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ON THE EFFECTS OF PROPIONATE AND OTHER SHORT-CHAIN FATTY ACIDS ON SODIUM TRANSPORT BY THE TOAD BLADDER

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SUMMARY

1. Propionate and other unbranched short-chain fatty acids, butyrate, pentanoate, hexanoate and octanoate were found to both stimulate and inhibit active sodium transport by the toad bladder, as measured by the short-circuit current (s.c.c.).

2. Stimulation alone followed addition of low concentrations of fatty acids (0.1–1.0 mM) to either the serosal or mucosal bathing medium; stimulation was also seen after an initial period of inhibition in response to higher concentrations (approx. 5 mM) of some compounds.

3. Inhibition alone followed addition of high concentrations (5–20 mM) of these compounds. The duration and magnitude of the inhibition varied with increasing concentration and chain length of the fatty acid, and was greater following mucosal addition than serosal addition.

4. The inhibitory effect of mucosal propionate increased with decreasing pH of the mucosal bathing medium.

5. Inhibition by the fatty acids was completely reversed upon removing the compound from the bathing medium, and stimulation characteristically followed.

6. In studies designed to evaluate the role of metabolism of the fatty acids in their mucosal inhibitory effects it was found that ^{14}C-labelled propionate, when added to the mucosal surface of the bladder, was converted to $^{14}CO_2$, and mucosal succinate and α-oxoglutaric acid at 20 mM inhibited the s.c.c. slightly. However, malonate did not interfere with inhibition by mucosal propionate and two non-metabolizable acids, dimethylpropionate and benzoate, induced inhibition (and no stimulation) of the s.c.c.

7. In the presence of an inhibitory concentration of fatty acid, the ability of the bladder to respond to added pyruvate was reduced in proportion to the reduction in the level of the s.c.c., whereas the natriferic response to vasopressin was largely intact.

8. We conclude that stimulation of sodium transport by propionate and other short-chain fatty acids is due to metabolism of the compounds and provision of energy to the sodium transport mechanism. The basis of the inhibition appears complex. It may in part depend on metabolism of the fatty acids and/or uncoupling of oxidative

phosphorylation, with resultant reduction in net ATP production for the sodium transport mechanism. However, the inhibition may also be caused in part by a direct effect on the mucosal entry of sodium into the transporting epithelial cells.

INTRODUCTION

Propionate has been shown to have variable effects on active sodium transport across the isolated urinary bladder of the toad [1–4]. In a previous paper from this laboratory [4] it was reported that addition of propionate simultaneously to the media bathing both surfaces of this tissue can, under varying experimental conditions, either stimulate or inhibit sodium transport. In an attempt to further investigate and characterize these effects of propionate, studies were performed in which propionate was added to the serosal and mucosal bladder surfaces separately. In addition, studies were carried out to determine whether the effects of propionate can be duplicated by other short-chain fatty acids.

All the unbranched short-chain fatty acids tested were found to stimulate sodium transport at low concentration and to reversibly inhibit transport at higher concentration, when added to either surface of the bladder; however, mucosal addition of the fatty acids was found to induce a greater degree of inhibition of sodium transport than serosal addition. Possible mechanisms contributing to the dual effects of propionate and other short-chain fatty acids are considered.

METHODS

Sodium transport across isolated hemibladders of the Colombian toad *Bufo marinus* was measured by the classical short-circuit technique of Ussing and Zerahn [5]. Paired freshly isolated hemibladders were employed in most experiments [4]: in a few experiments bladders were incubated overnight in aldosterone- and substrate-free Ringer's solution prior to short-circuiting ("overnight" bladders) [6]. A phosphate-buffered Ringer's solution of pH 6.4 [4] was employed except where noted. The Ringer's solution for experiments at pH 5.2 was prepared by adding HCl isosmotically to the phosphate- Ringer's solution. The experiments at pH 8.2 were performed with a bicarbonate- Ringer's solution [4].

Compounds to be tested were added to the medium bathing either the serosal or the mucosal surface of the experimental hemibladder: an equal volume of Ringer's solution was added to the medium bathing the opposite surface and to both bathing media of the control hemibladder. Compounds were added as sodium salts in solutions of pH and osmolality corresponding to those of the Ringer's solution employed: concentrations of compounds specified in the text refer to the final concentration in the bathing medium. Because of its low aqueous solubility, octanoic acid was made up directly in Ringer's solution: this octanoate Ringer's solution was then substituted for the solution bathing the appropriate surface of the hemibladder. In experiments in which the bathing media were replaced with fresh Ringer's solutions, the serosal and mucosal bathing media of each hemibladder were replaced simultaneously.

A quantitative index of the response to a compound was obtained by dividing the value of the short-circuit current (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:

$$(s.c.c._t/s.c.c._0)_{experimental}$$
$$(s.c.c._t/s.c.c._0)_{control}$$

Results in the text and tables are ordinarily expressed as the percentage deviation of this value from unity (percent change) ± the standard error for n, number 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 for paired hemibladders 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.

Methods employed in determination of the rate of conversion of ^{14}C-labelled compounds to $^{14}CO_2$ have been described previously [4]. In the present studies, [1-^{14}C]propionate was added to one surface of the bladder only. Paired hemibladders were prepared as bags with either the serosal or the mucosal surface innermost. Each bag was filled with 3 ml of Ringer's solution containing 5 mM propionate labelled with [1-^{14}C]propionic acid; it was then tied tightly closed and placed in a metabolic flask containing 25 ml of Ringer's solution. $^{14}CO_2$ production was measured over 1 h. To evaluate the possibility that propionate might diffuse into the external solution and accumulate in sufficient concentration to be metabolized significantly from the outer surface of the hemibladder, the final concentration of propionate in this solution was determined (as the difference between the total ^{14}C counts and the $^{14}CO_2$ counts). This concentration reached 0.07 ± 0.01 mM when the mucosal surface was innermost and 0.02 ± 0.01 mM when the serosal surface was innermost. Experiments previously performed with pieces of bladder tissue incubated with 0.01–0.1 mM propionate indicated that the presence of such low concentrations of propionate in the external solution could not have accounted for the rates of CO_2 production observed in the present studies.

Measurement and calculation of isotopic sodium flux in the presence of mucosal propionate were performed with ^{22}Na using standard methods [7, 8]. Paired, freshly isolated hemibladders were mounted in chambers and short-circuited. An aliquot of ^{22}Na was added to the mucosal bathing medium of one hemibladder and to the serosal bathing medium of its pair. Following an equilibration period of at least 20 min, unidirectional fluxes of sodium were measured for four successive periods of 30 min. Propionate, 20 mM, was then added to the mucosal bath; following a further equilibration period of 30 min, measurements were continued for three more 30-min periods.

The following compounds were obtained commercially and used without further purification: butyric acid, hexanoic acid, octanoic acid, benzoic acid, α-oxoglutaric acid, sodium succinate, sodium fumarate (Calbiochem, Los Angeles, Calif.); propionic acid, 2,2-dimethylpropionic acid (Eastman Organic Chemicals, Rochester, N.Y); sodium pyruvate, malonic acid, pentanoic acid (Sigma Chemical Co., St. Louis, Mo.); [1-^{14}C]propionic acid and [*carboxy*-^{14}C]benzoic acid (Amersham/Searle, Des Plaines, Ill.); 2,2-dimethyl [1-^{14}C]propionic acid (trimethyl [1-^{14}C]acetic acid, Mallinckrodt Chemical Works, St. Louis, Mo.); $^{22}NaCl$ (International Chemical and Nuclear Corporation, Irvine, Calif.); vasopressin (as Pitressin, Parke Davis and Co., Detroit, Mich.).

TABLE I

EFFECTS ON THE S.C.C. OF SHORT-CHAIN FATTY ACIDS WHEN ADDED TO THE SEROSAL BATHING MEDIUM

Compounds were added to the serosal bathing medium of one of each pair of freshly isolated hemibladders bathed in Ringer's solution of pH 6.4. Results are expressed as percent change in s.c.c. of the experimental hemibladders compared to their controls (see Methods). n.s., not significant.

Compound	Concentration	*n*	Percent change in s.c.c. after addition					
			7 min	15 min	30 min	60 min	90 min	120 min
Propionate	0.1 mM	7	− 1±2 n.s.	− 3±4 n.s.	+11±4 n.s.	+24±9 <0.05	+23±11 n.s.	+ 27±15 n.s.
	1.0 mM	6	− 1±2 n.s.	+ 9±3 <0.05	+20±5 <0.01	+30±5 <0.01	+31±7 <0.01	+ 28±12 <0.05
	6 mM	6	− 7±2 <0.02	− 1±4 n.s.	+22±11 n.s.	+49±17 <0.05	+71±21 <0.02	+ 75±21 <0.02
	6 mM*	6	+ 5±3 n.s.	+ 7±5 n.s.	+34±8 <0.01	+78±13 <0.01	—	+128±31 <0.01
	20 mM	8	−18±7 <0.05	−20±9 n.s.	−19±12 n.s.	−12±10 n.s.	−18±9 n.s.	− 17±10 n.s.
Butyrate	6 mM	8	− 8±2 <0.01	− 4±1 <0.01	+10±11 n.s.	+64±13 <0.01	+72±12 =0.001	+ 66±9 <0.001
Pentanoate	0.3 mM	5	+ 1±2 n.s.	+ 7±4 n.s.	+19±5 <0.02	+35±9 <0.02	+37±10 <0.02	+38±9 <0.02
	6 mM	6	−20±7 <0.05	−18±10 n.s.	−12±9 n.s.	−17±6 <0.05	−15±7 n.s.	− 12±8 n.s.
Hexanoate	0.3 mM	6	+ 1±1 n.s.	+ 7±2 <0.02	+24±7 <0.02	+39±10 <0.02	—	+ 56±17 <0.05
	6 mM	6	−15±3 <0.01	−15±4 <0.02	−18±6 <0.05	−31±7 <0.01	−35±9 <0.02	− 36±12 <0.05
Octanoate	0.1 mM	6	—	− 1±3 n.s.	+11±6 n.s.	+26±8 <0.05	—	+ 32±11 <0.05
	5 mM	7	−17±4 <0.01	−17±6 <0.05	−28±8 <0.02	−56±10 <0.01	−70±11 <0.001	− 72±15 <0.01

* "Overnight" bladder preparations.

TABLE II

EFFECTS ON THE S.C.C. OF SHORT-CHAIN FATTY ACIDS WHEN ADDED TO THE MUCOSAL BATHING MEDIUM

Compounds were added to the mucosal bathing medium of one of each pair of freshly isolated hemibladders bathed in Ringer's solution of pH 6.4. Results are expressed as described in Table I. n.s., not significant.

Compound	Concentration	*n*	Percent change in s.c.c. after addition						
			7 min	15 min	30 min	45 min	60 min	90 min	120 min
Propionate	0.1 mM	6	−5±2 n.s.	−10±3 <0.05	−15±3 <0.01	−11±4 <0.05	−15±7 n.s.	−11±8 n.s.	−6±8 n.s.
	1 mM	7	−3±3 n.s.	−3±3 n.s.	−5±6 n.s.	−24±12 n.s.	−35±15 n.s.	−50±18 <0.05	−62±19 <0.02
	6 mM	13	−15±2 <0.001	−15±3 <0.001	−15±4 <0.01	−13±5 =0.02	−3±8 n.s.	−17±13 n.s.	−28±17 n.s.
	6 mM*	6		−19±2 <0.001	−21±2 <0.001	−12±4 <0.05	−3±6 n.s.		−38±17 n.s.
	8.4 mM	7	−23±3 <0.001	−30±5 <0.001	−29±6 <0.01	−25±7 <0.02	−23±6 <0.02	−15±7 n.s.	−7±9 n.s.
	20 mM	12	−37±5 <0.001	−49±4 <0.001	−51±4 <0.001		−51±4 <0.001	−51±7 <0.001	−49±9 <0.001
Butyrate	6 mM	7	−31±9 <0.02	−34±8 <0.01	−33±8 <0.01	−33±9 <0.02	−34±11 <0.05		−34±13 <0.05
Pentanoate	0.3 mM	8	−2±2 n.s.	0±4 n.s.	−17±13 n.s.		−42±22 n.s.	−67±30 n.s.	−87±36 <0.05
	6 mM	6	−45±6 <0.001	−58±4 <0.001	−52±6 <0.001		−49±9 <0.01	−39±8 <0.01	−31±10 <0.02
Hexanoate	6 mM	6	−59±8 <0.001	−70±7 <0.001	−74±6 <0.001		−81±6 <0.001	−84±6 <0.001	
Octanoate	0.1 mM	5	−10±5 n.s.	−25±10 n.s.	−46±24 n.s.		−69±26 n.s.	−88±30 <0.05	−112±35 <0.05
	1.25 mM	6			−36±6 <0.01		−55±7 <0.001		
	5 mM	4	−68±7 <0.01	−79±5 <0.001	−88±3 <0.001		−96±1 <0.001	−99±1 <0.001	

* "Overnight" bladder preparations.

RESULTS

Effects of propionate and other short-chain fatty acids on the s.c.c.

Propionate and the other unbranched short-chain fatty acids tested, butyrate, pentanoate, hexanoate and octanoate, were found to both stimulate and inhibit the s.c.c. The response to the fatty acids varied with the concentration and chain length of the individual acids, as well as with the surface of the bladder to which they were added.

Stimulation

Stimulation alone of the s.c.c. followed addition of low concentrations (0.1–1.0 mM) of the acids to either the serosal or the mucosal bathing medium (Tables I and II); Fig. 1a depicts the mild stimulation observed when 0.1 mM propionate was added to either the serosal or the mucosal surface. The time course of the stimulation was similar to that seen after addition of other metabolic substrates [9]. Stimulation was also seen after an initial period of inhibition in response to higher concentrations of the shorter chain length acids (Figs 1b and 1c).

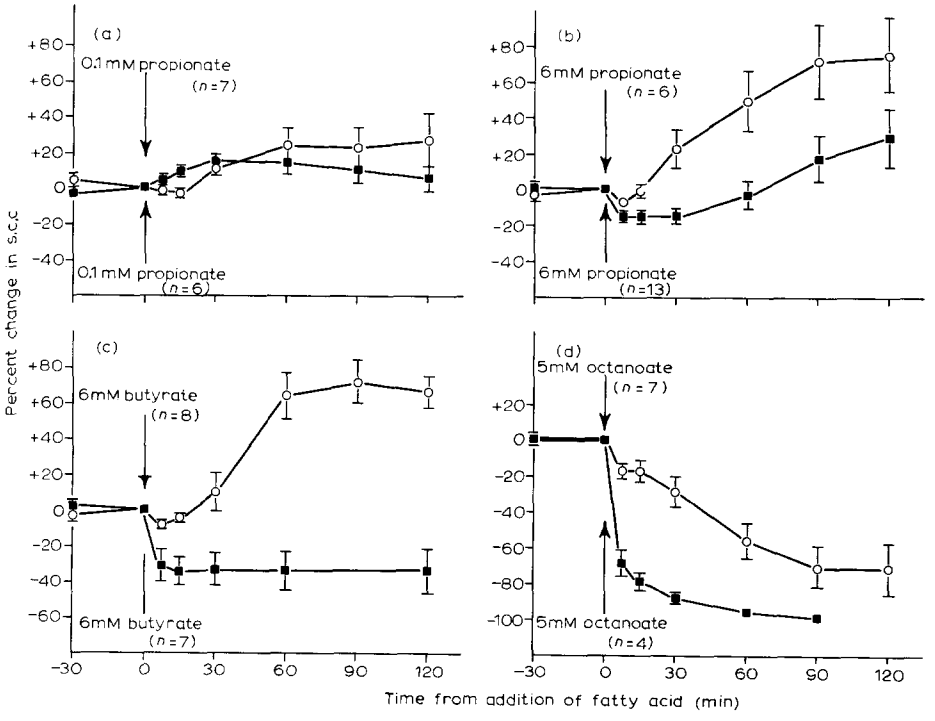

Fig. 1. Comparison of the effects of short-chain fatty acids on the s.c.c. when added to the serosal and the mucosal bathing medium. The four figures depict data from Tables I and II. At time zero, the fatty acid was added to the serosal (○—○) or mucosal (■—■) bathing medium of one of two paired hemibladders: the other hemibladder received an equal volume of Ringer's solution and served as a control. Each curve represents the mean percent change of the experimental hemibladders compared to their controls, as described in Methods.

Inhibition

Inhibition of the s.c.c. was observed when higher concentrations of the fatty acids were employed (Tables I and II, Figs 1b, 1c and 1d). The inhibition was rapid in onset, occurring within 2 min of addition of the test compound and reaching near-maximal values within 15 min. The duration and magnitude of the inhibition varied directly with increasing concentration and with increasing chain length of the fatty acids; at lower concentrations and/or shorter chain lenght, inhibition was short-lived and was followed by stimulation of the s.c.c. (Figs 1b and 1c, serosal addition), whereas at higher concentrations and/or longer chain length inhibition was persistent (Figs 1c, mucosal addition and 1d).

At any concentration, the extent of the inhibition induced by the fatty acids was considerably greater following mucosal addition than following serosal addition: thus, the most marked inhibition observed with the C_3–C_8 series of fatty acids followed mucosal addition of 5 mM octanoate ($-99 \pm 1\%$ at 90 min; Figs 1d and 4). At critical concentrations of the individual fatty acids, mucosal addition caused a persistent inhibition of the s.c.c., while serosal addition resulted in stimulation (e.g. 6 mM butyrate, Fig. 1c). The general observation that the fatty acids have greater inhibitory potency when added to the mucosal surface of the bladder was confirmed in paired experiments in which identical concentrations of fatty acid were added to

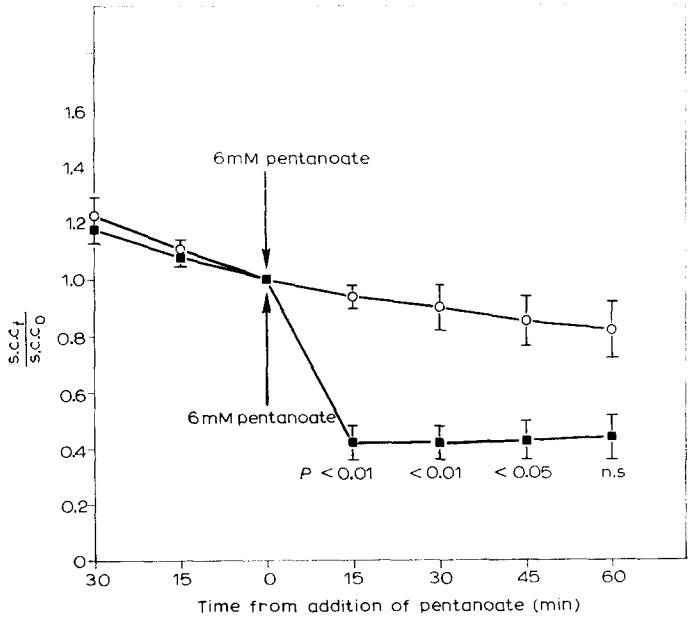

Fig. 2. Comparison of the effect of pentanoate on the s.c.c. when added to the serosal vs the mucosal bathing medium. At time zero, 6 mM pentanoate was added to the serosal (○—○) bathing medium of one of a pair of hemibladders and to the mucosal (■—■) bathing medium of the other hemibladder. The results are expressed as the mean values of the ratio $s.c.c._t/s.c.c._0$ for seven pairs of hemibladders. *P* values were calculated for the mean differences between paired hemibladders.

the serosal and mucosal bathing media, respectively, of paired hemibladders (Fig. 2).*

The serosal and mucosal effects of 6 mM propionate were similar in freshly isolated and overnight bladders (Tables I and II).

Effects of short-chain fatty acids on direct current resistance

In the above series of experiments, intermittent measurements of the transmembrane potential difference were made in addition to measurements of the s.c.c. and values for the direct current resistance were calculated. There was no consistent change in the direct current resistance upon addition of any of the fatty acids, whether they were added to the serosal or to the mucosal bathing media, or whether they stimulated or inhibited the s.c.c.

Effect of mucosal propionate on sodium flux

The s.c.c. has been shown to be an accurate measure of active sodium transport by the toad bladder under a variety of circumstances, including stimulation by metabolic substrates [10]. It seemed important to establish that the inhibition of the s.c.c. observed in the present studies in fact reflected inhibition of active sodium transport. Therefore, a series of experiments was carried out in which direct measurements of unidirectional sodium flux were made in paired hemibladders in the presence of mucosal propionate, using ^{22}Na. In eight hemibladders, for the period from 0.5 to 2 h following mucosal addition of 20 mM propionate, the s.c.c. declined by an average of 67 ± 10 μA and simultaneously the mucosal to serosal sodium flux (expressed in electrical units) dropped by 57 ± 13 μA (difference = 10 ± 7 μA, not significant); meanwhile, in the paired hemibladders, the serosal to mucosal flux did not change significantly (-3 ± 2 μA). These results indicate that inhibition of the s.c.c. by mucosal propionate, and by inference the other short-chain fatty acids, is indeed associated with an equivalent reduction in the rate of active transport of sodium.

pH dependence of the inhibitory effect of mucosal propionate

The inhibition of the s.c.c. induced by mucosal propionate was examined in bladders bathed in Ringer's solution of varying pH (Table III); the inhibitory effect of propionate was found to be strikingly pH dependent. Inhibition was not observed at pH 8.2, but was increasingly marked at pH 6.4 and 5.2. This relationship suggested that un-ionized propionic acid is the effective form of the molecule. When the concentrations in the mucosal bathing medium of the un-ionized and ionized forms of the acid were calculated (based on a pK of 4.87 at 25 °C [11]) and plotted against the percent inhibition of the s.c.c. at 15 min, there was no consistent relationship between the degree of inhibition and the concentration of propionate anion, but there was a striking curvilinear relationship between inhibition and concentration of the un-ionized acid (Fig. 3).

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* We considered the possibility that preferential binding by proteins of serosally added fatty acids might have resulted in a reduced concentration in the bathing medium and hence in a reduced inhibitory effect. However, calculation of the expected degree of binding, based on published data for octanoate binding to albumin [18], showed that this would have had an inconsequential effect on the free fatty acid concentration in the serosal bathing medium. Furthermore, in experiments in which serosal bathing solutions containing propionate were removed and added to the mucosal surface of fresh hemibladders the expected degree of inhibition of the s.c.c. was observed.

TABLE III

EFFECT OF pH ON RESPONSE OF THE S.C.C. TO MUCOSAL PROPIONATE

Propionate was added to the mucosal bathing medium of one of each pair of freshly isolated hemibladders. In the experiments at mucosal pH 5.2, the serosal pH was 6.4; in all other experiments mucosal and serosal pH were identical. Results are expressed as described in Table I. n.s., not significant.

pH	Concentration of propionate	n	Percent change in s.c.c. after addition				
			15 min	30 min	60 min	90 min	120 min
8.2	8.0 mM	14	+ 2 ± 5 n.s.	+ 10 ± 9 n.s.	+ 11 ± 9 n.s.	+ 12 ± 10 n.s.	+ 7 ± 8 n.s.
6.4	5.7 mM	13	− 15 ± 2 < 0.001	− 15 ± 3 < 0.001	− 3 ± 8 n.s.	− 17 ± 13 n.s.	+ 28 ± 17 n.s.
	8.4 mM	7	− 30 ± 5 < 0.001	− 29 ± 6 < 0.01	− 22 ± 6 < 0.05	− 15 ± 7 n.s.	− 7 ± 9 n.s.
	20.0 mM	9	− 49 ± 4 < 0.001	− 51 ± 4 < 0.001	− 51 ± 4 < 0.001	− 51 ± 7 < 0.001	− 49 ± 9 < 0.001
5.2	5.7 mM	6	− 61 ± 6 < 0.001	− 76 ± 3 < 0.001	− 87 ± 2 < 0.001	− 90 ± 3 < 0.001	

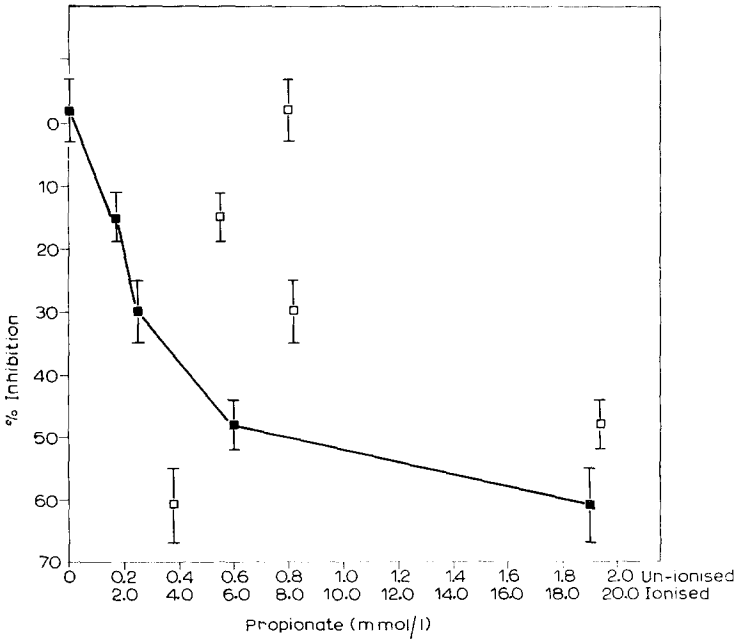

Fig. 3. Inhibition of sodium transport by propionic acid. On the abscissa are plotted the concentrations of the un-ionized (■) and ionized (□) forms of propionate in the mucosal bathing medium. On the ordinate is plotted the percent inhibition of the s.c.c. at 15 min after addition. Each point represents the mean of 6–14 experiments.

TABLE IV

REVERSIBILITY OF THE INHIBITORY EFFECT OF SHORT-CHAIN FATTY ACIDS ON THE S.C.C.

Compounds were added to either the mucosal or the serosal bathing medium of one of each pair of freshly isolated hemibladders bathed in Ringer's solution of pH 6.4. At the indicated time following addition of the compound, bathing media were replaced with fresh Ringer's solution free of the compound. Results are expressed as described in Table I; all values are related to the s.c.c. at the time of addition of the compound. n.s., not significant.

Compound	Concentration	Bathing medium	*n*	Minutes compound was present	Percent change in s.c.c. after removal					
					0 min	15 min	30 min	60 min	90 min	120 min
Propionate	8 mM	Mucosal	7	15	−25±3 <0.001	+ 3±5 n.s.	+16±7 n.s.	+26±8 <0.02	+ 29±9 <0.02	+ 31±10 =0.02
Propionate (mucosal) pH 5.2)	6 mM	Mucosal	6	90	−90±3 <0.001	−79±5 <0.001	−65±11 <0.01	−33±16 n.s.	+ 10±20 n.s.	—
Pentanoate	6 mM	Mucosal	6	120	−31±10 =0.02	—	+62±27 n.s.	+91±25 <0.02	+100±20 <0.01	+105±18 <0.01
	6 mM	Serosal	6	120	−12±8 n.s.	—	+30±12 n.s.	+77±12 <0.01	+111±10 <0.001	+127±15 <0.001
Hexanoate	6 mM	Mucosal	6	90	−87±5 <0.001	—	−35±18 n.s.	+24±16 n.s.	+ 54±12 <0.01	+ 51±20 <0.05
Octanoate	5 mM	Mucosal	4	90	−99±1 <0.001	—	−78±6 =0.001	−24±21 n.s.	—	+ 47±14 <0.05
	5 mM	Serosal	5	150	−73±16 <0.01	−67±21 <0.05	−32±35 n.s.	—	+120±51 n.s.	—
Propionate (8 mM propionate present in serosal medium throughout)	8 mM	Mucosal	7	15	−14±3 <0.01	− 5±4 n.s.	0±6 n.s.	+ 2±7 n.s.	+ 5±5 n.s.	+ 9±6 n.s.

Reversibility of the inhibitory effect of short-chain fatty acids, and "post-washout stimulation"

A series of experiments was carried out to determine if the inhibition of the s.c.c. induced by propionate and other short-chain fatty acids was reversible. At variable intervals of time after the test compound was added to either the serosal or the mucosal medium, the solutions bathing both sides of the bladder were removed and replaced with fresh Ringer's solution free of the compound. In all instances examined, the inhibiton of the s.c.c. induced by the fatty acids was reversible (Table IV, Fig. 4). Indeed, in the period which followed replacement of the Ringer's solution, the s.c.c. of the previously inhibited test hemibladders rose gradually to levels significantly above those of their controls ("post-washout stimulation").

It seemed likely that the stimulation observed after removal of fatty acid from the bathing medium was attributable to the presence in the tissue of a residual stimulatory concentration of the acid. Therefore mucosal-washout studies with propionate were repeated, but with a stimulatory concentration of propionate present in the serosal medium (and thus presumably in the tissue) of both experimental and control hemibladders throughout the course of the experiment: as seen in Table IV, the inhibition due to mucosal propionate was reversed following substitution of propionate-free Ringer's solution in the mucosal bath, but there was no subsequent rise of the s.c.c. of the experimental hemibladders above the level of their controls. The absence of "post-washout stimulation" under these conditions is consistent with the view that this stimulation is due to the presence of a residual stimulatory concentration of fatty acid within the tissue.

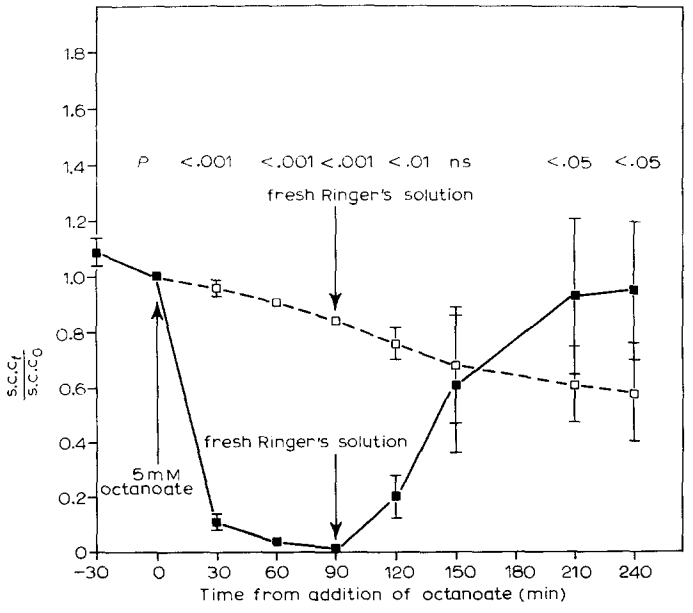

Fig. 4. Reversibility of the inhibitory effect of octanoate. At time zero, 5 mM octanoate was added to the mucosal bathing medium of one of each pair of hemibladders: 90 min later, all solutions were replaced with fresh, octanoate-free Ringer's solutions. The results are expressed as the mean values of the ratio $s.c.c._t/s.c.c._0$ for the experimental (■—■) and control (□ – □) hemibladders (n = 4).

Relationship between effects on sodium transport and metabolism of fatty acids and related compounds

(1) *Production of $^{14}CO_2$ from ^{14}C-labelled propionate added to separate bladder surfaces.* We have previously shown that ^{14}C-labelled propionate, when added to pieces of bladder tissue incubated in metabolic flasks, is converted to $^{14}CO_2$ at a substantial rate [4]. However, in an earlier study [9], substrates were found to be effective in stimulating sodium transport by the toad bladder only when added to the medium bathing the serosal surface of the tissue. In view of the observation that stimulation, as well as inhibition, of sodium transport can follow the addition of propionate and other short-chain fatty acids to either surface of the bladder, a series of experiments was performed in order to determine whether propionate can be metabolized when added to either surface.

Hemibladders were prepared as bags as described in Methods. Comparisons were made of $^{14}CO_2$ production when 5 mM propionate containing a tracer amount of [1-^{14}C]propionate was added to either the serosal or the mucosal bathing medium of paired hemibladders. As seen in Table V, 5 mM propionate was metabolized, whether added to the mucosal or to the serosal bathing medium, although mucosal addition resulted in a lower rate of metabolism than did serosal addition. As detailed in Methods, the rate of CO_2 production upon mucosal addition of propionate could not be accounted for by the accumulation of propionate in the serosal bathing medium and its consequent metabolism from the serosal surface.

(2) *Effects of malonate and tricarboxylic acid cycle intermediates on the s.c.c.* In our previous studies, malonate was found to interfere with the inhibitory effect of combined serosal and mucosal addition of propionate, indicating that the inhibition of sodium transport was dependent, at least in part, on the metabolism of propionate via succinate [4]. Since mucosal addition of propionate (or other fatty acid) leads to a greater degree of inhibition of sodium transport than serosal addition, we sought to assess the role of propionate metabolism in its mucosal effect. Accordingly, the effect of malonate on the response to mucosal propionate was examined. One of each of

TABLE V

$^{14}CO_2$ PRODUCTION FROM [1-^{14}C]PROPIONATE ADDED TO SEPARATE SURFACES OF THE BLADDER

Paired hemibladders were prepared as bags as described in Methods. Propionate was present at a total concentration of 5 mM.

Experiment	μmol/g dry weight per h		Mucosal / Serosal (%)
	Mucosal	Serosal	
A	4.5	7.6	59
B	5.5	5.7	97
C	3.5	6.4	56
D	6.8	10.6	64
E	5.4	11.3	48
F	5.1	10.8	47
G	7.2	13.6	53
Mean	5.4	9.4	61
S.E.	±0.5	±1.0	±6

ten paired hemibladders received 6 mM malonate in the serosal and mucosal bathing media: 60 min later, at which time the s.c.c. of the malonate-treated bladders had fallen by 32 ± 6 % in comparison with the controls, 6 mM propionate was added to the mucosal bathing media of all the hemibladders. There followed a definite inhibition of the s.c.c. in the malonate-free tissue and a somewhat lesser absolute effect in the malonate-treated bladders: 15 min after the addition of propionate, the s.c.c. of the former had declined by a mean value of 19 ± 3 μA, while the s.c.c. of the latter had fallen by 14 ± 2 μA (difference = 5 ± 2 μA, $n = 10$, $p < 0.05$). However, relative to the level of the s.c.c. at the time propionate was added, the percentage decline in the s.c.c. was equivalent in the two groups of hemibladders (−29 ± 3 % in the malonate-free tissue and −28 ± 3 % in the malonate-treated tissue, paired difference 1 ± 2 %, not significant). Thus, in contrast to its effect on the response to combined serosal and mucosal addition of propionate [4], malonate had relatively little effect on the inhibitory response to mucosal propionate.

As a further test of the possibility that the response to mucosal propionate is dependent on its metabolism via succinate, the effects of succinate when added separately to the serosal and mucosal bathing media were investigated. Whereas stimulation of the s.c.c. consistently occurred when 4 or 20 mM succinate was added to the serosal bathing medium at pH 6.4 [6], slight but consistent inhibition followed mucosal addition of 20 mM succinate at this pH (Table VI). However, the inhibitory effect of mucosal succinate did not increase with decreasing pH (Table VI), in contrast to the increased inhibitory effectiveness of propionate at lower pH.

In order to test the specificity of the inhibitory effect of succinate, the effects of the intermediates preceding and following succinate in the tricarboxylic acid cycle were examined. When added to the mucosal bathing medium 20 mM α-oxoglutarate consistently caused a slight inhibition of the s.c.c., which was sustained over a 2-h period: in contrast, 20 mM fumarate had no inhibitory effect but rather caused a minor degree of stimulation (Table VI).

TABLE VI

EFFECTS ON THE S.C.C. OF TRICARBOXYLIC ACID CYCLE INTERMEDIATES WHEN ADDED TO THE MUCOSAL BATHING MEDIUM

Compounds were added to the mucosal bathing medium of one of each pair of freshly isolated hemibladders. In all cases serosal pH was 6.4. Results are expressed as described in Table I. n.s., not significant.

Compound	Concentration	Mucosal pH	n	Percent change in s.c.c. after addition				
				15 min	30 min	60 min	90 min	120 min
α-Oxoglutarate	20 mM	6.4	6	11 ± 1 < 0.001	13 ± 2 < 0.01	12 ± 3 < 0.01	15 ± 3 < 0.01	15 ± 3 < 0.01
Succinate	20 mM	6.4	10	− 7 ± 2 < 0.01	− 8 ± 3 < 0.05	− 3 ± 5 n.s.	− 1 ± 7 n.s.	− 1 ± 7 n.s.
Succinate	20 mM	5.2	6	+ 2 ± 2 n.s.	+ 5 ± 3 n.s.	− 8 ± 5 n.s.		
Fumarate	20 mM	6.4	7	+ 1 ± 3 n.s.	+ 8 ± 5 n.s.	+ 19 ± 8 < 0.05	+ 29 ± 11 < 0.05	+ 29 ± 12 < 0.05

(3) *Studies with dimethylpropionate and benzoate.* In order to further evaluate the role of the metabolism of propionate and other fatty acids in their effects, the response of the s.c.c. to related non-metabolizable acids was examined. Since substituted fatty acids tend to be resistant to oxidation [12], it seemed probable that neither the fatty acid analogue 2,2-dimethyl propionate nor benzoate would be metabolized by the toad bladder. Studies with *carboxy*- ^{14}C-labelled dimethyl propionate and benzoate indeed established that the toad bladder does not convert these compounds to CO_2 in any significant amount (Table VII).

TABLE VII

METABOLISM OF ^{14}C-LABELLED BENZOATE, DIMETHYLPROPIONATE AND PROPIONATE

Labelled compounds were added to pieces of bladder tissue respiring in metabolic flasks as described previously [4]. Low total concentrations of the compounds were employed to maximize specific activity and thus maximize the number of counts obtained.

Compound	*n*	Concentration	μmol/g dry weight per h
[*carboxy*-^{14}C]Benzoate	4	0.003 mM	0.000±0.000
2,2-dimethyl[1-^{14}C]propionate	5	0.01 mM	0.004±0.003
[1-^{14}*C*]Propionate	4	0.01 mM	0.450±0.040

The effects of dimethylpropionate and benzoate on the s.c.c. when added to either the serosal or the mucosal bathing media are shown in Table VIII. In contrast to the stimulatory effect of serosal addition of 6 mM propionate, serosal addition of 6 mM dimethylpropionate caused a striking and persistent inhibition of the s.c.c.: mucosal addition of 6 mM dimethylpropionate resulted in an inhibition of the s.c.c. which was more persistent than that induced by the same concentration of propionate (cf. Table II). Similarly, both serosal and mucosal addition of 6 mM benzoate resulted in a striking inhibition of the s.c.c. Following removal of these compounds from the bathing media, return of the s.c.c. to approximately control levels occurred within 60–90 min: however, in contrast to experiments with the other fatty acids "post-washout stimulation" of the s.c.c. did not occur (Table VIII).

Effect of pyruvate on the s.c.c. in the presence of mucosal propionate or pentanoate

To test the ability of the sodium transport mechanism to respond to the addition of substrate after being inhibited by fatty acids, the response to pyruvate was examined in the presence of an inhibitory concentration of propionate or pentanoate. The results of studies with 6 mM propionate and 6 mM pentanoate were comparable and those with pentanoate are depicted in Fig. 5. The absolute rise in the s.c.c. in response to addition of serosal pyruvate was reduced by approximately one half in hemibladders exposed to 6 mM pentanoate in the mucosal bathing medium (absolute rise in s.c.c., 60 min after addition of pyruvate = 44 ± 17 μA in pentanoate-treated hemibladders and 92 ± 25 μA in control hemibladders, paired difference = 48 ± 16 μA, $n=8$, $p < 0.05$). However, the percentage rise in the s.c.c. relative to the level of the s.c.c. at the time pyruvate was added was equivalent in the fatty acid-treated and control hemibladders (+221 % in pentanoate-treated and +230 % in control hemi-

TABLE VIII

EFFECTS ON THE S.C.C. OF DIMETHYLPROPIONATE AND BENZOATE

6 mM dimethylpropionate or 6 mM benzoate was added to either the mucosal or serosal bathing medium of one of each pair of freshly isolated hemibladders at pH 6.4. At time 120 min, all bathing media were replaced with fresh Ringer's solution free of the compound. Results are expressed as described in Table I. n.s., not significant.

Compound	Bathing medium	*n*	Percent change in s.c.c. after addition						
			15 min	30 min	60 min	120 min	150 min	180 min	240 min
Dimethyl-propionate	Serosal	5	−29 ± 5	−34 ± 5	−44 ± 2	−51 ± 6	28 ± 12	−18 ± 14	−4 ± 21
			<0.01	<0.01	<0.001	<0.001	n.s.	n.s.	n.s.
	Mucosal	6	23 ± 5	23 ± 3	38 ± 3	46 ± 3	35 ± 6	−25 ± 6	15 ± 6
			0.01	<0.001	<0.001	<0.001	<0.01	<0.02	=0.05
Benzoate	Serosal	5	−5 ± 5	−14 ± 9	32 ± 10	−45 ± 9		15 ± 14	4 ± 12
			n.s.	n.s.	<0.05	<0.01		n.s.	n.s.
	Mucosal	6	−19 ± 2	31 ± 4	−61 ± 4	−84 ± 3	75 ± 4	53 ± 4	10 ± 8
			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	n.s.

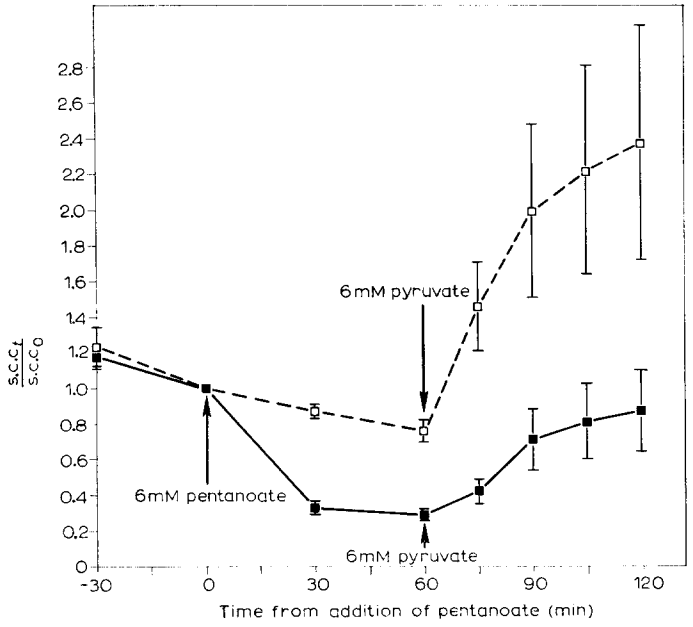

Fig. 5. Effect of pyruvate on the s.c.c. following inhibition by mucosal pentanoate. At time zero, 6 mM pentanoate (■—■) was added to the mucosal bathing medium of one of each pair of hemibladders: 1 h later, 6 mM pyruvate was added to all hemibladders ($n = 6$). The results are expressed as described for Fig. 4.

TABLE IX

EFFECTS OF MUCOSAL PROPIONATE AND OCTANOATE ON THE RESPONSE OF THE S.C.C. TO VASOPRESSIN

All experiments were performed with paired, freshly isolated hemibladders bathed in Ringer's solution of pH 6.4; propionate or octanoate was added to the mucosal bathing medium of one member of each pair and vasopressin 100 munits/ml was added to the serosal medium of all the hemibladders. *P* values refer to differences between the absolute response to vasopressin in paired hemibladders. n.s., not significant.

Compound added	Interval between additions	*n*	s.c.c. at time of addition of fatty acid (μA)	s.c.c. at time of addition of vasopressin (μA)	Maximum s.c.c. in response to vasopressin (μA)	*P*
Propionate, 20 mM	120 min	12	71±10	28±7	+47±6	
Control			76±13	53±7	+39±5	n.s.
Propionate, 20 mM	30 min	8	105±10	60±8	+32±4	
Control			104±13	98±13	+36±7	n.s.
Propionate, 8 mM	0 min	8	85±9	85±9	+35±9	
Control			96±12	96±12	+38±7	n.s.
Octanoate, 1.25 mM	60 min	6	159±27	56±9	+50±4	
Control			156±13	127±12	+55±9	n.s.
Octanoate, 5 mM	60–90 min	10	125±13	7±3	+30±4	
Control			126±14	100±11	+48±6	0.01

bladders, paired difference 9 ± 34 %, n 8, not significant). Thus, in the presence of fatty acid, the ability of the bladder to respond to added pyruvate was reduced in proportion to the reduction in the level of function of the sodium transport mechanism induced by the fatty acid.

Effect of vasopressin on the s.c.c. in the presence of mucosal propionate or octanoate

Experiments were performed to determine the response of the s.c.c. to vasopressin in the presence of an inhibitory concentration of either propionate or octanoate in the mucosal bathing solution. As seen in Table IX, 20 mM propionate produced an approx. 50 % depression of the s.c.c. but had no effect upon the subsequent absolute rise in the s.c.c. in response to vasopressin added either 30 or 120 min later. Since the value of the s.c.c. of the propionate-treated hemibladders was lower than that of the controls at the time of addition of vasopressin, the mean percentage rise in the s.c.c. was in fact much greater in the propionate-treated bladders than in the controls (for example, 319 versus 86 % ($p < 0.02$) when propionate had been present for 120 min). When 8 mM propionate and vasopressin were added simultaneously, inhibition was observed at 2 min only, before the effect of vasopressin became apparent; the subsequent peak response of the s.c.c. to vasopressin was identical, whether propionate was present or not.

When 1.25 mM octanoate had been present in the mucosal bathing medium for 1 h (resulting in 55 % inhibition of the s.c.c. in comparison to controls), subse-

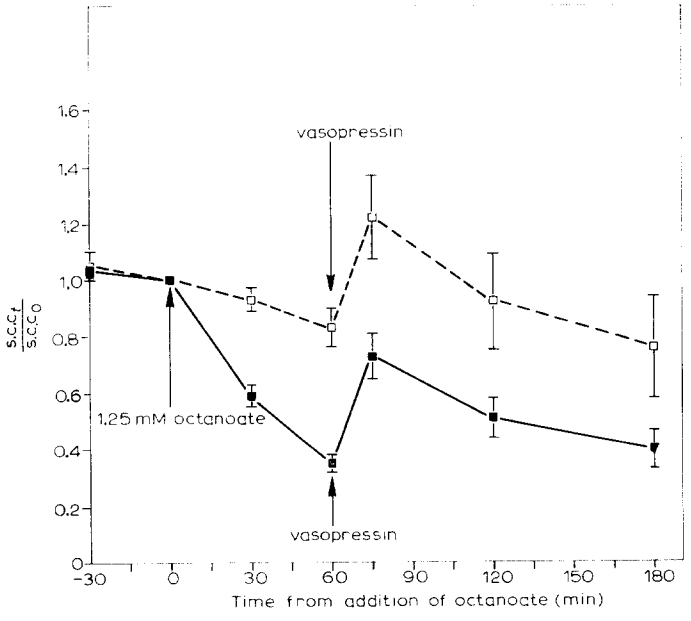

Fig. 6. Effect of vasopressin on the s.c.c. following inhibition by mucosal octanoate. At time zero, 1.25 mM octanoate (■—■) was added to the mucosal bathing medium of one member of each pair of six hemibladders: 60 min later vasopressin 100 munits/ml was added to the serosal bathing medium of all hemibladders. The results are expressed as described for Fig. 4. The time of the peak response to vasopressin (which occurred 9–15 min after addition) is plotted arbitrarily as the 75-min value.

quent addition of vasopressin again resulted in an identical absolute response, but greater relative response, of the s.c.c. (Fig. 6). However, when 5 mM octanoate had been present for 60–90 min (resulting in 95 % inhibition of the s.c.c.) the absolute response to vasopressin was moderately decreased in comparison to controls (Table IX).

DISCUSSION

These studies demonstrate that, in addition to propionate, other short-chain fatty acids can both stimulate and inhibit sodium transport by the isolated toad bladder. The straight chain fatty acids, from propionate to octanoate, stimulate sodium transport at low concentration and reversibly inhibit transport at higher concentration. The duration and magnitude of the inhibition vary directly with the concentration and chain length of the fatty acid and also with the surface of the bladder to which the acid is added, being consistently greater after mucosal than after serosal addition.

Fatty acids have been reported to exert a similar pattern of stimulatory and inhibitory effects, varying with concentration and chain length, in a variety of biological systems. For example, both short- and long-chain fatty acids have been shown to induce biphasic effects on respiration of mammalian and yeast cells [13–16] and on mitochondrial oxygen consumption, ATP-P_i exchange and ATPase activity (in association with uncoupling of oxidative phosphorylation) [15–19]. Short-chain fatty acids have been reported to inhibit phosphate uptake in yeast [14], glucose uptake in muscle [20] and amino acid uptake in bacteria [21] and are also known to act as anaesthetics [22, 23]. These various effects have been attributed to (a) the metabolism of the fatty acids [20, 24] and (b) non-specific interaction of the fatty acids with lipid and/or protein components of cellular membranes and/or enzyme systems [13–19, 21–23].

According to the model of sodium transport proposed by Koefoed-Johnsen and Ussing [25] for the epithelium of frog skin, and applied by Leaf [26] to the toad bladder, sodium enters the epithelial cell passively across its outward-facing (mucosal) membrane and is actively transported out of the cell across its inward-facing (serosal) membrane, through the activity of an energy-dependent sodium pump. Evidence that the sodium entry step is saturatable [27–29], and oxygen dependent [28] suggests that the entry step, like the exit step, involves an active energy-dependent component. Thus, according to current interpretations of the Koefoed-Johnsen and Ussing model [25], the effects of the fatty acids on sodium transport could be secondary to their effects on metabolism and/or be a consequence of a direct action on the sodium entry or exit steps.

Stimulation of sodium transport

The stimulation of the s.c.c. which followed serosal and mucosal addition of low concentrations of propionate and other short-chain fatty acids, or washout of higher concentrations of these acids, was similar both qualitatively and quantitatively to that seen after addition of other metabolic substrates [9]. Since propionate can be metabolized to CO_2 when added to either the serosal or the mucosal surface of the bladder, and since the toad bladder has been shown to metabolize other fatty acids

[9, 30], it seems reasonable to conclude that the stimulation of sodium transport observed following addition of the short-chain acids is due to an increase in the supply of energy available to the sodium transport mechanism as a result of their metabolism [4]. This conclusion is strengthened by the finding that stimulation of sodium transport did not occur following either addition or washout of the non-metabolizable compounds dimethylpropionate and benzoate.

Inhibition of sodium transport

The inhibition of the s.c.c. induced by higher concentrations of propionate and other short-chain fatty acids is more difficult to interpret. The evidence suggests that the fatty acids may interfere with the supply of energy to the sodium transport mechanism and may also directly reduce sodium entry into the epithelial cells.

(1) *Possible effect of fatty acids on supply of energy to the sodium transport mechanism.* In a previous paper [4], we reported evidence suggesting that a causal relationship exists between the metabolism of propionate and the inhibition of sodium transport which is observed on addition of propionate simultaneously to the media bathing both surfaces of the bladder. Propionate is apparently oxidized via succinate in the toad bladder, as in other animal tissues [4]: it is well established that a high rate of succinate oxidation is associated with an energy-dependent reversal of electron transport through Site I and conversion of NAD^+ to NADH [31]. We therefore proposed that the oxidation of added propionate by the toad bladder might lead to a reduction in the net amount of ATP available to the sodium transport mechanism. An energy-dependent reduction of NAD^+, comparable to that induced by succinate, has been described in association with fatty acid oxidation [32]. Therefore, it is possible that the inhibition of sodium transport induced by addition of the C_4- to C_8-fatty acids is in part a consequence of the metabolism of these compounds and attributable to mechanisms such as those previously proposed for propionate.

The findings (i) that propionate is readily metabolized by the bladder when added either to the mucosal or to the serosal surface at a concentration at which inhibition of sodium transport occurs and (ii) that mucosal addition of succinate or its precursor α-oxoglutarate, as well as propionate, can lead to inhibition of transport, are consistent with the view that both the mucosal and the serosal effects of propionate are to some extent dependent on propionate metabolism. However, the observations (i) that malonate has little, if any, effect on the response to mucosal propionate and (ii) that the non-metabolizable compounds dimethylpropionate and benzoate inhibit sodium transport, suggest that at least some portion of the inhibition induced by either serosal or mucosal addition of the fatty acids is independent of their own metabolism.

It is known that fatty acids can exert non-specific metabolic effects in other systems. In particular, both short- and long-chain fatty acids can uncouple oxidative phosphorylation [15–19]: according to the chemiosmotic hypothesis, fatty acids exert this effect by dissipating the H^+ gradient across mitochondrial membranes (ref. 33 and Cunnaro, J. and Weiner, M. W., personal communication). A decrease in the efficiency of oxidative phosphorylation clearly might contribute to the inhibition of sodium transport observed in our studies. The finding that the response of the bladder to exogenous pyruvate is reduced in the presence of inhibitory concentrations of fatty acid and, moreover, that the reduction in the response to this substrate is proportional

to the reduction in the level of function of the sodium transport mechanism is certainly consistent with this possibility. Indeed, our earlier finding that propionate stimulates oxygen consumption of bladder tissue while inhibiting sodium transport [4] could also be interpreted as evidence of an uncoupling effect. Alternatively, since fatty acids bind to proteins [34] and can inhibit the activity of a number of enzymes [14, 35], the fatty acids might interfere directly but non-specifically with the function of enzymes involved in oxidative metabolism and thus reduce the supply of energy available for sodium transport.

Since there is evidence to suggest that both the entry and exit of sodium involve energy-dependent processes, it would appear that a reduction in net ATP production by the bladder epithelial cells (whatever its precise cause) might secondarily interfere with either or both of these steps in the sodium transport mechanism.

(2) *Possible direct effect of fatty acids on mucosal entry of sodium.* In the context of the Koefoed-Johnsen and Ussing model [25], when an agent, such as propionate or the short-chain fatty acids, is more potent in affecting sodium transport when applied to the mucosal than to the serosal surface of the bladder it seems likely that the agent is acting preferentially at or near the mucosal surface and is directly affecting the entry of sodium into the epithelial cells. Previous investigators have presented evidence suggesting that an effect on sodium entry underlies the action on the bladder of amiloride [36, 28, 29], amphotericin [37], and an increased concentration of H^+ [38]: each of these agents is preferentially effective in altering sodium transport when applied to the mucosal side of the bladder. It therefore seems reasonable to infer that one action of the short-chain fatty acids is to directly reduce sodium entry across the mucosal surface of the bladder epithelial cells.

Since the inhibitory effectiveness of the short-chain fatty acids increases with increasing concentration of the non-polar (protonated) species and with increasing chain length, the inhibition of sodium transport appears to be dependent upon penetration of and/or interaction with a lipid barrier. The fatty acids might act at the mucosal membrane to reduce sodium entry into the cell through a mechanism analogous to their postulated mechanism of action in mitochondria (ref. 33 and Cunnaro, J. and Weiner, M. W., personal communication), that is, through dissipation of an H^+ gradient across the cell membrane. H^+ secretion at the mucosal surface of the bladder epithelial cells has recently been demonstrated in Colombian toads [39, 40]: dissipation of an existing H^+ gradient across the mucosal membrane might be expected to influence the rate of entry of sodium into the cells. Alternatively, fatty acids might induce alterations in the molecular configuration of the mucosal membrane and thereby decrease the permeability of the membrane to sodium. For example, the solubilization of fatty acids in the membrane might effect the structure and function of membrane proteins involved in sodium entry (either directly, by binding to such proteins, or indirectly, as a result of disordering of the phospholipid bilayers) or might influence the binding of calcium to membrane phospholipids. There is indirect evidence to suggest that short-chain fatty acids, in common with other anaesthetics, may reversibly reduce the rate of passive sodium entry into excitable tissues as a result of such non-specific perturbations of membrane structure and function [22, 23, 41–45].

Studies of the effects of the short-chain fatty acids on the response to vasopressin were performed in order to explore the possibility that the fatty acids interfere with sodium entry. Although the exact mechanism of the natriferic action of vaso-

pressin remains unclear, there is substantial evidence that the hormone promotes sodium entry across the mucosal surface of the epithelial cells [27, 46, 47]. Mendoza and coworkers [48, 49] have concluded that baseline and vasopressin-stimulated sodium transport may occur via independent but parallel pathways. The finding that propionate and octanoate, at concentrations which inhibit baseline sodium transport by 50 %, have little effect on the natriferic action of vasopressin is consistent with such a view. Our findings raise the possiblity that baseline sodium transport involves entry of sodium through hydrophobic (lipid bilayer) regions of the mucosal membrane, while vasopressin-stimulated sodium entry may occur through relatively hydrophilic (protein) regions in the membrane mosaic.

CONCLUSIONS

In summary, we have found that, in addition to propionate, other short-chain fatty acids may both stimulate and reversibly inhibit sodium transport by the isolated toad bladder. Our findings suggest that the observed stimulation of sodium transport is a function of the ability of the fatty acids to act as metabolic substrates and thus provide energy to the sodium transport mechanism of the bladder epithelial cells. On the other hand, it appears that the inhibition of sodium transport may reflect an action of the fatty acids at more than one site in the cellular machinery involved in the sodium transport process. Possible mechanisms contributing to the inhibition of transport include reversal of electron transfer through Site I in the electron transport chain as a result of fatty acid metabolism, uncoupling of oxidative phosphorylation and reduction in the rate of sodium entry across the mucosal membrane of the epithelial cells. The latter two actions may be dependent on the physicochemical properties of the short-chain fatty acids and may reflect the ability of fatty acids to reversibly perturb the structure and function of biological membranes.

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