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THE EFFECTS OF PROPIONATE ON SODIUM TRANSPORT BY THE TOAD BLADDER

EVIDENCE FOR A METABOLIC MODE OF ACTION

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SUMMARY

1. The effects of moderate concentrations (up to 20 mM) of propionate and related compounds on active sodium transport and metabolism in the freshly-isolated toad bladder have been studied.

2. Depending upon the concentration employed, propionate when added simultaneously to the serosal and mucosal bathing media of isolated bladders at pH 6.4 may either stimulate or inhibit sodium transport as measured by the short-circuit current (s.c.c.)*. The characteristic response to addition of 6–20 mM propionate in freshly-isolated bladders is a striking inhibition of the s.c.c.

3. Malonate interferes with both the stimulatory and inhibitory effects of propionate on the s.c.c. Propionate blocks the effects of malonate on the s.c.c. Succinate can mimic the dual effects of propionate under certain conditions.

4. Although 6 mM propionate inhibits the s.c.c., it has no net effect on CO_2 production and actually increases oxygen consumption.

5. Malonate reduces $^{14}CO_2$ production from ^{14}C-labelled propionate. Propionate prevents inhibition of oxygen consumption by malonate.

6. Propionate reduces the rate of conversion of ^{14}C-labelled pyruvate and acetate to $^{14}CO_2$.

7. It is concluded that both the stimulatory and the inhibitory effects of propionate may be consequences of the metabolism of propionate *via* succinate and that both depend, at least in part, on mechanisms affecting the supply of energy to the sodium pump in the epithelial cells; suggested mechanisms to account for the observed inhibition of sodium transport include changes in the concentration of CoA or its esters, reduction in the rate of oxidation of NAD-linked substrates, reversal of electron transport through Site I, and stimulation of gluconeogenesis.

INTRODUCTION

The rate of active sodium transport across the isolated toad bladder has been shown to be stimulated by the addition of certain metabolic substrates[1], which act synergistically with aldosterone[2–4].

Abbreviation: s.c.c., short-circuit current.

Fimognari *et al.*[4] reported that addition of propionate (4 mM) to the bathing media of the isolated overnight ("substrate-depleted") toad bladder resulted in stimulation of sodium transport as measured by the short-circuit current (s.c.c.). This effect of propionate was subsequently shown by Falchuk and Sharp[5] to be synergistic with the action of aldosterone. In a preliminary investigation of the effect of propionate on the s.c.c. in the freshly-isolated toad bladder it was found that addition of moderate concentrations of propionate (6–8 mM) simultaneously to the serosal and mucosal bathing media resulted in a rapid, marked decrease in the s.c.c.[6]. Singer *et al.*[7], using higher concentrations of propionate (up to 110 mM) in bladders of varying *in vitro* age, reported that an increase in the s.c.c. followed addition of propionate to the serosal bathing medium, whereas a slight decrease in the s.c.c. followed addition to the mucosal bathing medium. Thus both stimulation and inhibition of sodium transport across the toad bladder have been reported to occur following addition of propionate under varying experimental conditions.

A series of investigations was undertaken in order to further study these dual effects of propionate and, in particular, to elucidate the mechanism underlying the inhibition of sodium transport by propionate. Evidence is presented that propionate when added simultaneously to the serosal and mucosal bathing media affects sodium transport at least in part through mechanisms which depend on its metabolism and which affect the supply of energy to the sodium pump in the epithelial cells.

METHODS

Toads (*Bufo marinus*) were obtained from Tarpon Zoo, Tarpon Springs, Florida and originated from Colombia, South America. They were kept on moist dirt or sawdust, were not fed, and were sacrificed several days to several weeks after arrival in the laboratory.

In these studies no attempt was made to suppress the endogenous aldosterone secretion of the experimental animals prior to their sacrifice, nor was aldosterone added to the media bathing the isolated bladders. We infer that the rate of sodium transport in freshly-isolated bladders from Colombian toads is under the influence of endogenous aldosterone since freshly-isolated bladders from Colombian toads fail to respond to exogenous aldosterone, whereas after overnight incubation stimulation by aldosterone can be regularly demonstrated[8].

Measurement of the s.c.c.

Sodium transport was measured by means of the s.c.c. technique of Ussing and Zerahn[9]. After pithing the toad, each hemibladder was immediately mounted as a diaphragm between a pair of lucite or glass chambers; an equal volume of Ringer's solution (10–20 ml) was added to each chamber; air was bubbled through the solutions in the chambers throughout the course of the experiments. Measurements of the s.c.c. were carried out within a period of 6 h of mounting.

After an initial period of equilibration lasting 2–3 h, compounds to be tested were added simultaneously to the media bathing both the serosal and mucosal surfaces of one of each pair of hemibladders, while an equivalent volume of Ringer's solution was added to the media bathing the control hemibladders.

A quantitative index of the response to addition of a compound was obtained

by dividing the value of the s.c.c. recorded at time t after addition ($s.c.c._t$) by the value recorded just before addition ($s.c.c._0$) and factoring it by the equivalent values for the control hemibladder:

$$\frac{s.c.c._t}{s.c.c._0}\text{ (experimental)} \Big/ \frac{s.c.c._t}{s.c.c._0}\text{ (control)}$$

Results in the text and tables are expressed as the mean percentage deviation of this value from unity $\pm$ S.E. for n numbers of pairs studied; stimulation of the s.c.c. of the experimental hemibladders compared to the controls is indicated by + and inhibition by −. Values for P were calculated throughout using Student's t test, with 0.05 or less considered to be significant. Where the s.c.c. is expressed in μA, this is for an exposed area of 2.5 cm^2.

Measurement of oxygen consumption and CO_2 production

Oxygen consumption of isolated toad bladder tissue was measured by the Warburg technique as previously described[10]. Simultaneous measurements of the s.c.c. and of CO_2 production in isolated bladders were made using a conductometric technique based on that described by Maffly[11].

Measurement of conversion of ^{14}C-labelled substrates to $^{14}CO_2$

Hemibladders were washed in Ringer's solution, cut into a number of pieces equal to the number of metabolic flasks and gently blotted. One piece of each hemibladder was placed in each of 6–12 flasks containing several ml of Ringer's solution (with penicillin 0.1 mg/ml, streptomycin 0.1 mg/ml and polymixin B sulfate 0.01 mg/ml). Compounds to be tested were added, and the flasks were capped with rubber serum caps each of which held an empty plastic cup (Kontes Glass Company, Vineland, N.J.). After 1 h of shaking, 1 ml of 2 M HCl was added to the flasks and 0.2 ml hyamine hydroxide (Packard Instrument Company, Downers Grove, Ill.) was added to the plastic cups by hypodermic needle through the rubber caps. Following 90 min of further shaking the flasks were opened; the plastic cups were cut off and dropped into counting vials containing 20 ml Bray's solution[12], and radioactivity was counted in a liquid scintillation counter (Packard Instrument Company). Non-tissue blanks were run in all experiments and the blank counts, ranging between 1–5% ([U-^{14}C]pyruvate) and 10–35% ([1-^{14}C]propionate) of the counts from the experimental flasks, were subtracted from the latter. Results are expressed as μmoles of substrate converted to CO_2/g dry weight of tissue per h, assuming complete oxidation of the molecule contributing $^{14}CO_2$.

Solutions and chemicals

Most experiments were carried out using a phosphate-buffered Ringer's solution at pH 6.4 (Na^+, 111.0; K^+, 4.0; Ca^{2+}, 1.8; Cl^-, 113.0; HPO_4^{2-}, 2.0; and $H_2PO_4^-$, 2.0 mequiv per l; 220 mosM/kg water). In one group of experiments, HCO_3^--buffered Ringer's solution of pH 8.2 was employed (Na^+, 114.0; K^+, 3.4; Ca^{2+}, 5.4; Cl^-, 120.0; HCO_3^-, 2.4 mequiv per l; 228 mosM/kg water).

Substrates and inhibitors were made up in solutions of pH and osmolality corresponding to those of the Ringer's solution used. Adjustments of pH were made by addition of NaOH or HCl. Concentrations of substrates and inhibitors refer to final concentrations in the solution bathing the bladders.

The following compounds were obtained commercially and used without further purification: Sodium succinate and sodium fumarate (Calbiochem, Los Angeles, Calif.); sodium pyruvate and malonic acid (Sigma Chemical Company, St. Louis, Mo.); propionic acid (Eastman Organic Chemicals, Rochester, N.Y., and Matheson Coleman and Bell, Cincinnati, Ohio); sodium salts of [1-^{14}C]propionic acid, [U-^{14}C]pyruvic acid and [U-^{14}C]acetic acid (Amersham/Searle, Des Plaines, Ill.)

RESULTS

The dual effects of propionate on the s.c.c.

(i) Stimulation of the s.c.c. over a period of 1–3 h consistently occurred when 0.1 mM propionate was added to the serosal and mucosal bathing media of freshly-isolated bladders (Fig. 1, Table I).

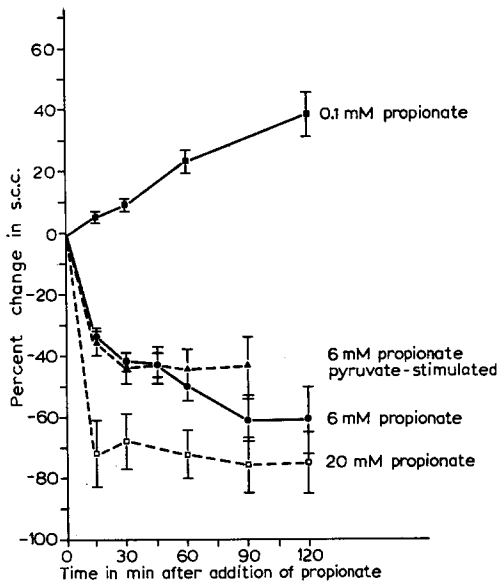

Fig. 1. The dual effects of propionate on the s.c.c. The s.c.c. was measured in paired isolated hemibladders; at time zero propionate was added simultaneously to the serosal and mucosal bathing media of one of each pair of hemibladders. The effects of propionate are expressed as the percent change in the s.c.c. of the experimental hemibladders after addition of propionate, relative to the spontaneous change in the s.c.c. of the paired control hemibladders (for details of calculation see Methods). In the "pyruvate-stimulated" preparations the s.c.c. of the paired hemibladders had been previously stimulated by the addition of 6 mM pyruvate. The direction and magnitude of the response varied with the concentration of propionate.

(ii) In contrast, rapid and marked inhibition of the s.c.c. followed addition of 6–20 mM propionate to the bathing media (Fig. 1, Table I). An example of this inhibitory effect is seen in Fig. 2a. At pH 6.4 mean inhibition at 30 min by 6 mM propionate was $42\pm3\%$ ($n=25$, $P<0.001$); at pH 8.2 inhibition was qualitatively similar but less marked (at 30 min $23\pm5\%$, $n=6$, $P<0.01$). In some instances the period of inhibition with 6 mM propionate was followed by a variable recovery of the s.c.c. over 1–3 h. Persistent and more marked inhibition occurred following addition of higher concentrations of propionate.

TABLE I

THE DUAL EFFECTS OF PROPIONATE ON THE s.c.c.

For details of the study and method of calculation, see legend to Fig. 1; n.s., not significant.

Propionate (mM)	*Bladder preparation*	*n*	*% change in s.c.c. after addition of propionate*					
			15 min	*30 min*	*45 min*	*60 min*	*90 min*	*120 min*
0.1	pH 6.4	7	+ 5 ± 2 n.s.	+ 9 ± 2 n.s.	—	+23 ± 4 $P<0.01$	—	+38 ± 7 $P<0.01$
6	pH 8.2	6	−13 ± 2 $P<0.01$	−23 ± 5 $P<0.01$	−15 ± 5 $P<0.05$	− 8 ± 6 n.s.	—	—
6	pH 6.4	25	−34 ± 3 $P<0.001$	−42 ± 3 $P<0.001$	−43 ± 4 $P<0.001$	−50 ± 5 $P<0.001$ (n = 19)	−61 ± 7 $P<0.001$ (n = 6)	−61 ± 11 $P<0.02$ (n = 4)
6	pH 6.4 pyruvate-stimulated	10	−36 ± 4 $P<0.001$	−44 ± 5 $P<0.001$	−43 ± 6 $P<0.001$	−45 ± 7 $P<0.001$	−43 ± 9 $P<0.01$ (n = 5)	—
6	pH 6.4 glucose-stimulated	6	−40 ± 6 $P<0.01$	−46 ± 6 $P<0.001$	−49 ± 6 $P<0.001$	−51 ± 6 $P<0.001$	—	—
20	pH 6.4	4	−72 ± 11 $P<0.01$	−68 ± 9 $P<0.01$	—	−72 ± 8 $P<0.01$	−76 ± 9 $P<0.01$	−75 ± 10 $P<0.01$

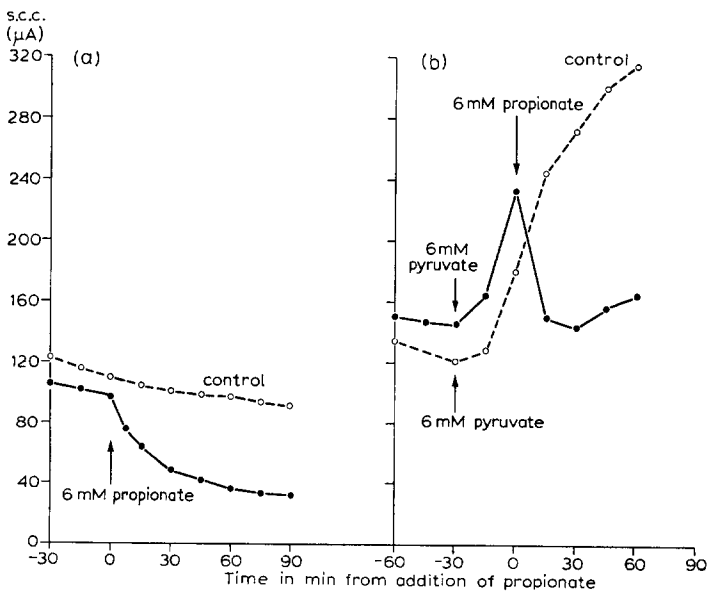

Fig. 2. Examples of the effect of 6 mM propionate on the s.c.c. (pH 6.4). At time zero 6 mM propionate (●—●) was added to both bathing media of one of a pair of freshly-isolated hemibladders. In (a) no substrate was present prior to time zero; in (b) the s.c.c. of both hemibladders had been stimulated by the addition of 6 mM pyruvate at time −30 min.

Striking inhibition was also consistently observed following addition of 6 mM propionate to bladders in which the s.c.c. had been previously stimulated by addition of 6 mM pyruvate or 10 mM glucose (Fig. 2b, Table I). Following inhibition of the s.c.c. by 6 mM propionate, addition of 6 mM pyruvate resulted in the usual brisk stimulation of the s.c.c.[1].

The interrelationships of the effects of malonate and propionate on the s.c.c.

Propionate is known to be metabolized by animal tissues, the main pathway involving its carboxylation and subsequent conversion to succinate[13]. In order to determine whether the dual effects of propionate on the s.c.c. are dependent on the metabolism of propionate *via* its conversion to succinate, the interrelationships of the effects of propionate and malonate—a specific inhibitor of succinate dehydrogenase (EC 1.3.99.1)—were examined.

TABLE II

THE EFFECT OF MALONATE ON THE s.c.c. (pH 6.4)

The s.c.c. was measured in paired isolated hemibladders; at time zero malonate was added simultaneously to the serosal and mucosal bathing media of one of each pair of hemibladders. Results are expressed as described in the legend to Fig. 1.

Malonate (mM)	*n*	*% change in s.c.c. after addition of malonate*		
		30 min	*60 min*	*90 min*
2	11	-14 ± 5 $P<0.05$	-28 ± 7 $P<0.01$	—
6	6	-16 ± 2 $P<0.01$	-36 ± 6 $P<0.01$	-44 ± 8 $P<0.01$

The effect of malonate on the s.c.c. (Table II). Inhibition of the s.c.c. was consistently observed over the period of study when 2–6 mM malonate was added to the serosal and mucosal bathing media.

The effect of malonate on the s.c.c. in propionate-treated bladders (Fig. 3). 6 mM propionate was added to one of each of 8 pairs of hemibladders. Following the expected inhibition of the s.c.c., 2 mM malonate was added to the bathing media of all bladders. While inhibition by malonate was seen in all the propionate-free bladders, it was absent or greatly reduced in all the propionate-treated bladders; as a result, 60 min after addition of malonate the mean s.c.c. of the propionate-treated bladders was significantly higher than that of their pairs. These results suggest that addition of propionate leads to an increase in the intracellular concentration of succinate which is sufficient to at least partially overcome the inhibitory action of malonate.

The effect of propionate on the s.c.c. in malonate-treated bladders. Edelman and Fimognari[14] reported that stimulation of the s.c.c. by propionate in overnight bladders was blocked in the presence of malonate (10 mM). In our studies similar results were obtained with freshly-isolated bladders when a low concentration of propionate was employed: 2 mM malonate was added to the bathing media of one

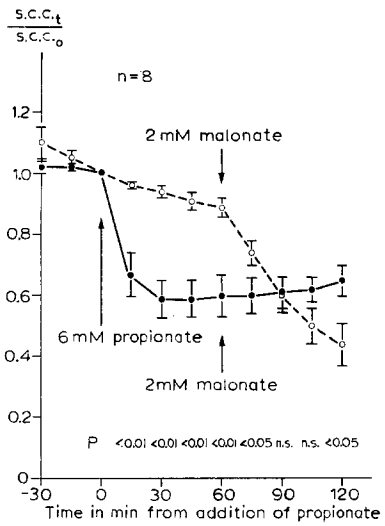

Fig. 3. The effect of malonate on the s.c.c. in propionate-treated bladders. At time zero 6 mM propionate (●—●) was added to the bathing media of one of each pair of hemibladders; 60 min later 2 mM malonate was added to the bathing media of all hemibladders. The effects are expressed as the mean values of the ratio ($s.c.c._t/s.c.c._0$) plotted for the experimental and control (○--○) hemibladders; *P* values were calculated for the mean differences between paired hemibladders. In the propionate-treated hemibladders the inhibitory effect of malonate was absent or greatly reduced.

of each pair of 6 hemibladders and 75 min later 0.6 mM propionate was added to the bathing media of all hemibladders; the stimulatory effect of this concentration of propionate was absent or reduced in all the malonate-treated bladders—45 min after addition of propionate the mean difference between the s.c.c. of the malonate-free and malonate-treated bladders (relative to the value at the time propionate was added) was $+17\pm5\%$ ($P<0.05$). These results provide evidence that the stimulatory effect of a low concentration of propionate is indeed dependent on the conversion of propionate to succinate and the subsequent oxidation of succinate.

A series of experiments was carried out to determine whether the inhibitory effect of higher concentrations of propionate is also affected by malonate. 2 mM malonate was added to both bathing media of one of each pair of freshly-isolated bladders. 75 min later, during the expected inhibition of the s.c.c., 6 mM propionate was added to the bathing media of all hemibladders. There followed a definite depression of the s.c.c. in the malonate-free tissue but there was a lesser effect in the malonate-treated bladders (Fig. 4): 60 min after addition of propionate, the s.c.c. of the former had declined by a mean value of $41\pm11\,\mu A$, the latter by $14\pm4\,\mu A$ (difference $=27\pm9\,\mu A$, $n=11$, $P<0.02$). Even relative to the values of the s.c.c. at the time propionate was added, propionate caused a greater inhibition when malonate was absent (35%) than when it was present (22%, $P<0.05$). Similar results were obtained in experiments with 6 mM malonate although the continuing decline of the s.c.c. due to the malonate inhibition minimized the relative effect: at 60 min the difference between the mean value of the s.c.c. of paired hemibladders was $13\pm5\,\mu A$, $n=6$, $P<0.05$. These results support the concept that inhibition of the s.c.c. by

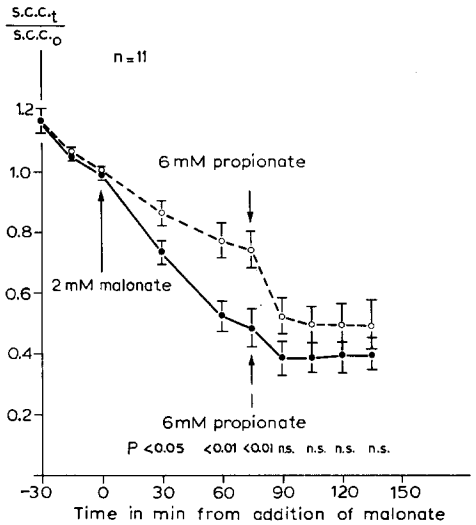

Fig. 4. The effect of 6 mM propionate on the s.c.c. in malonate-treated bladders. At time zero 2 mM malonate (●—●) was added to the bathing media of one of each pair of hemibladders; 75 min later 6 mM propionate was added to the bathing media of all hemibladders. Results are expressed as in Fig. 3. The addition of malonate caused a decline in the s.c.c. which was significantly greater than the spontaneous decline in the s.c.c. of the untreated controls. The inhibitory effect of propionate was greatly reduced in the malonate-treated hemibladders; as a result, following addition of propionate the significant difference between the mean values of the s.c.c. in the paired hemibladders was abolished.

propionate, like stimulation, is dependent upon the conversion of propionate to succinate and the subsequent oxidation of succinate.

The dual effects of succinate on the s.c.c.

The effects of succinate on the s.c.c. were examined as a further test of the possibility that the effects of propionate are dependent on its conversion to, and the subsequent oxidation of, succinate. It has been suggested that the failure to demonstrate an effect of succinate on sodium transport by the toad bladder[1,2,4] may reflect merely a failure of this dicarboxylic acid to penetrate in sufficient concentration into the epithelial cells at an unfavorable pH (pH 7.2–8.2)[1,15]. Therefore the effects of various concentrations of succinate were tested at pH 6.4 in an attempt to determine whether the dual effects of propionate can be reproduced under appropriate conditions by the addition of succinate.

(i) Stimulation of the s.c.c. consistently occurred when succinate was added to the bathing media at a concentration of 4 mM: mean stimulation at 45 min was $32\pm12\%$ ($n=6$, $P<0.05$). This stimulatory effect of succinate will be reported in detail elsewhere.

(ii) In contrast, 20 mM succinate had variable effects on the s.c.c. Overall in 13 experiments there was a mean increase in the s.c.c. of $20\pm8\%$ ($P<0.05$) 30 min after addition of succinate. However, out of the 13 experiments stimulation alone was seen in 8, whereas in the other 5 there was an initial period of inhibition, followed by variable recovery and stimulation. While the degree of inhibition was less marked,

the time course of the inhibition was similar to that seen after 6 mM propionate. Examples of these 2 types of response are seen in Figs 5a and 5b. When 20 mM succinate was added to bladders in which the s.c.c. had been previously stimulated by addition of 6 mM pyruvate (Fig. 6) slight but definite inhibition of the s.c.c. was present in 5 out of 8 bladders at 30 min (mean effect $-7\pm3\%$, $P<0.05$) and in all 8 bladders at 60 min (mean effect $-13\pm4\%$, $P<0.01$).

In order to test the specificity of the inhibitory effect of 20 mM succinate, and

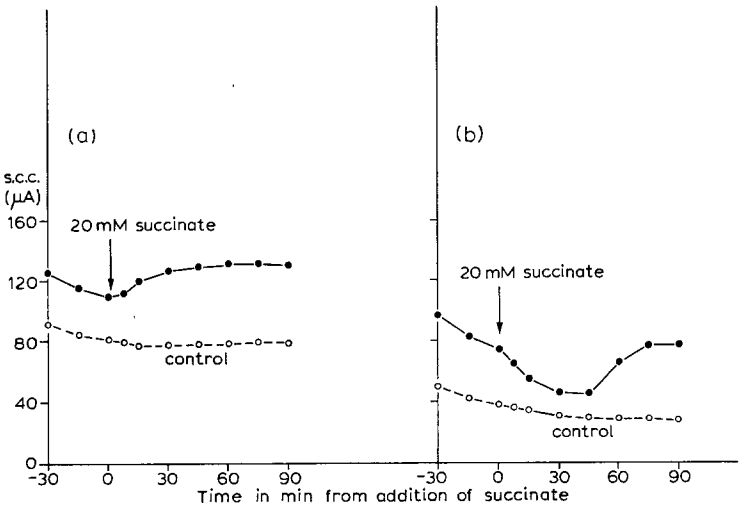

Fig. 5. The dual effects of 20 mM succinate on the s.c.c.; examples of (a) stimulation and (b) inhibition. In each experiment at time zero 20 mM succinate (●—●) was added to the serosal and mucosal bathing media of one of a pair of freshly-isolated hemibladders.

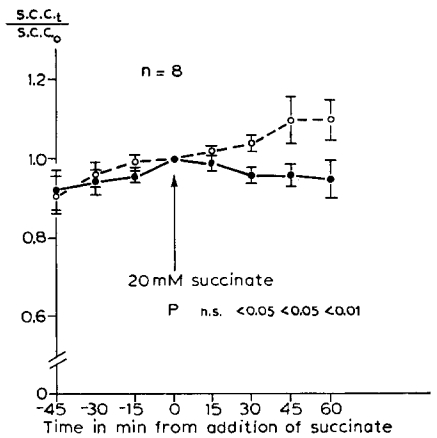

Fig. 6. The effect of 20 mM succinate on the s.c.c. in pyruvate-stimulated bladders. 6 mM pyruvate was added to the bathing media of all hemibladders 2 h prior to the addition of succinate. At time zero 20 mM succinate (●—●) was added to the serosal and mucosal bathing media of one of each pair of hemibladders. Results are expressed as in Fig. 3. Addition of succinate resulted in a significant decline in the mean value of the s.c.c.

to exclude the possibility that substitution of a high concentration of another anion for Cl^- might itself depress sodium transport, the effects of addition of the same concentration of 3 other tricarboxylic acid cycle anions were examined. In a total of 25 experiments with α-ketoglutarate ($n=11$), fumarate ($n=7$) and malate ($n=7$), no inhibition of the s.c.c. was seen; rather, a mild but inconsistent stimulation of the s.c.c. followed addition of these substrates (Taylor, A., Hess, J. J. and Maffly, R. H., unpublished).

Effects on oxygen consumption

Oxygen consumption of isolated toad bladder was measured for periods of 1 h before and after addition of propionate and malonate: (i) Addition of 6 mM propionate increased oxygen consumption over the ensuing 60 min by $10.4 \pm 2.9\%$ ($n=13$, $P<0.01$) compared to control tissue. (ii) 5 mM malonate added alone reduced oxygen consumption by $34.1 \pm 3.1\%$ ($n=10$, $P<0.001$). When added in the presence of 6 mM propionate, however, 5 mM malonate had minimal effect ($-7.7 \pm 4.4\%$, $n=14$, not significant).

Effects on CO_2 production

Simultaneous measurements were made of the effect of propionate on the s.c.c. and on the rate of CO_2 production. (The conductometric method for estimation of CO_2 production[11] has been recently modified in our laboratory by Dr Roderic E. Steele who kindly made the present measurements). Following 1 or more h of baseline control measurements, 6 mM propionate was added to the mucosal and serosal bathing media and measurements were continued for 1 h. In the absence of paired control hemibladders, the effect of propionate was taken as the difference between the observed values and the expected control values obtained by extrapolation of the baseline periods. In 8 experiments, over 60 min propionate induced a mean change in the s.c.c. of $-32.4 \pm 3.5\%$ ($P<0.001$) while the mean change in the rate of CO_2 production was $-4.1 \pm 3.4\%$ (not significant).

Production of $^{14}CO_2$ from ^{14}C-labelled propionate

In order to estimate the rate at which propionate is oxidized by the toad bladder, experiments were carried out with ^{14}C-labelled propionate. Isolated tissue was exposed to [1-^{14}C]propionate with a total concentration of propionate of 5 mM, and $^{14}CO_2$ was collected for 1 h as described in Methods. As shown in Table III, $^{14}CO_2$ was produced from propionate at a substantial rate. When 5 mM malonate was also present, $^{14}CO_2$ production from propionate was reduced by 25%.

At higher concentrations of propionate (10–20 mM) inhibition of conversion of [1-^{14}C]propionate to $^{14}CO_2$ was observed. In 6 paired experiments, CO_2 production from 20 mM propionate (calculated as μmoles/g dry wt per h) was $41 \pm 6\%$ less than from 1 mM propionate ($P<0.001$).

Effect of propionate on $^{14}CO_2$ production from ^{14}C-labelled pyruvate and acetate

Production of $^{14}CO_2$ from [U-^{14}C]pyruvate by isolated bladders was substantially decreased (26% over 1 h) in the presence of propionate. $^{14}CO_2$ production from [U-^{14}C]acetate was profoundly inhibited by propionate (85% over 1 h) (Table III).

TABLE III

CONVERSION OF ^{14}C-LABELLED SUBSTRATES TO $^{14}CO_2$ BY ISOLATED TOAD BLADDER TISSUE

Compound	*Concentration (mM)*	*n*	*Other compounds present*	*μmoles/g dry wt per h*	*% effect (paired experiments)*
[1-^{14}C]Propionate	5	9	None Malonate (5 mM)	6.27 ± 0.23 4.71 ± 0.20	−25 ± 3
[U-^{14}C]Pyruvate	5	8	None Propionate (5 mM)	15.78 ± 0.79 11.68 ± 0.48	−26 ± 3
[U-^{14}C]Acetate	5	6	None Propionate (5 mM)	2.76 ± 0.09 0.41 ± 0.02	−85 ± 1

DISCUSSION

Kaziro and Ochoa[13] have shown that in animal tissues the main pathway of propionate oxidation involves conversion of propionate, *via* propionyl-CoA and methylmalonyl-CoA, to succinyl-CoA. The net reaction is:

$$\text{propionate} + \text{CoA} + CO_2 + 2\ \text{ATP} \leftrightarrow \text{succinyl-CoA} + \text{AMP} + \text{ADP} + 3\ P_i \qquad (1)$$

Edelman and co-workers[4,14] employed propionate as a presumed precursor of succinate in the toad bladder because of failure to demonstrate an effect of exogenous succinate on the s.c.c.[1], a failure attributed to cellular impermeability to succinate. Propionate, because of its solubility in lipids and its single carboxyl group, is likely to penetrate all cell membranes more readily than succinate. Chappell and Haarhoff[16] showed that rat liver mitochondria are highly permeable to propionate, whereas permeability to succinate and other dicarboxylic acids could be demonstrated only under certain experimental conditions. In the toad bladder, Rosen *et al.*[17] found that the transepithelial permeability of non-ionized propionic acid is indeed relatively great. Thus the addition of 6–20 mM propionate to the media bathing the isolated toad bladder may be expected to result in propionate being present within the epithelial cells in relatively high concentration; the extent of the subsequent increase in the intracellular concentration of succinate will presumably be in part dependent on the intracellular concentration of propionate and on the availability of CoA and ATP.

Our studies provide substantial evidence that exogenous propionate is actively oxidized by the toad bladder, and *via* conversion to succinate: propionate stimulates oxygen consumption and is oxidized to CO_2; malonate inhibits CO_2 production from propionate; and propionate inhibits the effect of malonate on oxygen consumption.

Stimulation of sodium transport by propionate

A number of metabolic intermediates stimulate the s.c.c. when supplied to the toad bladder *in vitro*: glucose, pyruvate, lactate, β-hydroxybutyrate[1]; oxaloacetate[2];

acetoacetate[3]; succinate, α-ketoglutarate and acetate (Taylor, A., Hess, J. J. and Maffly, R. H., unpublished). Characteristically, the stimulatory response following addition of substrates to freshly-isolated[1] and overnight (aldosterone-stimulated)[18] bladders is leisurely, curving toward a maximum at 40–60 min and remaining at the elevated level for several or more hours. Where direct comparisons have been made, the rise in s.c.c. following addition of substrate has been shown to be equal to the rise in net transport of Na^+ measured isotopically[15]. *A priori* it seems reasonable to ascribe the effects on the s.c.c. of addition of metabolic intermediates to the consequences of the metabolism of those intermediates. Consistent with this inference is the observation that the rate of $^{14}CO_2$ production from exogenous substrates is greater in the presence of sodium transport than in its absence[19], indicating coupling between metabolism and transport. Although stimulation of the s.c.c. following addition of substrates could be due to an effect on metabolic processes other than those involved in energy production, for example protein or lipid synthesis, it seems most probable that the stimulation is a direct result of an increase in the supply of energy to the sodium pump. Falchuk and Sharp[5] concluded that stimulation of the s.c.c. by 10 mM propionate in aldosterone-treated bladders was the result of such an action. Since in our studies the stimulation of the s.c.c. by 0.1–0.6 mM propionate was similar qualitatively and quantitatively to that seen after addition of other metabolic substrates and it was blocked by malonate, we also conclude that the stimulation was the result of an increase in energy supply consequent to the metabolism of propionate.

Recently Singer *et al.*[7,20] concluded that the striking stimulation of the s.c.c. that they observed upon adding high concentrations (up to 110 mM) of propionate to the serosal bathing medium was not due to the metabolism of propionate. However the observed response to these high concentrations of propionate, as well as to several other anions tested, differed from the typical response of the toad bladder to known metabolic substrates (*vide supra*), as well as to propionate at lower concentration[5]: within several minutes of addition of the anion the s.c.c. began to rise abruptly, continued a steep rise to a maximum response at 20 min and thereafter sometimes declined sharply. Furthermore a reduced response was observed when the pH of the serosal bathing medium was lowered from 8.0 to 7.0; this finding is in direct contrast to our results with other substrates (Taylor, A., Hess, J. J. and Maffly, R. H., unpublished). It therefore seems likely that, as Singer *et al.*[7,20] concluded, they were studying a phenomenon different from that characteristically associated with metabolic stimulation of sodium transport in the toad bladder. We attribute the differences between our results and these authors' to the large differences in concentrations of propionate employed as well as to the dissimilarities in the pH of the bathing media, although it is possible that contributory factors included differences in the mode of addition of propionate (bilateral *versus* unilateral) and in the geographic origin of the toads (Colombia *versus* Dominican Republic[21]).

Inhibition of sodium transport by propionate

Propionate has been reported to inhibit a variety of metabolic functions in several tissues[22–27]; while the mechanisms involved have not been fully elucidated, inhibition has been attributed to various consequences of the metabolism of propionate. In the light of the evidence that exogenous propionate is metabolized by

the toad bladder, it seems reasonable to suspect that inhibition of the s.c.c. by propionate is also a consequence of its metabolism. Several mechanisms merit consideration.

Possible effect of propionate on the concentration of free CoA and CoA esters. In the conversion of exogenous propionate to succinate, tissue concentrations of free CoA may decrease as propionate is activated and converted first to propionyl-CoA and then to methylmalonyl-CoA and succinyl-CoA. Since the availability of free CoA in the mitochondria may be rate-limiting for both pyruvate and fatty acid oxidation[28], sequestration of CoA in the form of CoA esters might be responsible for a reduction in the rate of substrate oxidation[26,27]. Measurements of CoA concentrations by Weidemann and Krebs[27] in slices of rat kidney cortex indeed showed that on the addition of 5 mM propionate the concentration of free CoA decreased 5- to 10-fold; the authors considered that a consequent decrease in the rate of formation of acetyl-CoA accounted for the inhibition of respiration and gluconeogenesis observed. Evidence has also been obtained in other systems that propionyl-CoA, methylmalonyl-CoA and succinyl-CoA can themselves act as metabolic inhibitors[29,30]. Our findings that propionate decreased $^{14}CO_2$ production from [^{14}C]pyruvate and [^{14}C]acetate, and that with rising concentrations propionate inhibited its own conversion to CO_2 are consistent with the concept that propionate acts in the toad bladder by affecting the concentration of free CoA or CoA esters. However, the failure of propionate to inhibit oxygen consumption or total CO_2 production, the fairly brisk conversion of labelled propionate to $^{14}CO_2$, and the observation that the effect of malonate on the s.c.c. is antagonistic with that of propionate (whereas malonate should if anything increase the accumulation of CoA esters) argue against a change in the concentration of CoA or its esters as at least a sole explanation for inhibition of sodium transport by propionate.

Evidence that inhibition by propionate depends on the oxidation of succinate. There is substantial evidence that the striking inhibition of sodium transport seen in our experiments is at least in part a consequence of the conversion of propionate to succinate and the subsequent oxidation of succinate to fumarate: (i) Propionate, while inhibiting the s.c.c., stimulated oxygen consumption and did not inhibit CO_2 production. If propionate reduced the rate of sodium transport by an effect on sodium permeability or on the transport system through a mechanism not involving its own metabolism, a decline in tissue respiration would be expected. Since approximately 40% of the respiratory exchange of the toad bladder is related to sodium transport[31], the observed decrease of sodium transport of 35% induced by propionate should have reduced tissue respiration by about 15%. This was clearly not the case. Rather it can be inferred that the metabolism of the added propionate actually accounted for an increase of oxygen consumption of 25% and of CO_2 production of 15%. (Since CO_2 is utilized in the conversion of propionate to succinate, it is to be expected that the net increase in CO_2 production might be less than the increase in oxygen consumption).

(ii) Malonate reduced inhibition of the s.c.c. by propionate. At the concentrations used (2–6 mM) malonate is considered to be a specific inhibitor of succinate dehydrogenase[32] and inhibition of succinate oxidation by malonate has been demonstrated in the toad bladder by Falchuk and Sharp[5]. It should be noted that malonate did not completely protect the s.c.c. from inhibition by propionate. However, Falchuk

and Sharp[5] found in their studies that oxidation of succinate was not completely blocked by concentrations of malonate as high as 20 mM, and in our metabolic studies $^{14}CO_2$ production from propionate was reduced but not abolished by malonate. Thus the partial effect of malonate on inhibition of sodium transport by propionate mirrored the partial effect of malonate on the metabolism of propionate.

(iii) Succinate itself, at a concentration of 20 mM, was observed to induce inhibition of the s.c.c. with a time course similar to that seen with propionate. Although the inhibition following succinate addition was not as marked as that following propionate, and was not invariable, it appeared to be specific since it did not occur following addition of three other tricarboxylic acid-cycle substrates.

We conclude that these findings taken together are consistent with the concept that a causal relationship exists between the oxidation of propionate – *via* succinate – and inhibition of sodium transport.

Possible effects of propionate and succinate oxidation on the availability of ATP

A possible explanation for the inhibition of sodium transport by exogenous propionate is that the conversion of propionate to succinate leads to an increase in the rate of cellular oxidation of succinate and that this in turn results in reduced availability of energy for sodium transport. According to Krebs and co-workers[33,34] added succinate is oxidized by a variety of tissues in preference to other substrates, both endogenous and exogenous. A special feature of the oxidation of succinate, in contrast to that of other intermediates of the tricarboxylic acid cycle, is that electrons from succinate enter the electron transport chain at coenzyme Q or cytochrome *b* rather than at NAD^+[35]. It is well established that a high rate of succinate oxidation is associated not only with the passage of electrons down the electron transport chain to oxygen but also with an energy-dependent reversal of electron transport and conversion of NAD^+ to NADH[36]. Such specific succinate-linked reduction of NAD^+ has been described following addition of succinate to mitochondria and submitochondrial particles from a variety of tissues[37–41], as well as in the isolated squid axon[42]; the reduction of mitochondrial NAD^+ by succinate is malonate-sensitive[40,43]. The extent of the reduction of NAD^+ by succinate in mitochondria greatly exceeds that achieved through the usual NAD^+-linked substrates (*e.g.* pyruvate)[38,39,43].

Sharp and Leaf[15] (Sharp, G. W. G., personal communication) observed that oxygen consumption of isolated toad bladder mitochondria was selectively stimulated by succinate. Cannessa-Fischer and Davis[44] obtained spectrofluorometric evidence that addition of succinate to the toad bladder increased the tissue level of NADH; these workers noted that the characteristics of the succinate-induced reduction of pyridine nucleotide were similar to those reported for isolated mitochondria from other tissues. Thus it seems not unlikely that addition of exogenous propionate can lead to reversal of electron transport in the toad bladder, with reduction of NAD^+ to NADH.

The oxidation of propionate *via* succinate might interfere with the supply of energy to the sodium pump by (i) decreasing the production and/or (ii) increasing the utilization of ATP: (i) Preferential oxidation of succinate might simply decrease the rate of oxidation of NAD^+-linked substrates[33,34] and thus the overall rate of ATP production. Since only 2 molecules of ATP are formed for each molecule of succinate oxidized to fumarate whereas 3 molecules are formed from the oxidation

of NADH, substitution of succinate oxidation for NADH oxidation would decrease the rate of ATP production without a change in oxygen consumption. Furthermore ATP is utilized in the conversion of propionate to succinate (Eqn 1) and, as a result, in the oxidation of propionate to fumarate the net balance of high energy phosphate bonds is zero. Consequently, preferential oxidation of propionate to fumarate might well result in a decrease in the amount of phosphate-bond energy available for sodium transport.

Alternatively, preferential reduction of NAD^+ by succinate might interfere with oxidative processes specifically associated with the sodium transport system. Such a mechanism would be consistent with the hypothesis developed by Edelman and co-workers[4,14,45] that aldosterone-stimulated transport in the toad bladder is uniquely dependent on the oxidation of NADH and the ATP production linked to it. As an extension of this concept, we have considered the intriguing possibility that the inhibition of sodium transport might be specifically related to a decrease in the rate of formation of ATP at Site I in the respiratory chain resulting from reversal of electron transport at this site. Such a concept would imply some type of specific link between sodium transport and ATP produced at Site I, such as preferential utilization of Site I ATP by the transport mechanism. This possibility is at least compatible with the results of our studies of the effect of propionate on pyruvate oxidation, from which it can be inferred that addition of 6 mM propionate resulted in a 25% decrease over 1 h in the rate of formation of ATP at Site I.

(ii) The reversal of electron transfer and reduction of NAD^+ by succinate is itself an energy-dependent process, requiring the expenditure of one high-energy bond per molecule of NAD^+ reduced[36]. Utilization of energy in this process might be responsible for a reduction in the amount of ATP available for sodium transport.

Possible effect of propionate on gluconeogenesis. Exogenous propionate is known to be readily gluconeogenic in mammalian liver and kidney cortex[27,46,47], and the toad bladder has recently been shown to be capable of gluconeogenesis[48]. Thus stimulation of gluconeogenesis by propionate with resultant utilization of ATP and GTP might contribute to an overall decrease in the supply of energy to the sodium pump.

CONCLUSION

From the evidence presented we conclude that both the stimulatory and the inhibitory effects of propionate on sodium transport across the toad bladder may be consequences of the metabolism of propionate and that both depend, at least in part, on mechanisms affecting the supply of energy to the sodium pump in the epithelial cells. The precise mechanism of the inhibition of sodium transport by propionate remains undetermined; the relative contributions of the possible mechanisms discussed have yet to be evaluated. We consider it likely that the difference between our findings and those of Fimognari *et al.*[4,14]—who observed only stimulation of sodium transport upon addition of propionate to overnight ("substrate-depleted") bladders—is attributable to the difference in the *in vitro* age of the bladders employed. In experiments now in progress, we are therefore examining the dependence of the response to propionate on the metabolic state of the bladder. We are also evaluating the effects on sodium transport of separate serosal and mucosal addition of pro-

pionate; our results indicate that, under appropriate circumstances, separate serosal and mucosal addition of propionate can both stimulate and inhibit sodium transport (Hess, J. J., Taylor, A. and Maffly, R. H., unpublished). It is hoped that these various studies will help to clarify the mode of action of propionate in the toad bladder and, in particular, the apparent paradox that an intermediary of energy metabolism can inhibit the major energy-requiring process of the tissue.

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