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Selective effect of inhibitors on inner mitochondrial membrane channels

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The effect of amphiphilic cationic drugs on the channel activity of the mitochondrial inner membrane was examined with patch-clamp techniques. The therapeutic drugs amiodarone, propranolol and quinine reduced the probability of being open for the multiconductance channel (MCC) activity (levels from 30 pS to over 1 nS). While amiodarone decreased the probability of being open for the voltage dependent ~100 pS channel, it increased the conductance 42±20% (mean±SD, $n=6$) with no significant change in mean open time. Similar results were obtained with propranolol. These data indicate that the ~100 pS channel is distinct from MCC activity.

Mitochondrial channel; Patch-clamp; Pharmacology; Mitochondrial channel; Inner mitochondrial membrane

1. INTRODUCTION

The study of the mitochondrial inner membrane channels by patch-clamping has been handicapped by the lack of known pharmacological agents to affect their activity [1–4]. Amphiphilic drugs, many of which have medicinal value, were found to effect mitochondrial permeability in studies done with suspensions [5] and were therefore tested for their affect on channel activity. The present experiments examine the affects of amiodarone, propranolol and quinine on inner membrane channel activity. With each of these drugs we found an inhibition of the multiconductance channel (MCC) activity first described by Kinnally et al. [2]. Amiodarone decreased the likelihood of opening for the ~100 pS voltage dependent channel, and in addition it increased the single channel conductance. Similar results were obtained with propranolol. Recently, Szabó and Zoratti [6] have provided the only other pharmacological evidence for channel inhibition with patch clamping with a report that cyclosporin is effective in blocking MCC activity and does not inhibit the ~100 pS activity.

2. EXPERIMENTAL

Large mitochondria were isolated from the liver of normal mice of the Swiss-Webster strain as previously described [7]. The outer membrane was removed using the French-press method of Decker and Greenawalt [8] at 2000 psi. To study MCC activity, e.g. in the experiment of Fig. 1, mitoplasts were resuspended in 4 ml 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4. Patch-clamp experiments were carried out in 150 mM KCl, 5 mM HEPES, pH 7.4. Typically the medium's background calcium was approximately 10^{-6} M as determined by atomic absorption. To activate the ~100 pS channel [4] as in the experiments of Fig. 2 and Table I, mitoplasts were washed in 230 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4, containing 1 mM EGTA or alternatively 0.1 mM $CaCl_2$ and 1 mM EGTA (about 10^{-9} M free Ca^{2+}). After pelleting, they were then resuspended in 150 mM KCl, 5 mM HEPES, 1 mM EGTA, 0.95 mM $CaCl_2$ (about 10^{-7} M free Ca^{2+}), pH 7.4. Patch-clamping was carried out by placing on the slide approximately 5 μl of suspension and 500 μl of 150 mM KCl 5 mM HEPES 1 mM EGTA 0.95 mM $CaCl_2$ (about 10^{-7} M free Ca^{2+}), pH 7.4. The mitoplasts were given time to attach to the slide and then were extensively perfused with the medium. The patches were generally excised by first patching a mitoplast attached to the slide and then lifting the pipette away from the mitoplast. The inside-out patches made in this way displayed the same voltage dependence as attached patches. The voltages reported correspond to the bath (the matrix side of the mitoplast membrane). The pipettes ranged in resistance between 10 and 40 MΩ. Drugs were introduced to the bath by perfusion with approximately 5 ml of medium. The electronics used were previously described [2]. However, a digital data recorder from NeuroData Instrument Corporation (New York, N.Y.) (Neuro Corder model DR 384) and a Dagan model 3900A (Dagan Corporation, Minneapolis, MN) patch-clamp amplifier in the inside-out mode was used under voltage-clamp conditions. Data were recorded on a videocassette recorder at a bandwidth of 10 kHz. Analysis of stored signals was limited to a bandwidth of 1–5 kHz by a Frequency Devices Model 902, low pass filter using a 386 Zenith PC and Strathclyde PAT program (courtesy of J. Dempster, University of Strathclyde, U.K.).

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3. RESULTS AND DISCUSSION

In the present experiments, inner mitochondrial membrane patches were obtained from mitoplasts and only those which exhibited pronounced channel activity were selected. Under the conditions of these experiments, perfusion with the medium without drugs did not have a significant effect on channel activity.

Kinnally et al. [4] have shown that various conditions favor either the presence of multiconductance channel activity (MCC) (between 30-50 pS to approx. 1500 pS) or alternatively, the presence of the ~100 pS channel. We therefore chose conditions favoring the presence of either one or the other kind of activity to test the effect of inhibitors.

MCC is characterized by a very high, often greater than 1 nS, peak conductance. One of the predominant transition sizes seen with MCC is 550 pS [3]. As shown in Fig. 1A the current distribution had a fully open state, O_1, corresponding to over 11 pA at +10 mV (~1 nS) and a lower conductance open state, O_2, resulting from 550 pS transitions. The probability of occupying the fully closed state, C, was less than 0.01 and the probability of MCC being open (nP_o) approached 1. The fully closed state is often not observed in the absence of the drugs. Upon the addition of 4 μM amiodarone, the nP_o was reduced to 0 as indicated by a single peak, C, in the amplitude histogram of Fig. 1A. Fig. 1B shows representative current traces from data used to generate the diagrams of Fig. 1A. The effectiveness of amiodarone on the inhibition of channel activity was reflected in current traces and in the total amplitude diagrams (as in Fig. 1) in 22 of 23 independent experiments carried out at voltages ranging from ± 10 mV to ± 100 mV. The transitions effected include 30-65, 80-95, 120-140, 180, 210, 270-350, 450-550, 600-750, 900-1000 and 1300 pS. In 7 out of 14 patches which exhibited irregular current fluctuations (flickering) we observed a complete inhibition of activity by amiodarone. The flickering may be the result of rapid opening and closing of channels, the superimposition of the activity of several channels or alternatively noise due to an unstable seal. The inhibition in approximately half of the cases suggests that the flickering is a reflection of channel activity only 50% of the time.

MCC inhibition was obtained with propranolol (700 μM) and quinine (1.4 mM). Propranolol inhibited MCC activity in all of 7 independent experiments. The block was reversible in that removal of propranolol by perfusion with drug-free media restored activity (3 independent experiments). With the same patch we were able to carry out as many as 5 inhibition-reversal cycles by alternatively exposing the patch to propranolol and then removing it by perfusion. We obtained a block of

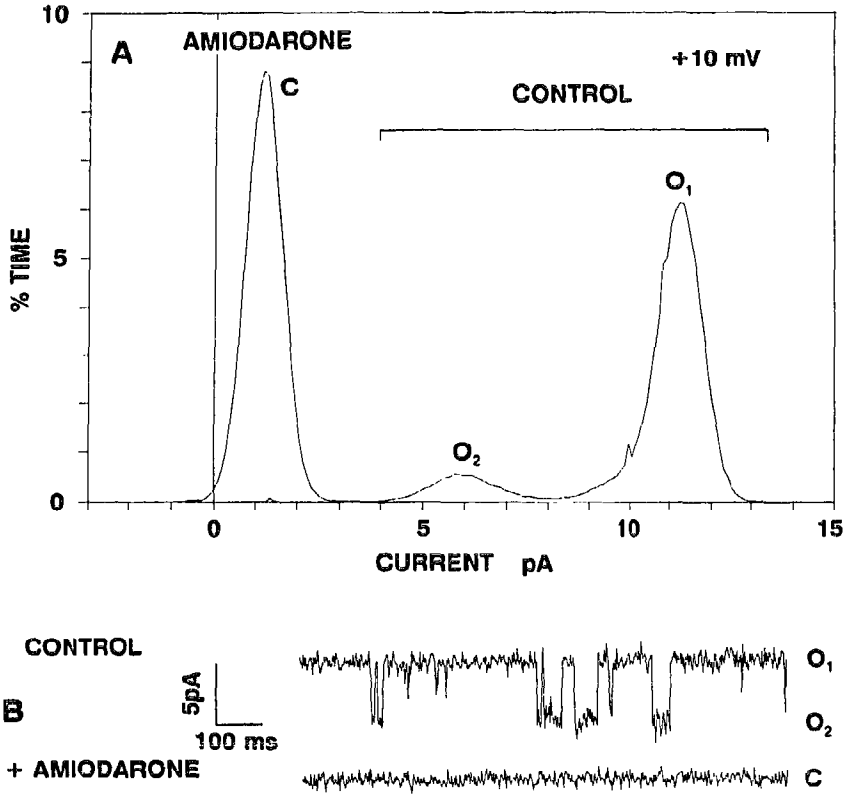

Fig. 1. Amiodarone shifts the current level occupancy of the multiconductance level activity. A. Amplitude diagram shows the current level occupancy as % of total time (bin width was 0.195 pA) at + 10 mV. The current analysis was limited to a bandwidth of 1 kHz with 2 kHz sampling. Both traces were analyzed for 34 s. C (closed state) corresponds to the lowest current level in the presence and absence of 4 μM amiodarone respectively. O_1 corresponds to the fully open channel and O_2 to the 550 ps transition current level in the control. Patch resistance in the presence of amiodarone was 8 GΩ. B. Current traces with time in the absence (control) and presence of amiodarone. O_1, O_2 and C are as defined above. Current traces are part of the data used to generate amplitude diagrams in A and show transitions of approximately 550 pS in control and no transitions in the presence of amiodarone.

multiconductance activity with quinine in 4 of the 5 patches. As with propranolol, the inhibition was reversible.

Fig. 2 shows the effect of amiodarone at +40 mV on the voltage dependent channel described by Sorgato et al. [1]. This activity was distinguished from MCC conductance levels by its strong voltage dependence, conductance and other channel characteristics (see Table I). In Fig. 2, the unitary conductance shifted from 92 pS to 113 pS and the nP_o (probability of being open) decreased 42% from 0.73 to 0.42 after the addition of amiodarone. The conductance increased and nP_o decreased upon the addition of amiodarone in 6 out of 6 independent observations.

The current level of the apparent closed state is reduced (in some experiments only 1–2 pA). This observation suggests a conductance pathway (e.g. MCC) which remains predominantly open in the absence of the drugs. Table I summarizes some of the properties of the channel activity. Those properties most notably affected by amiodarone are a 76 ± 19% (mean ± SD, $n = 6$) decrease in probability of opening (nP_o) and an increase in single channel conductance. The 53 ± 22% decrease in burst length coupled with a similar decrease in openings per burst contribute to the observed dramatic increase in mean closed time. No significant change in mean open time was observed. Similar results were obtained with propranolol (2 experiments) in that the conductance of the ~100 pS channel increased while the probability of opening (nP_o) decreased.

The single channel conductance was 102 ± 11 pS and increased 42 ± 20% to 145 ± 23 pS with the addition of amiodarone. The increase in conductance can be interpreted in two different ways. Amiodarone could simply increase the ~100 pS channel conductance by about 40%. Alternatively, it could inhibit the ~100 pS channel and induce a new higher conductance channel. We consider the first alternative more likely since (a) the mean open time and the flickering character in the presence and absence of the inhibitor was not significantly different, (b) the channel activity displayed the characteristic asymmetric dependence on voltage (i.e. more open at positive and closed at negative voltages), and finally, (c) we have never observed the appearance of this activity when amiodarone was applied to patches showing no channel activity, not ex-

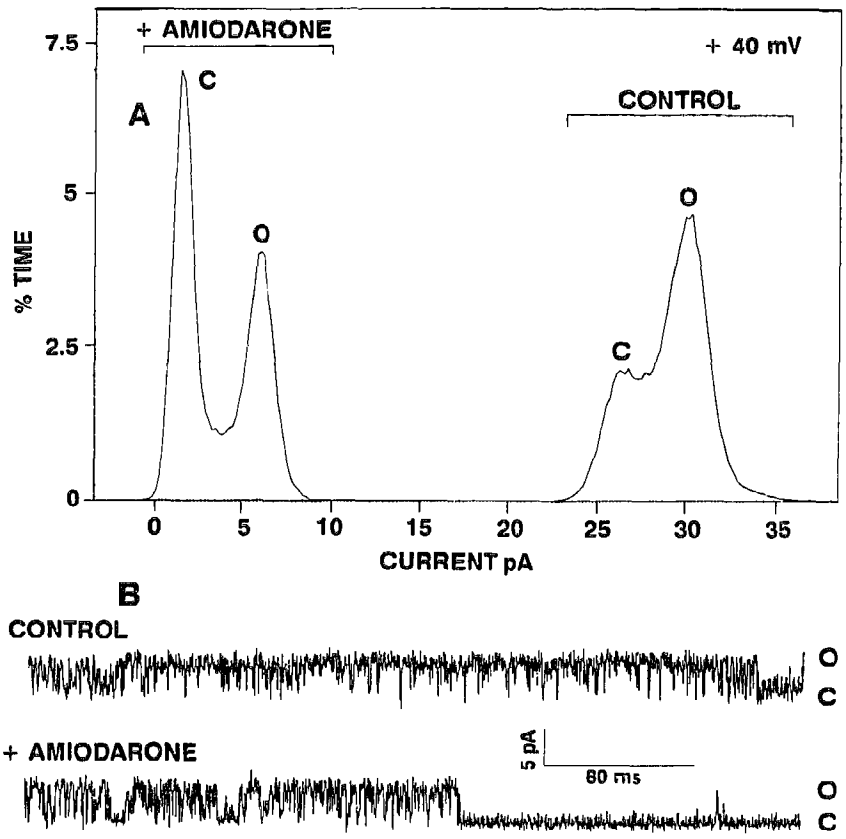

Fig. 2. Amiodarone shifts the current level distribution of the ~pS activity. A. Amplitude diagrams showing occupancy of current level as % of the total time with and without μM amiodarone (bin width was 0.195 pA) at +40 mV. Both current traces were analyzed at a bandwidth of 2 kHz with 5 kHz sampling for 29 s. C (closed state) corresponds to the lowest and O (open) to the highest current level with and without amiodarone. B. Current traces show transitions corresponding to 92 pS in the control and 113 pS in the presence of amiodarone. The patch resistance was 4 GΩ in the presence of 4 μM amiodarone.

Table I

Amiodarone		affects	IMM channel characteristics*				
		(mV)	Cond. (pS)	nP_o	Open t (ms) no. events)	Closed t (ms) (no. events)	Burst length (ms)	open per burst
1	Control	+40	92	0.73	2.07 (10022)	0.756 (10022)	403.0	115.0
	Exp.		113	0.42	1.78 (6623)	2.49 (6622)	255.0	80.9
2	Control	+40	118	0.042	0.819 (974)	14.3 (1090)	24.4	10.6
	Exp.		156	0.005	0.832 (305)	197.0 (396)	8.4	2.4
3	Control	+30	104	0.090	1.30 (1345)	13.2 (1406)	70.3	28.6
	Exp.		176	0.021	3.74 (647)	189.0 (652)	133.0	27.7
4	Control	+30	110	0.30	1.66 (1682)	3.7 (1781)	51.7	16.1
	Exp.		143	0.04	2.26 (472)	42.7 (687)	40.7	9.3
5	Control	+40	92	0.040	0.82 (852)	14.8 (945)	30.2	15.9
	Exp.		126	0.003	1.05 (268)	231.0 (337)	8.3	2.3
6	Control	+20	93	0.66	1.52 (988)	2.2 (1019)	46.1	9.3
	Exp.		156	0.21	2.03 (691)	4.9 (709)	11.3	4.7

*Control represents values before and Exp. represents after the addition of 4 μM amiodarone. Conductance was calculated from the open state peak of total current amplitude histogram and voltage. nP_o, the mean probability of the channel being open, was the ratio of time spent at the open state current level and total time of the record. Mean open time (open t) was the mean duration of open state transition. Closed time (closed t) was mean duration of closed state transition. Burst length was the duration of bursts with a 5 ms maximum closed interval and excluded single openings. Openings per burst corresponded to the average number of transitions to open state per burst and excluded single openings. For each experiment data were recorded with 10 kHz bandwidth. Offline computer analysis was limited to a 2 kHz bandwidth with a sampling interval of 0.2 ms for experiments 2-5, and 0.25 ms for experiment 1 and 6. Settling time was 3 points and the threshold for transition was 50% open current level.

hibiting the ~100 pS activity or from liposomes (data not shown). It is not likely to correspond to the 120–150 pS activity we have observed as a subconductance level of MCC as the flickering characteristics and voltage dependence are different and it has also never been observed after the addition of amiodarone independent of the ~pS activity.

Amiodarone and propranolol are antiarrhythmic [9,10]. Amiodarone has been found to block Na^+ [11] and Ca^{2+} [12] channels in cultured cardiomyocytes. The Na^+ channel block occurs with a decrease in probability of opening and no change in open time or unitary conductance [11]. These data were interpreted to suggest that amiodarone interacts with the inactivated state of the Na^+ channel. The behavior of the ~100 pS channel could be interpreted in a similar manner. However, the increase in unitary conductance induced by amiodarone indicates further complexities. Amiodarone could also be modifying the open state(s) such that the conductance increases. Alternatively, it could decrease the current through an already open lower conductance level accounting for the shift in the C (closed) peaks with the addition of amiodarone. A shift in the current level of the closed state was observed in all cases and was sufficient to account for the increase in unitary conductance in 83% of the patches. A behavior similar to our second alternative was observed with VDAC which exhibited a decrease in 'close state' conductance in the presence of a modulator protein [13]. A combination of both mechanisms is also possible. We cannot, at this time, exclude the possibility that this channel activity is a normal subconductance level of a larger channel. We feel that this is evidence that the ~100 pS channel is capable of displaying subconductance levels but that normally transitions of ~100 pS are predominant. It is thought that the ~100 pS and MCC activities represent distinct classes of channels. Evidence in support of this conclusion includes a variety of observations. Their relative distribution can be varied with isolation protocols based on the effect of calcium [4]. MCC activity and the ~100 pS activity were reconstituted from different membrane fractions in experiments of Moran et al. [14]. Finally, cyclosporin A [6] was shown to block MCC but not the ~100 pS channel. The present study provides further evidence that there are two distinct classes of channels in the inner mitochondrial membrane.

The patch clamp identification of three new inhibitors by this work supplies an important tool for the study of inner membrane channels. The possible role(s) of channels in such functions as metabolism (e.g. in regulating coupling), intermitochondrial communication [15] cell death [16] and protein import [17] may be examined.

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REFERENCES

[1] Sorgato, M.C., Keller, B.U. and Stühmer, W. (1987) Nature 330, 498–500.
[2] Kinnally, K.W., Campo, M.L. and Tedeschi, H.J. (1989) J. Bioenerg. Biomembr. 21, 497–506.
[3] Petronilli, V., Szabó, I. and Zoratti, M. (1989) FEBS Lett. 259, 137–143.

[4] Kinnally, K.W., Perini, S. and Tedeschi, H. (1990) Biophys. J. 57. 390a.
[5] Beavis, A.D. (1989) J. Biol. Chem. 264, 1508–1515.
[6] Szabó, I. and Zoratti, M. (1991) J. Biol. Chem. 266, 3376–3379.
[7] Bowman, C.L. and Tedeschi, H. (1983) Biochim. Biophys. Acta 731, 261–266.
[8] Decker, G.L. and Greenawalt, J.W. (1977) J. Ultrastr. Res. 59, 44–56.
[9] Mason, J.W. (1987) New Engl. J. Med. 316, 455–466.
[10] Barrett, A.M. and Cullum, V.A. (1968) Br. J. Pharm. 34, 43–55.
[11] Kolhardt, M. and Fichtner, H. (1988) J. Membr. Biol. 102, 105–119.
[12] Nishimura, M., Follmer, C.H. and Singer, D.H. (1989) J. Pharm. Exp. Therap. 251, 650–659.
[13] Holden, M. and Colombini, M. (1988) FEBS Lett. 241, 105–109.
[14] Moran, O., Sandri, G., Panfili, E., Stümher, W. and Sorgato, M.S. (1990) J. Biol. Chem. 265, 908–913.
[15] Amchenkova, A., Bakeeva, L.E., Chentsov, Yu., Skulachev, V.P. and Zorov, D.B. (1988) J. Cell Biol. 107, 481–495.
[16] Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R.D. and Korsmeyer, S.J. (1990) Nature 348, 334–337.
[17] Pfanner, N., Rassow, J., Wienhues, U., Hergersberg, C., Söllner, T., Becker, K. and Neupert, W. (1990) Biochim. Biophys. Acta 1018, 239–242.