μl of the indicated Cl^- salt buffered by 5 mM Tris/HEPES to a pH of 8.8. The concentration of HEPES was minimized because it has been shown to block certain Cl^- channels (Yamamoto & Suzuki, 1987), and occasionally Tris-HCl was substituted. Unless otherwise noted, the solutions also contained 2 mM $CaCl_2$. The contents of both chambers were changed, when required, by perfusion. All potentials are quoted as *cis-trans*. The *cis* chamber, to which vesicles were added, was voltage-clamped at a range of holding potentials relative to the *trans* chamber, which was grounded. We used either a Biologic RK-300 patch-clamp amplifier (Intracel, UK), or a home-made current-to-voltage converter, connected to the chambers by chlorided Ag wires and agar salt bridges containing 3 M CsCl. Liquid junction potentials were nulled by applying an appropriate offset potential after current-clamping the bilayer, or by balancing the opamp in the current-to-voltage converter just before the bilayer was cast. Membrane thinning was monitored by observing the formation of a black membrane and by measuring the membrane capacitance (which reached 250–300 pF). None of the membranes used showed spontaneous channel-like activity, and all had conductances of <5 pS. In subsequent experiments, bilayers were stable under voltage clamps of up to ±100 mV, provided these were only applied for ~30 sec, and could often be held for long periods at potentials of ±50 mV without evidence of breakdown or thickening. Transmembrane currents were digitized and recorded on videotape using an Instrutech VR10 data recorder or a modified digital audio processor.

Data Analysis

Single channel data were low-pass filtered at cut-off (corner) frequencies of 40–200 Hz (−3dB point of an 8-pole Bessel filter). The data were sampled at no less than 10× the filter cut-off frequency for further computer analysis. This was carried out as described in Results, using pCLAMP and Axotape software (Axon Instruments), supplemented by our own programs. For steady-state, appropriately filtered data, channel amplitudes could easily be measured to an accuracy of 0.05 pA where required. Current/voltage (I/V) curves were either fitted directly (by nonlinear least-squares regression) to the GHK current equa-

tion (Hodgkin & Katz, 1949), or relative ionic selectivities were simply calculated from reversal potentials determined by eye, applying the modified GHK voltage equation (Hodgkin & Katz, 1949):

$$E_r = -RT/zF \cdot \ln\{(P_{C^+}[C^+]_c + P_{Cl^-}[Cl^-]_t/(P_{C^+}[C^+]_t + P_{Cl^-}[Cl^-]_c)\}$$

rearranged as:

$$P_{Cl^-}/P_{C^+} = \{n - \exp(E_r/K)\}/\{n \cdot \exp(E_r/K) - 1\}$$

P_{C^+} and P_{Cl^-} represent the absolute permeability of the relevant cation or of Cl^- respectively, *c* and *t* stand for the *cis* and *trans* compartments respectively, and *R*, *T*, *F* and *z* have their usual significance ($K = RT/zF$); *n* is the *trans* : *cis* concentration ratio. The concentrations of all the monovalent salts (except choline Cl) were corrected for activity by using values taken from standard tables. Significance was assessed by using unpaired *t*-tests.

Materials

Collagenase D was obtained from Boehringer. Lyophilized lipids from Avanti were transported to the laboratory on dry ice and stored at −70°C under N_2. Fresh *n*-decane (Sigma) dispersions were prepared daily from a working stock in (Analar, BDH) $CHCl_3$ (this stock was kept at −70°C and renewed fortnightly). Other chemicals were obtained from BDH/Merck, Janssen or Sigma, and were of the best available grades.

Abbreviations

POPE: palmitoyl-oleoyl phosphatidylethanolamine; POPS: palmitoyl-oleoyl phosphatidylcholine; SITS: 4-acetamido-4′-isothiocyanostilbene-2,2′ disulfonic acid.

Results

Bilayer Incorporation of Anion Channels

Bilayers were cast in symmetric solutions, and the salt concentration in each chamber was then increased as required by additions from a 3.0 M stock solution. In initial experiments, mitoplast membrane vesicles (final protein concentration: 1–5 μg/ml) were stirred into the *cis* chamber in the presence of an osmotic gradient of KCl, *cis*>*trans* (e.g., 300 *vs.* 150 mM, pH 7.4). The appearance of channels was accelerated in the presence of 2 mM Ca^{2+}, and by forming steeper osmotic gradients. However, these protocols resulted in the incorporation of complicated, mainly cationic, multichannel activity which was difficult to analyze.

Raising the pH of the solutions to 8.8 substantially reduced the rate of channel incorporation, and increased the proportion of anionic conductances obtained. When this modification was combined with the use of Cl^- salts of relatively large cations, especially choline$^+$ (and also Tris$^+$), we regularly observed anion-selective channels which remained unchanged, and uncontaminated by cationic currents, even if the pH was subsequently reduced to 7.4. Again, the rate of channel incorporation could be increased by adding 2 mM $CaCl_2$ to the bilayer solutions, and by using steep osmotic gradients (e.g., 750 *vs.* 150 mM choline Cl, pH 8.8). Under optimal conditions, single channels (or, very occasionally, two or even three at once) became incorporated into the bilayer within 40 min, and most were Cl^- selective (i.e., negative currents flowing from *cis* to *trans* at a holding potential of zero mV in the presence of a *cis*>*trans* concentration gradient). Following the appearance of channel activity, the *cis* chamber was usually perfused with 10 volumes of *trans* solution to remove added vesicles. Reversal potentials were routinely determined in symmetric conditions, before re-establishing an ionic gradient, and the residual junction potentials (which were corrected for in subsequent measurements) were noted to be +0.4 ± 2.5 mV (mean ± SD, $n = 7$).

We could identify two distinct types of multisubstate Cl^- channel activity. Either one or the other species predominated in individual mitoplast preparations. When disruption had been achieved by mild sonication, we could often observe *in*termediate-conductance *m*itoplast *a*nion *c*hannels (INMAC) with multiple substates, while more vigorous sonication appeared to correlate with the subsequent appearance of low-conductance (*s*mall) *m*itoplast *a*nion *c*hannels (SMAC) which displayed only two, well-defined substates. The two types of channel activity are compared under similar ionic conditions, and with similar filtering, in Fig. 1. Although the channels appeared to have some substates in common, more clearly seen when the various sublevels were carefully compared on both fast and slow time scales, the larger conductances do not appear to result from the simultaneous incorporation of two, low-conductance channels. In such a case (as shown in Fig. 2), the individual channels can be distinguished quite easily in at least parts of the record, although transitions between substates do complicate the pattern.

Both species of channel (especially INMAC activity) tended to inactivate irreversibly within about 10–15 min. of incorporation. This did not appear to be due to membrane thickening, as SMAC activity could occasionally be observed for up to 1 hr. Further efforts were made to optimize conditions for the preferential incorporation of either INMAC or SMAC activity, and although some modifications were undoubtedly helpful (e.g., 750 : 150 mM choline

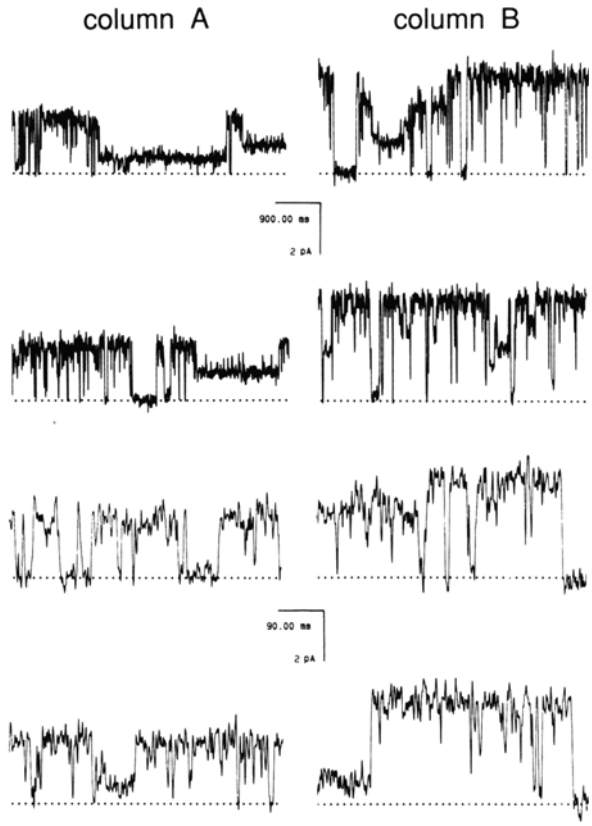

Fig. 1. Comparison of anion channel activities. (*Column A*) Low-conductance channel activity. (*Column B*) intermediate-conductance activity. Data from two experiments, both carried out in 650 *vs.* 50 mM (*cis vs. trans*) choline Cl, holding potential −40 mV. The closed levels are marked (...), and to facilitate comparisons, the upper and lower panels show different time scales (low-pass filtered at 50 and 200 Hz, respectively).

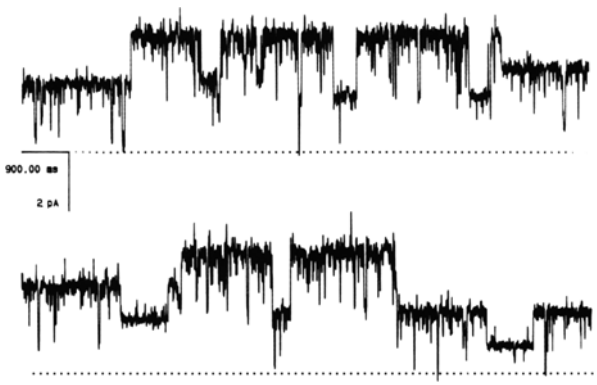

Fig. 2. Simultaneous incorporation of two low-conductance channels. From an experiment in 550 *vs.* 50 mM (*cis vs. trans*) choline Cl, pH 7.4, holding potential −40 mV. The closed levels are marked (...). Filtered at 50 Hz to facilitate comparison with Fig. 1, upper panel.

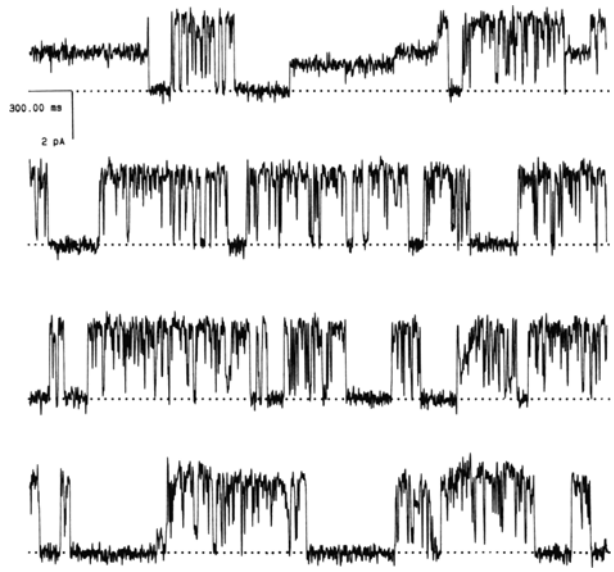

Fig. 3. Multisubstate anion channel: Single channel recording. Recording at −40 mV with 750 mM choline Cl *cis vs.* 150 mM *trans*. The closed state is indicated (...), and openings are shown as upwards deflections.

Cl, and all-POPE bilayers, increased the rate of appearance of INMAC activity, while 550 : 50 mM choline Cl favored SMAC activity), the major factor appeared to be the treatment of membranes during preparation, as described earlier. Overall, SMAC activity was obtained much more frequently than INMAC activity.

Intermediate-Conductance Anion Channels (INMAC)

Currents recorded from INMAC at a single holding potential are presented in Fig. 3, and an amplitude histogram of identified current levels from the same data is shown in Fig. 4. At least 4 nonequally spaced substates are obvious, and in every channel examined, transitions appeared to occur freely between the various current levels and the closed and fully open states. Substates consistently occurred at ~30, ~45, ~60 and ~80% of the fully open level, and could often be distinguished at ~15% (e.g., *see* Fig. 3). The last observation is important, as it provides further evidence that INMAC activity is unlikely to be a simple combination of two of the lower-conductance channels. Even for a channel with just four substates, the number of possible transitions is obviously very large (5! = 60 pairs of transitions), and a more detailed analysis of substate transitions was not attempted.

Current-voltage (I/V) relations were obtained

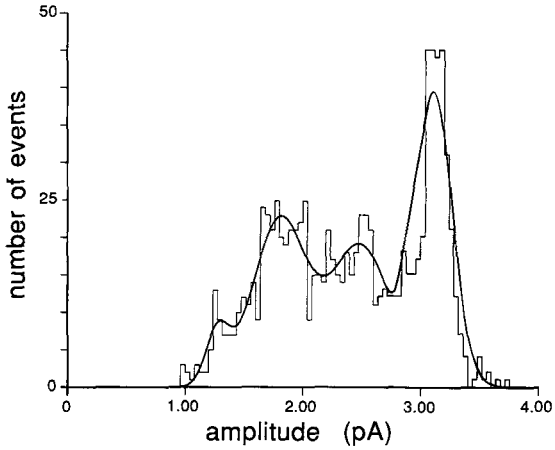

Fig. 4. Multisubstate anion channel: Amplitude histogram. The recording shown in Fig. 3 was filtered at 100 Hz, and identified open states fitted to four Gaussian curves. Note that at least one additional substate (of ~ 1 pA) has escaped detection.

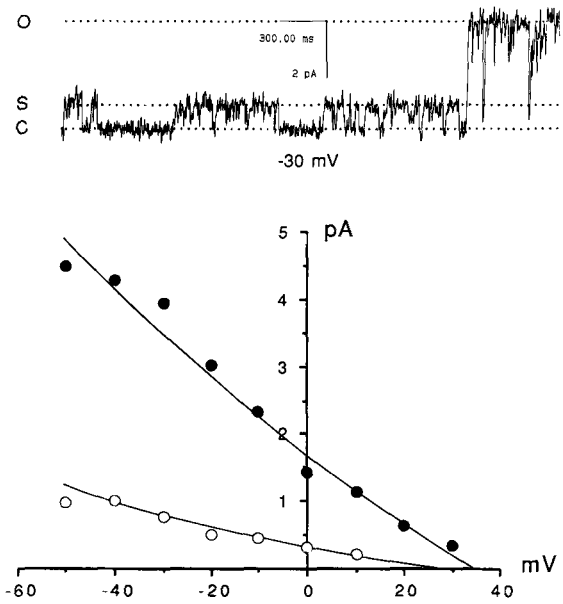

Fig. 5. Multisubstate anion channel: *I/V* relations. Data recorded at a range of potentials in 750 *vs.* 150 mM choline Cl (*cis vs. trans*). Part of the recording at −30 mV is shown to illustrate a particularly clear distinction between the main open state (*O*) and lowest subconductance state (*S*). The closed level (*C*) is also shown. The *I/V* plots of the main state (●) and the lowest subconductance state (○) are both fitted to the GHK current equation (mean permeability ratio of Cl^- : $choline^+$ = 12.7).

for the fully open state of INMAC, determining channel amplitudes either from amplitude histograms or by cursor measurements on long (~5 sec) current traces. Both methods gave equivalent results. It was not always possible to identify every substate level at each holding potential, but the lowest amplitude substate could usually be identified. When fully open and substate amplitudes were combined on a single *I/V* plot (e.g., Fig. 3), the fits (in this case directly to the GHK current equation, Hodgkin & Katz, 1949) converged to similar reversal potentials. This behavior was also seen following a wide variation in concentration gradients (from 3 : 1 to 11 : 1), or the particular salt (choline Cl, KCl or Tris Cl) used. These observations, together with the similarity of the pattern of transitions obtained from many (>25) different channels, suggested that the different current levels represented true substates of a single type of channel, rather than multiple channels. The limiting slope conductance of INMAC in asymmetric choline Cl (300 : 50 mM, *cis* : *trans*) was 57 ± 8.1 pS (mean ± SD, n = 4).

Lower-Conductance Mitoplast Anion Channels (SMAC)

Most of the channels obtained from vigorously sonicated preparations were of comparatively low conductance (26 ± 3.9 pS, mean ± SD, n = 7, in 300 : 50 mM choline Cl gradients, *cf.* INMAC above). They invariably displayed two distinct substates, as shown in Figs. 1 and 2, and in more detail in Fig. 6. These occurred at 27 ± 6.1% and 50 ± 5.7% of the fully open level (mean ± SD, n = 30 channels).

The amplitude histogram for 102 sec of the data from which Fig. 6 is extracted is shown in Fig. 7. In asymmetric conditions both substates showed the same reversal potential as the main-state conductance (*results not shown*). The spacing of these substates suggested a multisubunit type of substate behavior, which is investigated in detail in the accompanying paper (Hayman & Ashley, 1993). The low-conductance channel, like INMAC activity, was also poorly selective between anions and cations (Fig. 8).

The two types of channel activity (INMAC and SMAC) might be related in a more subtle way, perhaps representing various subunits of a single multibarrelled anion channel disrupted to a greater or lesser extent by sonication. In an attempt to determine clearly whether they were distinct channels, we compared their anion *vs.* cation selectivities by measuring reversal potentials in asymmetric choline Cl and KCl, using the modified GHK voltage equation as described in Materials and Methods. For INMAC, $P_{Cl^-}/P_{choline^+}$ was 13 ± 1.1 (mean ± SD, n = 4), and the corresponding value for SMAC was 11 ± 2.6 (mean ± SD, n = 10). The uncorrected means were not significantly different (unpaired t-test, $P > 0.05$). The corresponding values for P_{Cl^-}/

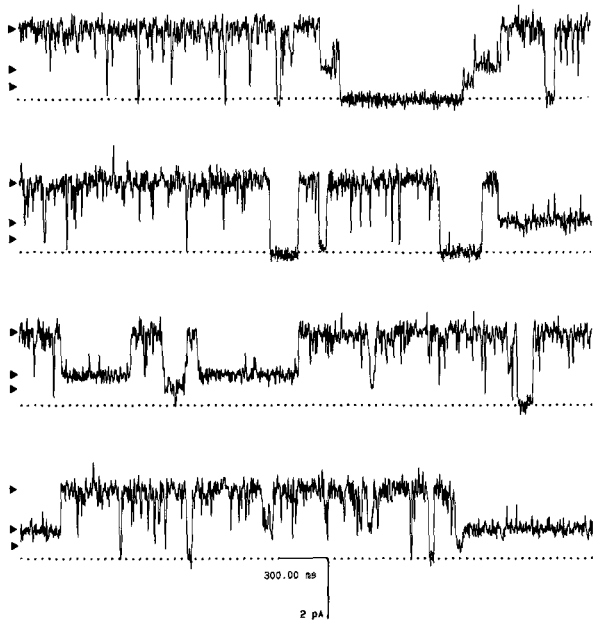

Fig. 6. Low-conductance anion channel: Single channel recording. Data recorded at −40 mV in 550 mM choline Cl *cis vs.* 50 mM choline Cl *trans*. The closed levels are marked (...), and the open states are indicated (▶), including substates at ~25 and ~50% of the main state conductance.

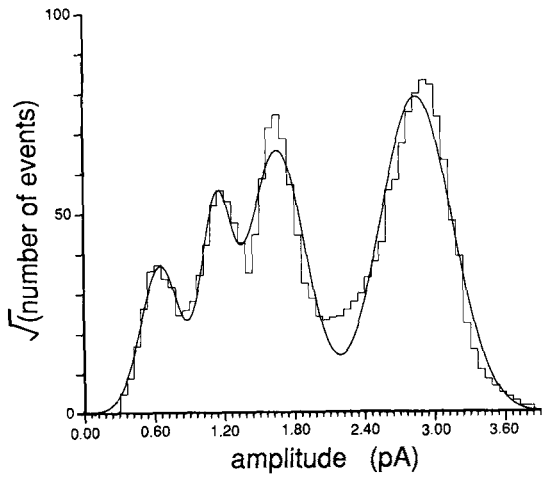

Fig. 7. Low-conductance anion channel: Amplitude histogram. All-points amplitude histogram of 102 sec of the data from which the traces in Fig. 6 are examples, fitted to four Gaussian curves representing (reading from left to right) closed, ~25%, ~50%, and fully open (100%) states. Note square root scale on ordinate to prevent compression of closed and substate frequencies.

P_{K^+}, 8.8 ± 0.7 (mean ± SD, n = 6) and 7.1 ± 1.1 (n = 4), did differ significantly at the 5% level (unpaired t-test, $0.05 > P > 0.01$).

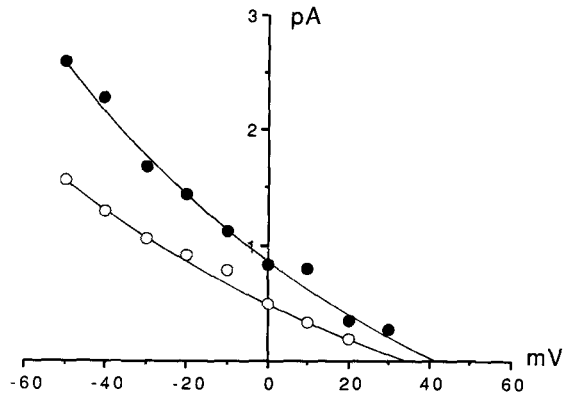

Fig. 8. Low-conductance anion channel: I/V relations. Data obtained from single channels exposed to 11 : 1 (●) and 6 : 1 (○) *cis : trans* gradients of choline Cl (550 *vs.* 50 mM, and 300 *vs.* 50 mM, respectively), fitted to the GHK current equation with anion : cation permeability ratios of 8.5 and 9.1 respectively. Not corrected for ionic activities.

Table 1. Relative anion selectivities of SMAC

A^-	E_r (mV, ± SD)	P_{A^-}/P_{Cl^-} (± SD)	n
(Cl^-)	(0)	(1.00)	(−)
NO_3^-	11.6 ± 3.1	1.01 ± 0.30	4
Br^-	4.0 ± 1.3	0.91 ± 0.30	5
I^-	6.0 ± 1.7	0.87 ± 0.25	4
SCN^-	4.9 ± 0.9	0.79 ± 0.15	4

Anion (A^-) *vs.* Cl^- permeability ratios were determined from reversal potentials obtained in 300 mM KCl *vs.* 300 mM KA salts (all converted to activities).

Anion Selectivity and Conductance of SMAC

More detailed studies were confined to SMAC, partly because of its simpler substate behavior, and also because INMAC activity was obtained much less frequently. Having determined the relative permeability of Cl^- to K^+, an anion selectivity sequence was obtained after establishing a concentration of 300 mM KCl *trans* and replacing the *cis* solution by 300 mM K^+ salts of other anions (A^-). Relative anion selectivities were calculated from reversal potential measurements (obtained from I/V relations) by solving the GHK voltage equation for a single unknown, the permeability ratio A^-/K^+. Activity corrections were applied routinely, but were essentially only important for KNO_3 *vs.* KCl. It was usually possible to perfuse the *trans* chamber again once or twice without breaking the bilayer, and reversal potentials obtained for different salt-pairs bathing the same channel were similar to those obtained from measurements made on different channels. The results from a number of experiments are summarized in Table 1.

The conductance of SMAC was examined over

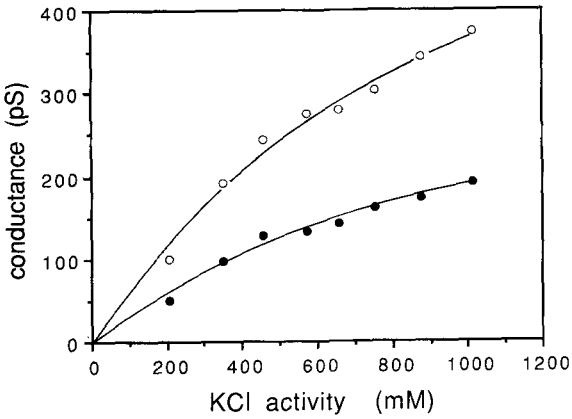

Fig. 9. Low-conductance anion channel: Conductance *vs.* activity curve. Conductance values for each activity (main state ○ and ~50% substate ●) are all averaged from at least two experiments, except for the last three points, which were obtained from a total of two experiments. The curves are direct fits to: g/g_{max} = [KCl]/(K_m + [KCl]), where g_{max} (maximum conductance) = 800 and 420 pS (main state and substate, respectively), and K_m = 1.13 and 1.17 M (main state and substate, respectively).

a wide range of symmetric KCl activities (Fig. 9), and did not appear to saturate even when the concentration of KCl was increased towards 2.0 M. We could not consistently achieve higher concentrations without breaking the membranes. It is notable that the substate conductances increased proportionately (remaining at a quarter and a half of the main-state conductance) during these experiments. The behavior of the 50% level is shown in detail in Fig. 9.

Inhibitor Studies of INMAC and SMAC

INMAC activity occasionally disappeared within a few minutes of reducing the pH in both chambers to 5.5, but this could not be reliably distinguished from the spontaneous inactivation mentioned earlier, since after realkalinization the channel often failed to reappear (*see* Table 2). INMAC activity also seemed to be independent of voltage, and was unaffected by 2 mM ATP, 2 mM Ca^{2+} or Mg^{2+}, or by 500 μM concentrations of quinine, propranolol, amitriptyline (*see* Antonenko et al., 1991), H_2O_2 (Crompton et al., 1987) or SITS (4-acetamido-4′-isothiocyanostilbene-2,2′ disulfonic acid, Knauf & Rothstein, 1971). All these reagents were added to both chambers, except H_2O_2, which was only added to the cis chamber as it is highly membrane permeant (Halliwell, 1989). SMAC activity was also insensitive to the same concentrations of ATP, quinine, propranolol, amitriptyline, H_2O_2 and SITS. Its activity was unaffected by reducing the pH of the bathing solutions to 5.5, but it did show divalent cation-dependent rectification, mainly at negative holding potentials, and voltage-dependent substate behavior. These features are investigated in detail in the following paper (Hayman & Ashley, 1993). The inhibitor profiles, and other major properties of the two types of channel activity, are compared in Table 2.

Discussion

This paper provides evidence for the presence of up to two types of anion channel in cardiac mitoplast preparations. The preparations seem to have been relatively uncontaminated by outer membrane fragments (a major component of the original mitochondria) because of the absence of VDAC (the voltage-dependent outer membrane anion channel, Colombini, 1986) in our experiments. There is strong circumstantial support, but no definite proof, for the suggestion that the channels originated from the inner mitochondrial membrane, rather than from other microsomal membranes. In particular, they seem to be distinct from previously described microsomal (including endoplasmic and sarcoplasmic reticulum) Cl^- channels (Tanifuji, Sokabe & Kasai, 1987; Rousseau, Roberson & Meissner, 1988; Ashley, 1989), and also from rat liver nuclear membrane Cl^- channels (Tabares, Mazzanti & Clapham, 1991). It is also worth remarking that none of these other channels (or similar channels) were seen in > 100 experiments. The anion channels described in this report may therefore be a novel type of channel which has escaped previous detection in patch-clamp studies because of relatively low abundance, or relative inaccessibility to patch-clamp electrodes, although they do appear to become incorporated into bilayers by the fusion of membrane vesicles (i.e., their appearance is "quantal" and they have a preferred orientation). However, this may not be a random process, as not all membrane vesicles can be assumed to have an equal (and of course extremely small) chance of fusing, and it remains possible that all the channels seen arose from highly fusogenic membrane contaminants, or from proteins that are not currently thought to operate as channels at all in vivo (*cf.* de Meis & Inesi, 1992). The inner mitochondrial membrane contains a variety of translocators which could conceivably be modified and behave as channels in bilayers.

Some of the channel activity described here, especially INMAC activity, has features in common with the voltage-dependent channel originally described in mouse by Sorgato et al. (1987), which has been shown to co-purify with bovine heart F_0 (Sorgato et al., 1989). In particular, INMAC shows

Table 2. Comparison of anion channel properties

Property	INMAC	SMAC
Conductance		
300 *vs.* 50 mM choline Cl	~60 pS	~25 pS
symmetric 150 mM KCl	~50 pS	~100 pS
Substates		
number identified	>4	2
approximate amplitudes (%)	15, 30, 45, 60, 80	25, 50
Voltage dependence	Minimal or absent	In substates[a]
Anion *vs.* cation selectivity		
P_{Cl^-}/P_{K^+}	~9	~7
$P_{Cl^-}/P_{choline^+}$	~13	~11
Relative anion selectivity	ND	Poor
Ca^{2+} or Mg^{2+} dependence (2 mM)	0/5	Increased conductance[a]
pH dependence (8.8 → 5.5 → 8.8)	3/6[b]	0/3
Ineffective activators/inhibitors[c]		
H_2O_2 (500 μM)	(2)	(2)
ATP (2 mM)	(2)	(2)
propranolol (500 μM)	(3)	(4)
quinine (500 μM)	(3)	(4)
amitriptyline (500 μM)	(3)	(3)
SITS (100–500 μM)[d]	(3)	(3)

[a] In each of > 20 experiments. *See* Hayman & Ashley, 1993.
[b] Including two examples of INMAC which were *irreversibly* inactivated during exposure to low pH.
[c] Listing only those experiments (number of experiments in parentheses) in which reagents were added to both *cis* and *trans* chambers (except for H_2O_2).
[d] 4-acetamido-4′-isothiocyanostilbene-2,2′ disulfonic acid.

a similar conductance, and is selective for anions over cations. However, inhibitor studies are not helpful: for example, Sorgato et al. (1989) reported that (0.2 mM) quinine had no effect on the channel when applied to preparations clamped in "whole-mitoplast" configuration (consistent with our observations), while Kinnally, Antonenko and Zorov (1992) showed the drug had clear effects on the channels (increased conductance, reduced P_o) at concentrations similar to those used by us here. (It is convenient to state here that H_2O_2, added in the absence of transition metals, will have acted largely as an oxidizing agent, rather than a free-radical provider, in our system.) The absence of the marked voltage-dependent gating usually associated with the inner membrane anion channel (Sorgato et al., 1987) may have been attributable to differences in preparation methods (e.g., sonication in our study), or exposure to Ca^{2+} (*see* Kinnally et al., 1991), or the types of membrane lipids used. An inner membrane anion channel in brown fat cells (Klitsch & Siemen, 1991), similar in many respects to the channel of Sorgato et al. (1987), showed much less voltage dependence, and clear evidence of substates (though not as marked as those associated with INMAC in this study).

Although their substate behavior did appear to be quite distinctive, and INMAC activity did not appear to be a simple summation of SMAC activity, both species of channel were only poorly selective between anions and cations, even between Cl^- and large cations (such as $choline^+$). The difference (significant at the 5% level) in P_{Cl^-}/P_{K^+} for INMAC and SMAC activity suggests they may be distinct molecules with different pores, but an extensive survey of anion *vs.* cation selectivities, covering a wide range of salts, was not undertaken. The permeation behavior of SMAC, which was investigated in more detail, was also unusual in two other important respects: it discriminated very poorly between different anions, and had a very low apparent affinity for permeant ions. The relative anion selectivity sequence (permeability sequence: NO_3^- = $Cl^-(1.0)>Br^->I^->SCN^-(0.8)$) of SMAC was certainly not a simple reflection of hydration energies, and did not correspond to any of the sequences constructed for anion binding sites (Wright & Diamond, 1977). In fact, selection between the different anions was almost negligible. On the other hand, the channels had only moderate conductances at low salt activities, inconsistent with a very wide, poorly selective pore. The permeation mechanism may, for example, involve cationic occlusion of a negatively charged binding site in the pore (Franciolini & Petris,

1990), or ions may not interact with specific binding sites at all, but permeate by a quite different mechanism (e.g., diffusion in a neutral "central cylinder" of solution, Zambrowicz & Colombini, 1993). The reasonable, but inappropriate, fits to the GHK equations would not rule this out. In fact, a simple analysis of channel conductance behavior, assuming that permeant ions bound to a single (?uncharged) site, gave rise to an anomalously low apparent binding affinity, in excess of 1.0 M (Fig. 9), which is quite unlike the values of 10–50 mM common in other ion channels.

The physiological role (if any) of mitochondrial ion channels, particularly those demonstrated to be present in the inner membrane, remains largely obscure. There is, however, very good evidence for the involvement of one or more channels in mitochondrial Ca^{2+} metabolism. In particular, changes in cytosolic and matrix [Ca^{2+}] have been related to alterations in mitochondrial volume, which may in turn physically regulate the activity of membrane-located enzyme complexes involved in the oxidation of fatty acids and other substrates (Halestrap, 1987). These volume changes may follow pore formation. Specifically, increasing cytosolic [Ca^{2+}] leads to elevation of matrix [Ca^{2+}] and also matrix [pyrophosphate] (by an unknown mechanism). The binding of excess (nucleotide-free) phosphate and pyrophosphate to the (dimeric) adenine nucleotide translocase may then cause the protein to function as an ungated K^+-selective pore (Davidson & Halestrap, 1990). In the presence of an anion uptake pathway, such as that demonstrated by Garlid and Beavis (1986), net salt and water entry may then induce mitochondrial swelling, and thereby regulate key enzymes. Much more severe swelling in supraphysiological cytoplasmic [Ca^{2+}] (1–10 μM) corresponds to the evolution of a large, nonspecific, Ca^{2+}- Pi- and respiration-dependent pore (Hunter, Haworth & Southard, 1976; Crompton et al., 1987). Nonspecific pore formation can be prevented by cyclosporin A (which binds to peptidyl-prolyl *cis-trans* isomerase, cyclophilin), as shown by Fournier, Ducet and Crevat (1987). The activity of the multiconductance channel described by Kinnally et al. (1989) and by Petronilli et al. (1989) is reduced by cyclosporin (Szabò & Zoratti, 1991), strongly suggesting that it is in fact formed by the translocase.

There is no similarly compelling evidence for a functional role for any other channel in the inner membrane, or indeed any evidence yet that these channels operate in vivo. Finally, while some of the properties of the channel activity described in this report resemble in some respects those of the voltage-dependent inner membrane anion channel of Sorgato et al. (1987), the channels do not appear to correspond to the pH-sensitive anion pathway of Garlid and Beavis (1986), although it remains possible that factors responsible for its pH dependence might have been removed during reconstitution.

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