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Identification of Anion and Cation Pathways in the Inner Mitochondrial Membrane by Patch Clamping of Mouse Liver Mitoplasts

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Summary. Alkalinization of the matrix side of the mitochondrial inner membrane by pH shifts from 6.8 to 8.3 caused a reversible increase in current of 3.2 ± 0.2 pA (mean ± SE, $n = 21$) at ± 40 mV measured using patch-clamp techniques. The current increase was reversed in a graded fashion by the addition of Mg^{2+} as well as a reduction in pH. Detection of single-channel events was done at 0.5, 1 and 2 M KCl. The single-channel amplitude in 0.15 M KCl corresponds to approximately 15 pS. Reversal potentials derived from whole patch currents indicated that the inner mitochondrial membrane was primarily cation selective at pH 6.8 with a $P_K/P_{Cl} = 32$ ($n = 6$). Treatment with alkaline pH (8.3) increased the current and anion permeability ($P_K/P_{Cl} = 16$, $n = 6$). The membrane becomes completely cation selective when low concentrations (12 μM) of the drug propranolol are added. The amphiphilic drugs amiodarone (4 μM), propranolol (70 μM) and quinine (0.6 mM) blocked almost all of the current. The pH-dependent current was also inhibited by tributyltin. These results are consistent with the presence of two pathways in the inner mitochondrial membrane. One is cation selective and generally open and the other is anion selective and induced by alkaline pH. The alkaline pH-activated channel likely corresponds to the inner membrane anion channel postulated by others from suspension studies.

Key Words ion transport · inner mitochondrial membrane channel · propranolol · amiodarone · patch clamp

Introduction

Recently the native mitochondrial inner membrane was studied by patch-clamp techniques (Sorgato, Keller & Stühmer, 1987; Kinnally, Campo & Tedeschi, 1989; Petronilli, Szabó & Zoratti, 1989). These studies have revealed the presence of currents associated with individual channel activity (Sorgato et al., 1987; Kinnally et al., 1989, Petronilli et al., 1989). Sorgato et al. (1987) have identified a channel with a unit conductance of approximately 100 pS in 0.15 M KCl, whereas Kinnally et al. (1989) and later Petronilli et al. (1989) have observed multiple conductance channel (MCC) activity. In particular, Kinnally et al. (1989) reported prominent conductances of 45, 120–150, 350 and approximately 1,000 pS. Similar levels were observed in the inner membrane reconstitution studies of Moran et al. (1990).

The present study using patch clamping examines in detail the conductance properties of the inner mitochondrial membrane under conditions (alkaline pH, Mg^{2+} depletion, addition of amphiphilic cationic drugs or tributyltin) which have been associated with permeability transitions in earlier studies of mitochondrial suspensions (Selwyn, Dawson & Fulton, 1979; Warhurst, Dawson & Selwyn, 1982; Garlid & Beavis, 1986; Beavis & Garlid, 1987, 1988; Beavis, 1989; Beavis & Powers, 1989). Under these conditions, we have observed changes in inner membrane conductance that could be accounted for by an opening or closing of channels or groups of channels. In 0.5 to 2 M KCl, we have observed alkaline pH-induced single-channel activity. Measurements of macroscopic currents as a function of voltage (i.e., *I-V* curves) were used to characterize the permeability of mitochondrial inner membranes. Shifts in reversal potentials (voltage at zero current level) indicated by displacement of the *I-V* curves in response to ionic gradients across the membranes provide direct information about the relative permeability of the membrane to various ions. In the present experiments we have obtained evidence in mitoplasts for a cation-selective conductance pathway that is normally present and an anion-specific pathway which is induced by alkaline pH.

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Materials and Methods

Isolation of Mitochondria and Preparation of Mitoplasts

Large mitochondria were isolated from the liver of normal mice by the method previously described (Bowman & Tedeschi, 1983) except that the isolation mediums were 0.25 and 0.5 M sucrose with 0.25 mM EDTA[1] and 5 mM HEPES at pH 7.4 or 230 mM mannitol, 70 mM sucrose, 0.25 mM EDTA, and 5 mM HEPES at pH 7.4. No differences in mitoplasts prepared with the different media were noted. The mitoplasts were prepared by the method of Decker and Greenawalt (1977). The mitochondria in the pellet were resuspended in 15 ml of 460 mM mannitol, 140 mM sucrose, 10 mM HEPES, pH 7.4, and subjected to 2,000 psi using a French press to remove the outer membrane. The resulting mitoplasts were centrifuged at 10,000 × *g* for 5 min and resuspended in approximately 3 ml of 0.15 M KCl, 5 mM HEPES, pH 7.4.

Chemicals

Propranolol and amiodarone were purchased from Sigma (P-0884 and A-8423, respectively), quinine was from Matheson Coleman and Bell (6970) and tributyltin chloride was from Aldrich (T5,020-2).

Patch-Clamping Procedures

The manipulations were carried out while viewing the preparation with a Zeiss Opton inverted microscope equipped with differential interference optics (DIC) and a heat filter, and providing a magnification of 480×.

The mitoplasts were sealed to the tip of glass micropipettes by applying a slight negative pressure to the pipette after it touched a mitoplast attached to the slide. The resistances of the patch generally ranged from 3 to 50 GΩ. Less frequently high GΩ seals were obtained spontaneously, i.e., without the application of negative pressure, with the same results. Inner mitochondrial membrane patches were excised by drawing the patching pipette away from the whole mitoplast. In previous experiments (Kinnally et al., 1989) the sidedness of these excised membrane patches was the same as that of attached patches, as shown by the voltage dependence of the channels in both configurations. These observations suggest that the patches have an inside-out configuration.[2]

Generally, 1 ml of external medium was used as a bath and the perfusions were done with 5 ml carried out on the bath (matrix) side of the membrane. In most experiments, the pipette and initial medium contained 0.15 M KCl, 10 μM $CaCl_2$, 5 mM HEPES, pH 6.8. The reference electrode was a Ag, AgCl wire connected to the bath through a 2% agar bridge containing the original medium (generally 0.15 M KCl, 10 μM $CaCl_2$, 5 mM HEPES, pH 6.8) to avoid junction potential changes produced by the dilution of Cl^- in some of our experiments. In the experiments with higher KCl concentrations (*see* Figs. 3 and 4), the KCl concentration was held constant in the entire system (pipette, bath and agar bridge). Where a gradient was introduced the measured junction potential was negligible. The patch pipettes had resistances ranging from 20 to 40 MΩ at 0.15 M KCl. At high salt solutions the pipette resistances were usually of the same value if normalized to 0.15 M KCl concentration.

In this study, the polarity of the voltages is reported in relation to the pipette unless otherwise stated (i.e., $V = V_{pipette} - V_{bath}$). All procedures were carried out at room temperature (about 26°C).

The current of the excised membrane patch was monitored under voltage-clamp conditions using a Dagan 3900 (Dagan, Minneapolis, MN) patch-clamp system. The currents and voltages were displayed on a Hitachi digital storage oscilloscope (model VC 6020) and a Tektronix 5111 (Tektronix, Beaverton, OR) storage oscilloscope with 5A19N and 5A21N amplifiers. Current and voltage records were stored on video tape using a digitizer (VR 10 digital data recorder, Instrutech, Mineola, NY). The signals were stored at a 10 kHz bandwidth. Subsequent analysis of the stored signals was at a bandwidth of 1 or 2 kHz for analysis. Occasionally, a bandwidth of 30 to 50 Hz was used for displaying the data (*see* figure legends). A low-pass,eight-pole Bessel filter (Model 902, Frequency Devices, Haverville, MA) was used to obtain different bandwidths. Analysis was done with two types of software, IPROC (courtesy of C. Lingle, Washington University) and Strathclyde Electrophysiological Data Analysis package (courtesy of J. Dempster, University of Strathclyde, Glasgow, U.K.) using IBM 386-compatible computers.

Current-Voltage (IV) Curves

The current-voltage records of membrane patches were recorded on the storage oscilloscope while varying the voltage manually. Ionic gradients were obtained by replacing the usual medium outside the pipette by perfusion of the chamber with 30 mM KCl,

[1] *Abbreviations used:* EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β amino-ethyl ether) N,N′-tetraacetate.

[2] At this time we cannot discard the possibility that the excised patches are in a vesicular rather than a planar configuration. Some of our results, however, suggest that the latter is the case. We have never seen a vesicle at the magnification used. In addition, treatment of the whole mitoplast with Ca^{2+} chelators induces predominantly the presence of the ~100 pS channels. However, introduction of the Ca^{2+}chelators after excising the patch has no effect. This observation suggests that in the presumed patch the surface exposed to the medium is the matrix side of the membrane. It is generally accepted that vesicles do not form if excision is done at very low calcium levels. Our results are the same when the calcium concentration is at 10^{-9} M and lower, suggesting a maintenance of planar configuration. Furthermore, the presence of a small symmetric vesicle which could not be distinguished from an excised patch would have a characteristic electric behavior. When several channels are present at both sides of the vesicle, it would produce a symmetric *I-V* curve since the polarity of the opposed membranes is reversed. In the presence of one or a few channels, the chances that they would be on one side or the other of the vesicle would be equal. Therefore, the voltage dependence would differ from experiment to experiment. Several of the channels studied can be demonstrated to invariably have a sharp voltage dependence, closing at either negative or positive potentials reproducibly (*see* Kinnally et al., 1989). Furthermore, comparison by others of results obtained with excised patches and whole mitoplast configurations (Sorgato et al., 1987) is consistent with this interpretation.

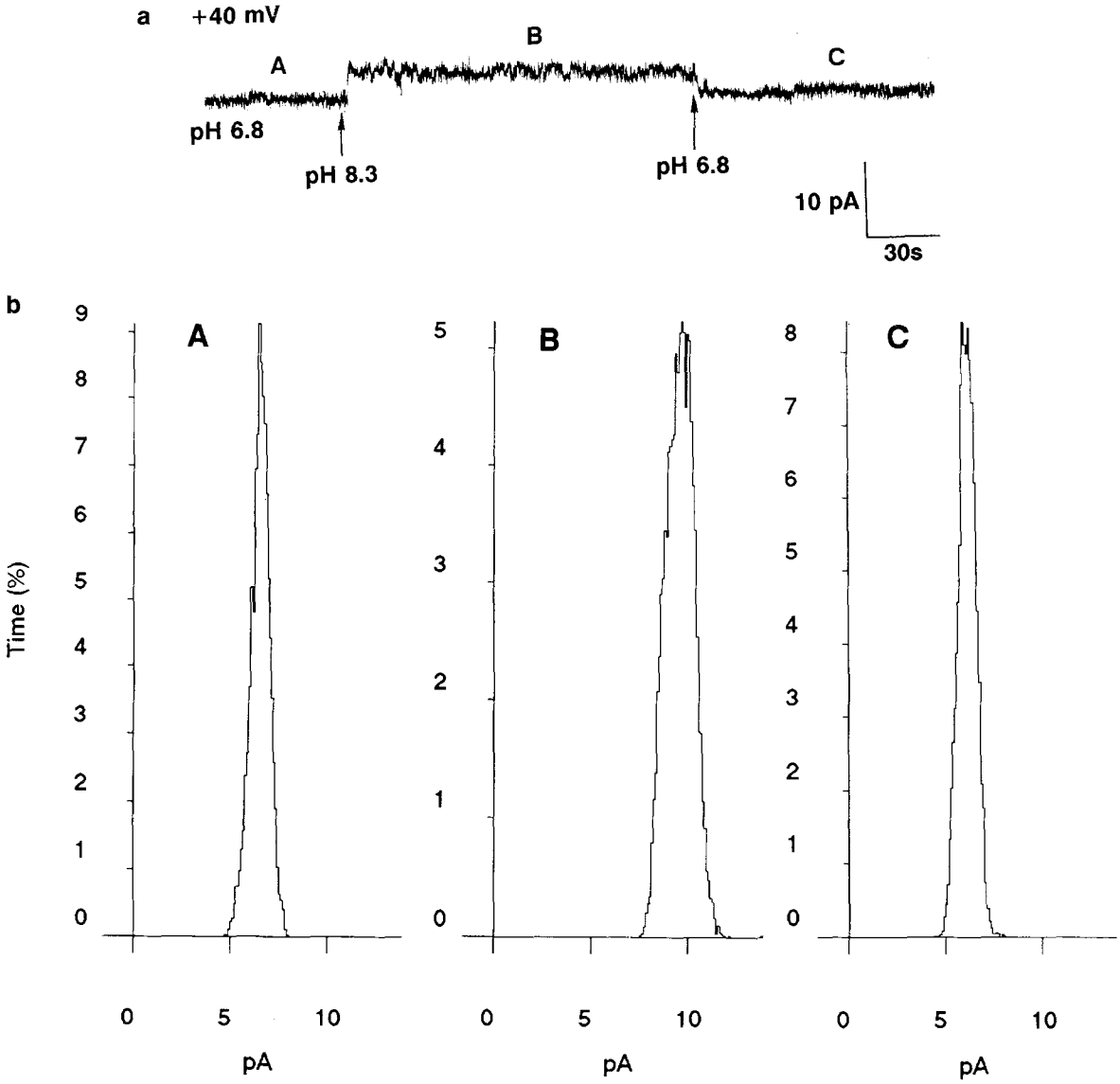

Fig. 1. (*a*) Record of a mitoplast excised patch illustrating the current changes induced by variations in pH. The patch was initially in symmetrical 150 mM KCl, 5 mM HEPES, 10 μM $CaCl_2$, pH 6.8 (*A*), then perfused on the bath (matrix) side at pH 8.3 (*B*), followed by a return to pH 6.8 (*C*). The current was recorded with a bandwidth of 50 Hz. (*b*) Total current amplitude distribution of the data of *a* showing the peak current (the current level of highest occupancy determined with a bin width of 0.1 pA), determinations which were used to calculate the conductance of the pathway (*see* text). *A*, *B* and *C* are from the corresponding sectors of *a* (*A:* pH 6.8, *B:* 8.3 and *C:* 6.8). The data was analyzed at a bandwidth of 2 kHz.

240 mM sucrose, 5 mM HEPES, 10 μM $CaCl_2$ at pH either 6.8 or 8.3. With this experimental design and the conventions used, a displacement of the *I-V* curve towards the negative voltage range corresponds to an increase in cation-selectivity in relation to the anion. Permeability ratios for various ions were calculated from the reversal potentials using the Goldman-Hodgkin-Katz equation (*see* Hille, 1984, pp. 230–243).

Results

The effect of alkalinization of the matrix face of the inner membrane on the current is shown in Fig. 1*a*. At the times indicated by arrows the bath medium was replaced by perfusion with another at a different pH. A shift from pH 6.8 to 8.3 ($A \rightarrow B$) resulted in a current increase of 3.2 pA at +40 mV. This shift is completely reversible [compare $A \rightarrow B$ to $B \rightarrow C$] when the pH is returned to pH 6.8. The same patch can be cycled repeatedly between these two pHs with essentially reversible current shifts. The mean conductance change was 80 ± 5.5 pS (mean ± SE, $n = 21$) for patches clamped at ±40 and ±60 mV and was independent of voltage at least up to ±60 mV. Current amplitude distributions (illustrated in

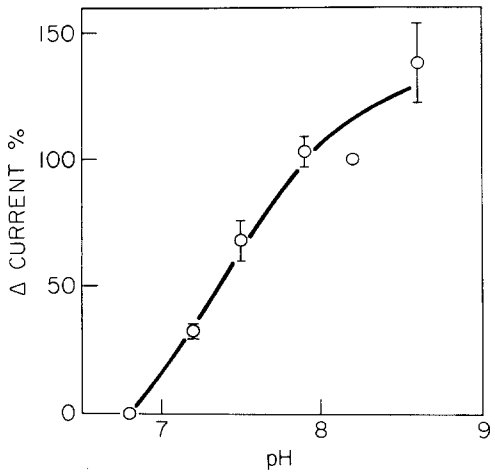

Fig. 2. pH dependence of the current through the membrane patch expressed as % of the total current difference between pH 8.2 and 6.8 at varied pH. The pH was changed by perfusion of the bath solution. Conditions are the same as in the legend of Fig. 1. The data represents mean ± SE of five independent patches. A curve is a best fit with an assumed half maximum pH of 7.4.

Fig. 1*b*) were compiled for pH-induced transitions and used to compute the conductance change (21 determinations, 18 different patches). The width of the current distribution at 8.3 is greater than at 6.8 (Fig. 1*b*) and is suggestive of channel activity.

A more detailed analysis of the pH dependence of the current is shown in Fig. 2. The current increased gradually with pH and began to level off at pH > 8 (*see* Fig. 2). A rough estimate of pH producing 50% of the effect was pH 7.4.

pH-Dependent Single-Channel Activity

The pH-dependent single-channel activity was resolved in 0.5 to 2 M KCl as transition size increases with ionic strength. The amplitude histogram of Fig. 3*a* shows the stepwise occupancy of current levels observed with time when the bath medium was changed from pH 6.8 to 8.2 in 1 M KCl. No change was observed for the first 10 sec of perfusion. However, in the next 30 sec the current level occupation shifted from the closed state to reveal four higher current levels likely corresponding to the presence of four channels in this patch. In Fig. 3, the total current increase after the perfusion with pH 8.2 was approximately 15 pA at +40 mV which would correspond to four channels with unit currents of 3 to 4 pA each (*see below*). The current traces with time of Fig. 3*b* show that while little or no activity was observed at pH 6.8, transitions of 3.6 ± 0.3 pA were seen at +40 mV with alkaline pH at 1 M KCl. On the average, the pH-induced single-channel transitions were 92 ± 2 pS (n = 4 patches, mean ± SE) at ±40 mV in 1 M KCl. Occasionally (n = 2), larger pH-induced transitions were seen at other salt concentrations (e.g. 240 pS in 0.5 M KCl). At physiological (0.15 M KCl) salt concentrations the pH-induced current shift, such as that shown in Fig. 1, was observed in 90% of the patches (n = 25). Some decrease in the probability of opening of the alkaline pH-activated channels by high ionic strength is suggested as the pH-dependent current shift was seen in only 54% (n = 33) of the patches at 0.5 to 2 M KCl. The dependence of the single-channel conductance on KCl concentration is shown in Fig. 4. Extrapolation of this transition data indicates a single-channel conductance of 15 pS at physiological (0.15 M KCl) salt concentrations.

Effect of Divalent Cations

The addition of Mg^{2+} to the bath was found to reverse the alkaline pH-induced current (n = 4). As shown in Fig. 5, the Mg^{2+} concentration needed for 50% reversal was approximately 0.3 mM. The effect of Mg^{2+} on the current, like pH, is reversible (*not shown*) and the same patch can be cycled repeatedly. In four independent experiments inclusion of 0.2 mM EGTA (in the absence of Ca^{2+}) in the bath had no effect on the current shift.

Effect of Inhibitors

As shown in the Table, the conductance of the patches is reduced by the amphiphilic drugs amiodarone, propranolol and quinine. Figure 6 shows that amiodarone almost completely eliminated all current, in this case 90%, through the patch regardless of voltage. In parallel controls (*not shown*) we never observed a significant change in current with perfusion. In addition, a 70 ± 10% (mean ± SE, n = 3) inhibition of alkaline pH-induced current at pH 8.3 was observed with low concentrations of tributyltin.

Ion Selectivity

Figure 7, curve *1* corresponds to a current voltage (*I-V*) relationship for the inner mitochondrial membrane with symmetrical solutions (predominantly 0.15 M KCl at pH 6.8). As expected, in the absence of an ion gradient, the reversal potential is 0 mV. Neither pH or Ca^{2+} gradients (e.g., pH 8.3 or the absence of Ca^{2+} on the bath side) were found to have an effect on the reversal potential (*not shown*) if the KCl concentration was symmetrical. After establishing a fivefold gradient (by substitution of the 0.15 M KCl medium in the bath by perfusion with

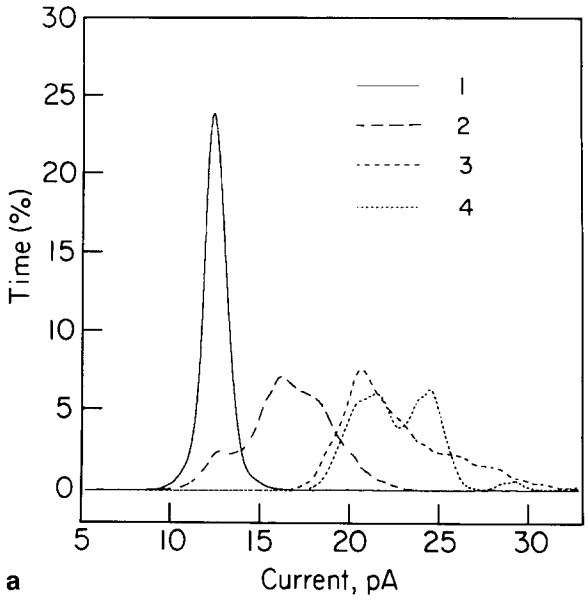

Fig. 3. Alkaline pH-induced current transitions. (*a*) Amplitude histogram showing the time course of the current shift. 1 M KCl, 10 μM $CaCl_2$, 5 mM HEPES at pH 6.8 was initially present throughout the system (pipette, agar bridge and bath). At time = 0, the pH was changed by perfusion with the solution at pH 8.2. Curves indicate occupancy of current levels using a bin width of 0.4 pA at (*1*) 0–10 sec, (*2*) 10–20 sec, (*3*) 20–30 sec, (*4*) 30–40 sec after perfusion. Voltage was +40 mV and is reported in relation to the matrix side. The current was recorded at a bandwidth of 5 kHz. (*b*) Sample current trace showing single-channel transitions at 1 M KCl, pH 8.2, and none at 6.8 at +40 mV. At pH 8.2, the mean open time was 4 msec and unit conductance was 91 ± 2 pS (n = 4).

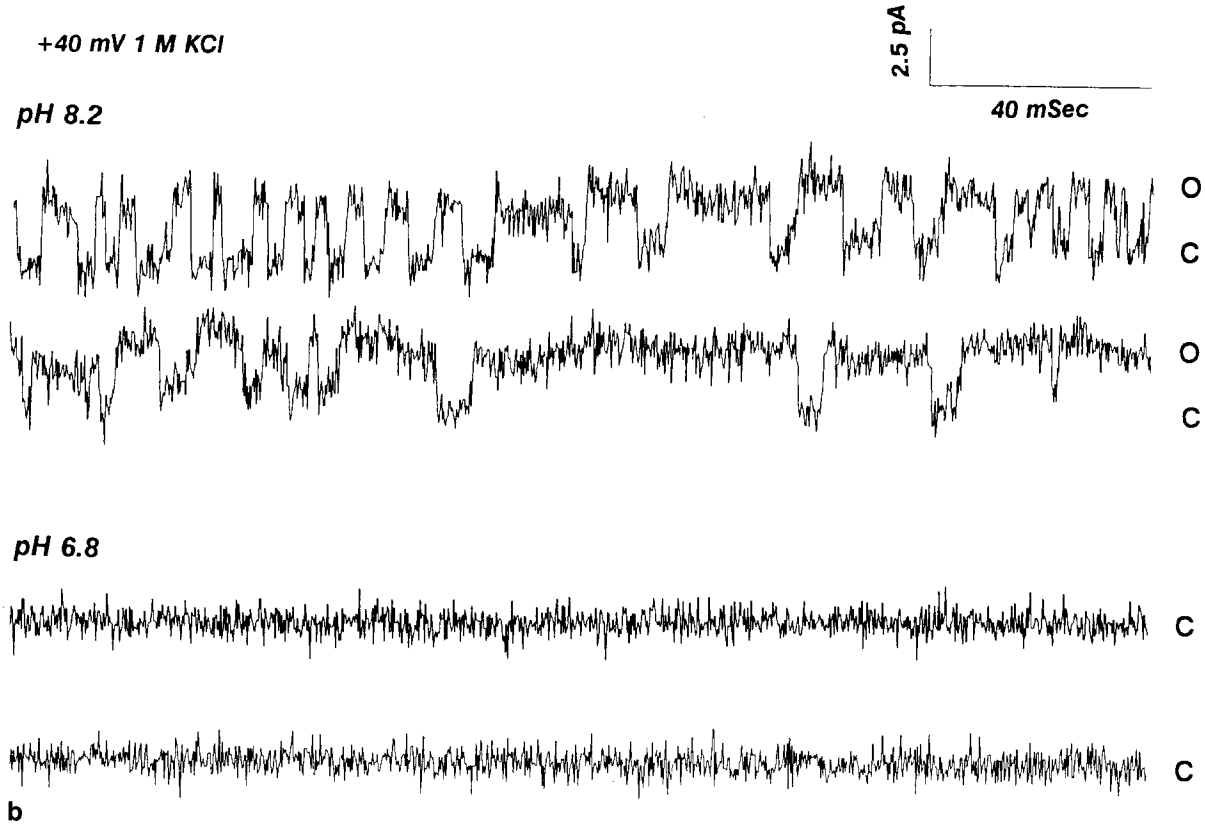

Table. Current inhibition by amphiphilic drugs

Drug	Concentration (μM)	n	Inhibition (% of total current before addition)
Amiodarone	0.4	2	60, 80
	4	8	88 ± 7[a]
Propranalol	70	3	63 ± 9[a]
	700	1	82
Quinine	600	3	93 ± 4[a]

[a] Mean ± SE.

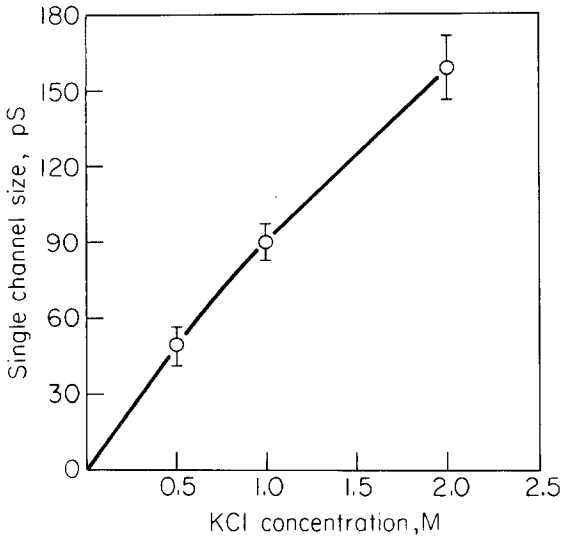

Fig. 4. Effect of KCl concentration on the alkaline pH-induced channel size. The experimental conditions were the same as in the legend to Fig. 1*b* except for the KCl concentrations. Each point represents a minimum of three independent patches at each KCl concentration (mean ± SE).

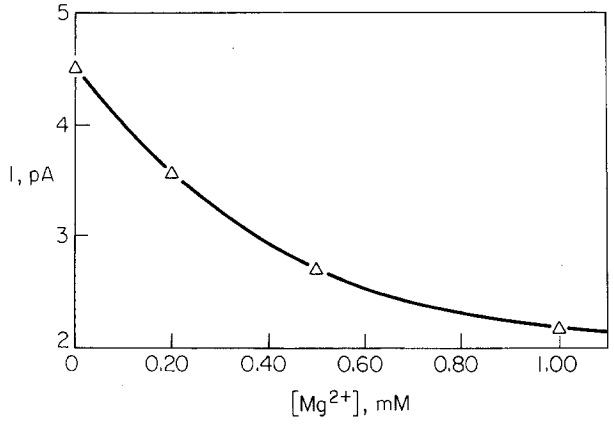

Fig. 5. Mg^{2+} reversal of the pH-induced current increase. At pH 8.3 the patch was perfused with increasing concentrations of Mg^{2+}. The initial current level at pH 6.8 and +40 mV was 2.0 pA. Conditions are the same as in the legend of Fig. 3 except that the KCl concentration was 0.15 M.

30 mM KCl, 240 mM sucrose, 5 mM HEPES, 10 μM $CaCl_2$, pH 6.8) *I-V* curve *3* was observed. The shift of the reversal potential to negative values (−36 mV) indicates that the membrane contains an open, primarily cation-selective current pathway. Increasing the pH from 6.8 to 8.3 (curve *2*) in the presence of the same gradient resulted in a less negative reversal potential (−31 mV), suggesting a decreased selectivity of the mitoplast membrane. Curve *4* shows that the addition of 12 μM propranolol induced an almost

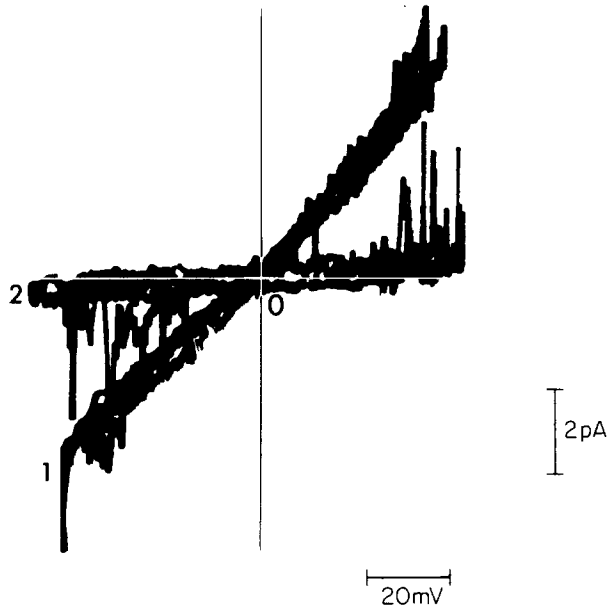

Fig. 6. Oscilloscope tracing showing *I-V* curves illustrating the amiodarone-induced inhibition of the membrane current. Curves *1* and *2* are respectively the *I-V* curves before and after perfusion with the medium containing 4 μM amiodarone. The solution is the symmetrical 150 mM KCl medium at pH 6.8. The current was recorded at a bandwidth of 30 Hz.

perfect cation selectivity (reversal potential of −41 mV). Higher concentrations of propranolol appear to inhibit the cation-selective current as well (*see* the Table). Similarly, the addition of Mg^{2+} (to the matrix side) at pH 8.3 also shifts the reversal potential to more negative values, indicating a decrease in anion selectivity (*not shown*). The statistical analysis of this kind of experiments showed that the reversal potential shifts from 36.8 ± 1.5 to 33.6 ± 1.7 (mean ± SE, $n = 6$) when the pH was changed from 6.8 to 8.3.

Discussion

The results show that anion permeability is increased with alkalinization of the matrix side of the inner membrane. While the reversal potential determinations indicate the membrane is invariably cation selective, the ratio of relative permeability P_K/P_{Cl} of 32 at pH 6.8 is reduced to 16 at pH 8.3. (*see* Materials and Methods). The anion permeability increase is likely due to the opening of several channels with a unit conductance of 15 pS in physiological salt. The introduction of Mg^{2+} as well as acidification of medium reversed the current shift in a graded fashion, as expected from patches with multiple channels.

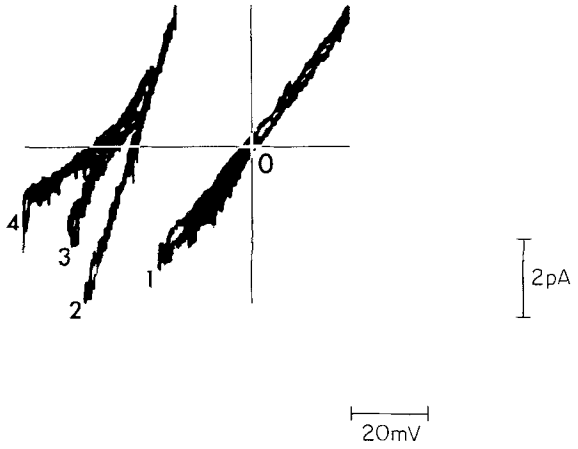

Fig. 7. Oscilloscope tracings of current-voltage (*I-V*) curves used to determine reversal potentials for a patch under varied conditions. Initially the patch was under symmetrical conditions (150 mM KCl, 5 mM HEPES, 10 μM $CaCl_2$) at pH 6.8 (curve *1*) and $E_{rev} = 0$. Curve *2* was recorded after changing the bath medium (the matrix side of the excised patch) to 30 mM KCl, 240 mM sucrose, 5 mM HEPES, 10 μM $CaCl_2$, pH 8.3, and the $E_{rev} = -31$ mV. Curve *3* corresponds to perfusion of the membrane patch with the same medium as curve *2* but at pH 6.8, $E_{rev} = -36$ mV; and curve *4*, corresponds to a perfusion under the same conditions of curve *3* but with the addition of 12 μM propranolol, $E_{rev} = -41$ mV. Under our conditions, a negative reversal potential indicates cationic selectivity. The current was recorded at a bandwidth of 30 Hz.

The open probability as a function of pH and Mg^{2+} is reflected by the % change in current in Figs. 2 and 5. The alkaline pH-induced transition observed at 0.15 M KCl was due to the opening of several channels which were resolved in 0.5 to 2 M KCl. The invariance of the conductance increase of 80 ± 5.5 pS (mean ± SE) reflects the constancy of the channel density within the patch. Since the estimated channel size is 15 pS in 0.15 M KCl the average number of channels per patch is estimated to be 6. These data indicate there are on the order of 250 to 500 of these channels in the inner membrane of each mitochondrion.

These observations can be most simply interpreted by the presence of two pathways. One is cation selective and open at pH 6.8 and the other corresponds to anion-selective channels induced to open by alkaline pH. This interpretation, based on the effect of pH on current levels and reversal potentials, is supported by the effect of propranolol. In low concentrations, propranolol makes the membrane patch completely cation selective, suggesting that this drug is acting differentially on two distinct pathways. However, at higher concentrations, the amphiphilic cationic drugs eliminated most of the current, probably nonselectively. In addition, tributyltin selectively inhibits the alkaline pH induced pathway. It is also possible to interpret the results by postulating a single channel whose subconductance levels are regulated by pH and Mg^{2+} and differentially sensitive to drugs. These modulators may affect the amplitude as well as the probability of opening.

Several direct correlations can be made from these results to the suspension studies of Beavis and Powers (1989) and others (e.g. Selwyn et al., 1979; Beavis & Garlid, 1987) who have postulated the existence of an inner membrane anion channel. These include, (i) pH profile (Beavis & Garlid, 1987), (ii) Mg^{2+} inhibition at similar concentrations (Beavis & Garlid, 1987; Beavis & Powers, 1989), and (iii) pharmacology including amiodarone, propranolol, quinine (Beavis, 1989) and tributyltin (Powers & Beavis, 1991) as inhibitors of the proposed inner membrane anion channel. However, in our hands, the amphiphilic drugs inhibit even at pH 6.8, suggesting that the effects of the drugs may be on more than one pathway. We have found amiodarone, propranolol and quinine are effective blockers of the other two classes of inner mitochondrial membrane, i.e., MCC activity and the ~100 pS channel (Antonenko et al., 1991).

We simultaneously studied the cation and anion currents through the membrane. The pH-dependent single-channel events detected most probably correspond to inner membrane anion channels postulated by Selwyn et al. (1979) and Garlid and Beavis (1986). At this point, the physiological roles of the cation and anion pathways are not known. Future studies of the effects described in this article would hopefully allow us to define their role.

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