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RELATION OF THE OXIDOREDUCTION LEVEL OF ELECTRON CARRIERS TO ION TRANSPORT IN SLICES OF AVIAN SALT GLAND

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SUMMARY

1. A study has been made of the effects on electron carriers of agents which simultaneously affect respiration and ion transport in avian salt-gland slices.

2. Ouabain caused changes in the oxidoreduction state of the electron carriers which were consistent with a transition of the mitochondria of the slices towards State 4.

3. Methacholine caused reduction of all the electron carriers examined, in medium containing 5 mM K^+. The reduction of cytochromes was reversed by ouabain and so appears to be related to the stimulation of ion transport by methacholine.

4. The reduction of nicotinamide nucleotides induced by methacholine was not inhibited by ouabain or by iodoacetate. It was inhibited by 3 agents which also prevent the succinate-dependent reduction of nicotinamide nucleotides in the slices—malonate, dicoumarol and a K^+-free medium.

5. It is suggested that methacholine has 2 different effects in the slices: (a) stimulation of ion transport with consequent release of phosphate acceptor and transition of the mitochondria towards State 3; (b) stimulation of a reaction leading to reduction of nicotinamide nucleotides which is not related to an effect on ion transport. The latter reaction may be the reversal of electron transfer.

INTRODUCTION

The secretion of NaCl by the nasal gland of the sea gull is stimulated by cholinergic agents and inhibited by ouabain[1,2]. These substances also affect slices of the salt gland *in vitro*, cholinergic agents causing a temporary stimulation of ^{24}Na efflux and a stimulation of respiration, while ouabain inhibits both ion movements and respiration[3–5]. These results suggest that the respiration of the salt-gland cells is partly controlled by the activity of the ion-transporting mechanism (*cf.* refs. 6–8).

There is evidence that the coupling of respiration to ion transport, in other tissues, is mediated by ADP released by the transport mechanism[9,10]. Since it is known that activation of respiration by ADP in isolated mitochondria, including those from salt gland, leads to characteristic changes in the oxidation–reduction steady state of components of the respiratory chain[11,12], the study of electron carriers in the whole cells offers a means of examining the mechanism of respiratory control exerted by the ion-transporting mechanism. In the present work, relevant information

has been obtained by examining the effects of a cholinergic drug, methacholine (α-acetyl-β-methylcholine), and of ouabain, on electron carriers in slices of the tissue. A preliminary account of some of this work has been published[13].

METHODS

The experimental methods and incubation procedure have been described previously[14,15]. The reduction level of the nicotinamide nucleotides was followed in the differential spectrofluorimeter described by CHANCE and co-workers[16,17], and cytochromes were measured in the split-beam spectrophotometer of YANG AND LEGALLAIS[18]. Fluorescence changes are expressed as a percentage of the aerobic level of fluorescence[14]. Values given in the text and tables represent mean ± S.E. with the number of observations in parentheses.

RESULTS

Ouabain

At a concentration of 0.05 mM, ouabain inhibits the respiration-dependent movements of Na^+ and K^+ in slices of salt gland[3]. In slices utilising endogenous substrate, this concentration always caused a clear reduction of nicotinamide nucleotides and cytochrome *b*, and usually some oxidation of cytochromes (*c plus* c_1) and

TABLE I

THE EFFECT OF OUABAIN ON RESPIRATORY PIGMENTS IN THE PRESENCE OF ENDOGENOUS SUBSTRATE OR ADDED SUCCINATE

Paired slices were incubated in a continuous flow of medium (30°; gassed with O_2) in the slice holder of the fluorimeter. When the fluorescence trace attained a steady level after a particular treatment, the holder was temporarily transferred to the spectrophotometer for estimation of the absorption changes. In Expt. 1, the sections i and ii were separated by a period of washing in the basic medium which was continued until the fluorescence trace returned to its initial (untreated) level. The basic medium contained (in mM): Na^+, 170.0; K^+, 5.0; Mg^{2+}, 1.0; Ca^{2+}, 1.2; Cl^-, 130.7; SO_4^{2-}, 1.0; phosphate, 24.5 (pH 7.4). In the succinate medium, 30 mM sodium succinate replaced an osmotic equivalent amount of NaCl. The values given represent the changes in oxidoreduction level induced by each treatment, positive values indicating reduction, and negative values oxidation.

Treatment	*Nicotinamide nucleotides (% change of fluorescence)*		*Cytochrome (μmoles/kg wet wt.)*	
	Maximum	*Net*	*c plus* c_1 *Net*	*a plus* a_3 *Net*
Expt. 1				
(i) Ouabain (0.05 mM)	10.9 ± 1.9	4.2 ± 2.6	− 2.5 ± 5.4	− 6.9 ± 4.3
(ii) Succinate (30 mM)		69.8 ± 9.0	44.4 ± 6.5	34.2 ± 4.7
plus ouabain		20.7 ± 5.6	−13.7 ± 3.6	− 6.6 ± 2.4
(*n*)	(13)	(13)	(8)	(8)
Expt. 2				
Succinate (30 mM)		72.1 ± 17.6		
plus ouabain (0.05 mM)		30.4 ± 9.8		
Sum		102.5 ± 11.6		
plus dicoumarol (0.4 mM)		−42.4 ± 8.2		
(*n*)		(4)		

(*a* plus a_3), so that it induced a 'crossover point' (ref. 11) between cytochromes *b* and *c* (Table I and Fig. 1). The response of the nicotinamide nucleotides was biphasic, the fluorescence trace passing through a maximum before attaining a new steady state.

Treatment of the slices with succinate caused a reduction of nicotinamide nucleotides (due to reversal of electron transfer[19]) and of cytochromes; subsequent addition of ouabain caused further reduction of nicotinamide nucleotides but oxidation of cytochromes (*c plus* c_1) and (*a plus* a_3) (Table I). That the responses of these pigments to ouabain were now clearer than with endogenous substrate alone is probably due to the greater saturation of the respiratory chain with electrons in the presence of succinate, since the respiration rate is then doubled[4]. The site of the crossover point induced by ouabain in this case was variable, cytochrome *b* sometimes becoming reduced (Fig. 2A) and sometimes oxidised (Fig. 2B).

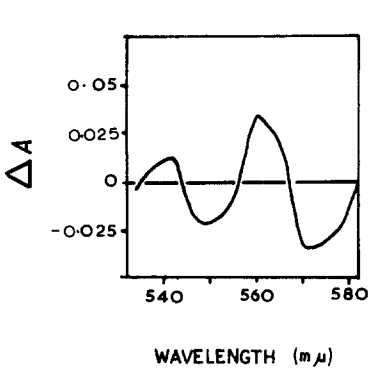


Fig. 1. Difference spectrum of a salt-gland slice before (baseline) and after treatment with 0.05 mM ouabain. The trough at 550 mμ and the peak at 562 mμ are in the regions of the α-bands of cytochromes (*c plus* c_1) and *b*, respectively.

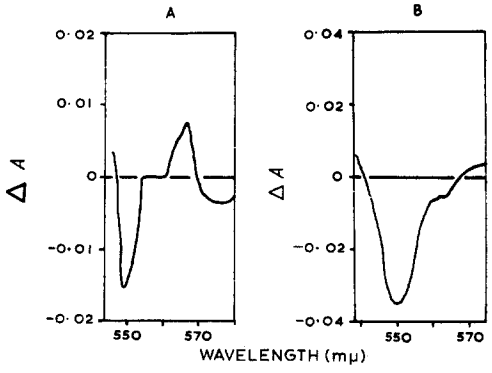
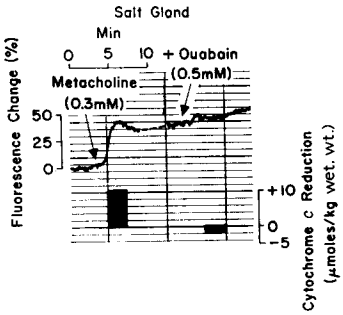


Fig. 2. Difference spectra of salt-gland slices after treatment with 30 mM succinate (baselines) and after further addition of 0.5 mM ouabain.

These observations are consistent with the idea that ouabain induces a transition of the mitochondria in the slices from a state approaching the active State 3 (ref. 11) towards the resting State 4. However, that the actual situation may be more complex is suggested by Expt. 2 of Table I. Here the uncoupling agent, dicoumarol, caused only a partial reversal of the sum of the fluorescence changes induced by succinate and ouabain together. This result was unexpected, since the succinate-induced reduction of nicotinamide nucleotides in the slices[19], and the State 3–State 4 transition in isolated mitochondria[11], are both completely reversed by the concentration of dicoumarol used here.

Methacholine

If the effects of methacholine on electron carriers are solely due to a transition of the mitochondria of the slice towards State 3 because of the stimulation of ion transport then, by analogy with isolated mitochondria[11,12], they would be expected to include oxidation of NADH and reduction of electron carriers on the oxygen side of a crossover point in the respiratory chain. However, Figs. 3 and 4 show that all



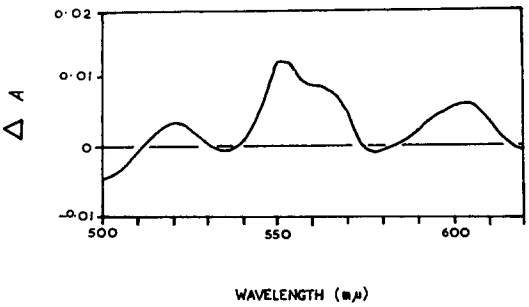


Fig. 3. Effects of methacholine and ouabain on fluorescence and cytochrome (*c plus* c_1). The vertical bars represent the change in reduced cytochrome *c* content (μmoles/kg wet wt.) 6 min after addition of methacholine and 9 min after further addition of ouabain to the medium, relative to the content before methacholine treatment.

Fig. 4. Difference spectrum of a salt-gland slice before (baseline) and after treatment with 0.3 mM methacholine. The peaks at 520, 550 and 605 mμ are in the regions of the β cytochrome bands, the α-band of cytochrome (*c plus* c_1) and the α-band of cytochrome (*a plus* a_3), respectively; the shoulder in the region of 563 mμ is probably due to the α-band of cytochrome *b*.

the electron carriers examined were reduced upon treatment with methacholine. Similar results were obtained in the presence of added succinate, except that the increase of fluorescence induced by methacholine was then usually rather less (Table II, Expt. 1). A characteristic feature of the response to methacholine was that the fluorescence trace passed through a maximum before attaining a new steady state (Figs. 3 and 6). The values for the fluorescence response to methacholine given in Table II refer to the final steady-state level. Subsequent treatment with ouabain led to changes similar to those seen in the State 3–State 4 transition in mitochondria, the cytochromes

TABLE II

EFFECTS OF METHACHOLINE ON RESPIRATORY PIGMENTS

Details as for Table I.

Treatment	*Nicotinamide nucleotides (% change of fluorescence)*	*Cytochrome (μmoles/kg wet wt.)*	
		c plus c_1	*a plus* a_3
Expt. 1			
(i) Methacholine (0.3 mM)	16.2 ± 2.9 (15)	5.4 ± 1.9 (5)	6.2 ± 3.4 (5)
(ii) Succinate (30 mM)	46.9 ± 7.2 (16)	28.4 ± 9.9 (6)	26.2 ± 6.6 (6)
plus methacholine	9.1 ± 3.4 (16)	11.3 ± 5.8 (6)	3.7 ± 5.2 (6)
Expt. 2			
Methacholine (0.3 mM)	25.1 ± 5.3 (16)	7.3 ± 2.2 (12)	4.3 ± 2.5 (12)
plus ouabain (0.05 mM)	9.4 ± 2.5 (16)	− 4.3 ± 2.2 (12)	−4.9 ± 2.4 (12)
Expt. 3			
(i) Methacholine (0.3 mM)	14.6 ± 5.2 (14)	6.0 ± 3.7 (9)	4.5 ± 2.2 (8)
plus malonate (20 mM)	−16.3 ± 4.3 (14)	−10.7 ± 2.5 (9)	−9.6 ± 2.5 (8)
(ii) Malonate	−19.4 ± 9.4 (10)	−18.3 ± 5.5 (6)	−5.9 ± 6.2 (6)
plus methacholine	− 4.9 ± 4.3 (10)	1.7 ± 4.3 (6)	1.6 ± 5.2 (6)

reduced by methacholine becoming completely (cytochromes *c* and *a*) or partly (cytochrome *b*) reoxidised, and the nicotinamide nucleotides becoming further reduced (Table II, Expt. 2). Ouabain is also known to inhibit the methacholine-induced stimulation of respiration in slices of this tissue[4]. These effects of ouabain suggest that the respiratory stimulation and the reduction of cytochromes caused by methacholine are due to a transition of the slice mitochondria towards State 3, which shows a crossover point on the substrate side of cytochrome *b*, and which results from the effects on ion transport. However, the fluorescence increase which is caused by methacholine, and which is not reversed by ouabain, cannot be accounted for in this way, and experiments were therefore done with metabolic inhibitors in order to obtain information on the reactions involved.

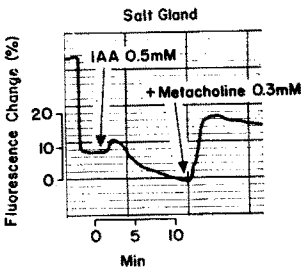


Fig. 5. Effect of methacholine on slice fluorescence in the presence of iodoacetate (IAA).

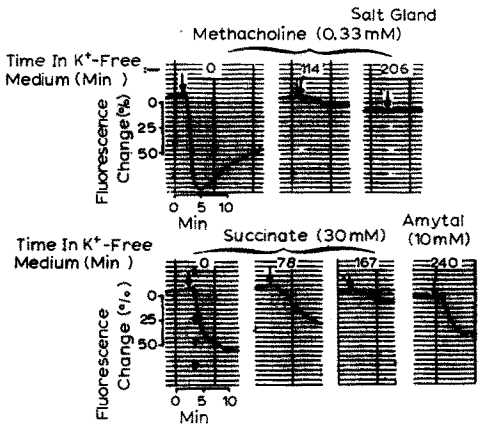


Fig. 6. Effects of methacholine and succinate on the fluorescence of a single slice after different periods in K^+-free medium. The responses of the slice to succinate and methacholine were first determined in the medium containing 5 mM K^+ (see Table I). After each treatment the slice was washed with plain medium (no succinate or methacholine) until the fluorescence trace returned to the original base line. The medium was then changed to K^+-free medium (K^+ replaced by Na^+) and thereafter the effects of agents in this medium were examined as indictated; after each treatment, the slice was washed with plain, K^+-free medium.

In the experiment illustrated in Fig. 5, iodoacetate (0.5 mM) caused a net oxidation of nicotinamide nucleotides, presumably due to the inhibition of glyceraldehyde-3-phosphate dehydrogenase[15], but did not prevent a 19 % increase of fluorescence intensity upon subsequent addition of 0.3 mM methacholine. In 4 such experiments the mean fluorescence increase was 36.1 ± 14.2 %. Comparison with the mean value of 25.2 ± 3.4 % (78) for all experiments in the absence of iodoacetate, suggests that the effect of methacholine on the nicotinamide nucleotides was not due to reactions of glycolysis. On the other hand, malonate (20 mM) completely prevented the response of all respiratory pigments to methacholine when added to the slices before this agent, and reversed the methacholine effects when added subsequently (Table II, Expt. 3). The effects of methacholine on the respiratory pigments are thus dependent upon the activity of succinate dehydrogenase. However, methacholine does not appear simply to stimulate succinate oxidation, since it causes a relatively greater percentage reduction of the nicotinamide nucleotides reducible by

anaerobiosis than of the cytochromes, whereas treatment of the slices with succinate causes a reduction of nicotinamide nucleotides which is approximately equivalent, percentage-wise, to that of the cytochromes (Table III). It is therefore suggested that methacholine caused a preferential stimulation of the transfer of electrons from succinate to nicotinamide nucleotides. In common with the increase of fluorescence induced by succinate[19], that induced by methacholine (23.4 ± 11.4 % increase in 5 experiments) was considerably reversed (13.1 ± 2.3 % decrease) upon addition of dicoumarol at a concentration of 0.2 mM.

TABLE III

PERCENTAGE REDUCTION OF ELECTRON CARRIERS INDUCED BY METHACHOLINE AND SUCCINATE

The reduction of each of the electron carriers is expressed as a percentage of the reduction induced in the same slice by terminal respiratory inhibition with anaerobiosis or 10 mM Na_2S (ref. 15).

Treatment	*Nicotinamide nucleotides*	*Cytochrome (c plus c_1)*	*Cytochrome (a plus a_3)*
Methacholine (0.3 mM)	31.8 ± 5.6 (17)	13.4 ± 4.9 (12)	14.4 ± 8.2 (12)
Succinate (30 mM)	55.0 ± 6.7 (17)	50.5 ± 6.9 (14)	43.1 ± 5.9 (14)

Effect of alkali metals in the medium

It has been reported previously that methacholine causes a greater stimulation of ^{24}Na efflux from salt-gland slices in K^+-free medium than in medium containing 5 mM K^+ (ref. 3). The experiments described above suggested that the methacholine-induced increase of fluorescence was independent of the effects of methacholine on ion transport, and related to the oxidation of succinate. The effect of a K^+-free medium on the reduction of nicotinamide nucleotides by methacholine and succinate was therefore studied. Fig. 6 shows that the effects of both substances on the fluorescence of the slices were slowly inhibited after the medium bathing the slices was changed to one without K^+. In a series of similar experiments, the response to methacholine was completely abolished after about 120 min in the K^+-free medium. The response to succinate was not quite abolished, but levelled off at the low value of 8.7 ± 2.8 (3) % fluorescence increase, after about 140 min. Companion slices incubated in 5 mM K^+ medium throughout showed no significant alteration in either response during the experiments. After 240 min the slice in K^+-free medium in Fig. 6 still responded normally to the addition of 10 mM Amytal, with an increase of 36 % in fluorescence intensity compared to 43 % for the companion slice in the 5 mM K^+ medium. Thus, the inhibition of the succinate- and methacholine-induced responses was not shared by the reduction of mitochondrial nicotinamide nucleotides by electrons from endogenous, NAD-linked substrates.

The slow decline of the effects of succinate and methacholine after removal of K^+ from the medium probably indicates that the inhibition is due to a loss of intracellular K^+ or gain of intracellular Na^+, rather than directly to the absence of extracellular K^+. During 60 min in K^+-free medium the slices lose some 75–80 % of their K^+ content and show a 30–40 % increase in Na^+ content[3].

In 2 experiments, substitution of Li^+ for the Na^+ of the medium caused no inhibition of the fluorescence response to either succinate or methacholine within a period of 210 min.

DISCUSSION

This work represents an attempt to relate studies of the control of respiration by ADP in isolated mitochondria to the control exerted by an energy-requiring process (ion transport) in the whole cell. Even though the salt gland is particularly suited to this type of study because of its high content of mitochondria[20], it is to be expected that changes observed in mitochondrial experiments will be modified by a variety of factors in the more complex cellular systems. Such factors could, for example, include interactions between processes occurring at different morphological regions of the cell, and multiple actions of a single chemical at different sites. The observed effects of ouabain on electron carriers in the slices are of the type to be expected when the mitochondrial respiration becomes less active as a result of the limitation of phosphate acceptor[11]. Methacholine treatment leads to an increase in the rate of respiration[4] and to a reduction of cytochromes *b*, (*c plus* c_1) and (*a plus* a_3), all of which are compatible with a transition towards State 3 and which, in view of their inhibition by ouabain, appear to result from the effects of methacholine on ion transport. All these results, therefore, can be readily accounted for in terms of the known interactions of phosphate acceptor with the respiratory chain, and suggest that this interaction may also account for the control of respiration by ion transport in the whole cells of the salt-gland slices.

Certain observations, however, cannot be accounted for in these terms. One is the failure of dicoumarol to cause complete reversal of the increase of fluorescence caused by succinate *plus* ouabain in Expt. 2 of Table I; another is the finding in other work that, in a K^+-free medium, cholinergic drugs stimulate ^{24}Na efflux (ref. 3) but not respiration[4]. No information is at present available which allows an explanation of these observations.

A third observation which differs from the findings anticipated from the hypothesis that the stimulation of ion transport by methacholine leads to an activation of respiration by a State 4–State 3 transition of the mitochondria, is that methacholine caused a reduction of nicotinamide nucleotides. In this case some evidence is available to suggest a possible explanation. Thus, the experiments with iodoacetate and malonate indicate that reactions of the citric acid cycle rather than of glycolysis are responsible, and the results with Amytal in the K^+-free medium show that it is not due to reactions catalysed by NAD-dependent dehydrogenases of the mitochondria. On the other hand, the methacholine-induced reduction does show properties in common with the succinate-linked reduction of NAD, being sensitive to malonate, dicoumarol and the absence of K^+, and it is therefore tentatively suggested that methacholine stimulated the reversal of electron transfer in the salt-gland slices. However, no direct information on the sensitivity of reversed electron transfer to cholinergic agents, or to the relative proportions of Na^+ and K^+ in the medium, is available from studies with isolated mitochondria.

The question as to whether the methacholine-induced reduction of nicotinamide nucleotides is related to the effect of this agent on ion transport in the slices must, on the present evidence, be answered in the negative. Thus, the reduction was not inhibited by ouabain, which does inhibit active movements of ions, but was inhibited in a K^+-free medium in which the active efflux of sodium is stimulated[3]. Methacholine would thus appear to offer a possible example of a substance which has two separate

effects in the cells and thereby gives a different result from that anticipated on the basis of the simple hypothesis. The biphasic nature of the fluorescence response to the drug could then conceivably be explained as the resultant of two processes: (1) a stimulation of the reaction leading to the reduction of nicotinamide nucleotides (whether or not this is due to reversal of electron transfer); (2) an oxidation of NAD resulting from the increased availability of phosphate acceptor from the ion-transporting mechanism. If the latter effect continues for a longer time than the former, it could account for the second (decreasing) phase of the fluorescence response. In support of this idea, the duration of the stimulation of ^{24}Na efflux after methacholine treatment[3] corresponds roughly to the time required for completion of the oxidative phase of the fluorescence changes.

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