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IONIC AND METABOLIC REQUIREMENTS FOR TETRAETHYLAMMONIUM TRANSPORT IN MOUSE KIDNEY CORTEX SLICES

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Abstract—^{14}C-Tetraethylammonium (TEA) accumulates in mouse kidney cortex slices incubated in Krebs–Ringer bicarbonate buffer (37°, pH 7.4) aerated with O_2–CO_2 95:5 v/v per cent. The metabolic inhibitor cyanide inhibits accumulation and stimulates TEA exit. Inhibition of TEA uptake in absence of extracellular Na^+ (replaced by Li^+) has a rapid onset and is easy to reverse. It is apparently due to a diminished TEA influx since efflux is unaffected. With increasing external concentrations of TEA Na^+ dependent TEA uptake reaches a saturation level and constitutes a decreasing fraction of the total uptake. TEA uptake is inhibited when active Na^+ transport is depressed (removal of extracellular K^+ or addition of ouabain), but only the Na^+ dependent part of the uptake is ouabain sensitive. The latter inhibition of TEA uptake has a slow onset and is difficult to reverse which is compatible with the idea that it may be secondary to changes in the intracellular electrolyte concentrations in time brought about when active Na^+ extrusion is impaired. A rise in the intracellular level of Na^+ might thus by analogy with the effect of extracellular Na^+ stimulate TEA efflux and thereby decrease TEA accumulation. The data seem to be consistent with the hypothesis that maintenance of a Na^+ gradient across the cell membrane is important for accumulation of TEA although alternative interpretations cannot be excluded. Furthermore, TEA uptake was inhibited in absence of external Ca^{2+}.

The kidney cortex slice is a commonly used preparation *in vitro* for studying cellular transport processes involved in active secretion of organic cations in the proximal tubulus of the kidney [1]. We have previously attempted to define some of the ionic and metabolic requirements for organic cation transport in mouse kidney cortex slices using tetraethylammonium (TEA) as a transport substrate [2]. This prototype organic cation which is secreted in the proximal tubulus of the kidney is well suited for transport studies since it accumulates in the kidney tissue without undergoing metabolic transformation [3, 2]. The possibility that any effects on ^{14}C-TEA transport should be secondary to changes in ^{14}C-TEA metabolism can therefore be disregarded. Our previous results can be summarized as follows: (1) No accumulation of TEA occurs in the tissue in the absence of metabolic energy [2]. (2) The capability of the tissue to accumulate TEA is diminished in the absence of external Na^+. The partial inhibition of TEA uptake seen when Na^+ is replaced by Li^+ or sucrose turns into a complete inhibition when K^+ is used as a substitute for Na^+ [4]. (3) There is a decreased accumulation of TEA in the tissue under experimental conditions causing impairment of active Na^+-transport, i.e. absence of external K^+ or presence of ouabain [4–6].

The present study represents a continuation of these investigations. Attempts were made to characterize the metabolic and ionic requirements for individual transport components, i.e. influx and efflux. The present data are moreover evaluated in terms of the Na^+ gradient hypothesis [7].

MATERIALS AND METHODS

Tetraethyl-*l*-^{14}C ammonium bromide (spec. act. 2.8 mCi/mM) was supplied by New England Nuclear Corp., Boston, MA, U.S.A. The following unlabeled compounds or drugs were used: Tetraethylammonium chloride (Merck), potassium cyanide (Merck), *g*-strophantin (Merck) and ethacrynic acid (Merck Sharp and Dohme).

Male albino mice (25 g) of a single strain (NMRI) were received from Moellegaard A/S Scanbur Ejby, Denmark.

Incubation procedure. Cortical slices from mouse kidneys were prepared and used as previously described [8, 9]. Immediately after preparation the slices were placed in Erlenmeyer flasks (three to four slices weighing 50 mg in each flask), containing ice-cold Krebs–Ringer bicarbonate incubation buffer (100 ml) with 11 mM glucose. The tissue was stored (½ hr) under these conditions until start of incubation (normally tissue was stored and incubated in the same buffer). The cationic composition of the buffers used was as follows: Standard buffer: 125 mM Na^+, 25 mM K^+, 2.5 mM Ca^{2+}, 1.2 mM Mg^{2+}. Na^+-free buffer: Na^+ in standard buffer replaced by 125 mM Li^+. K^+-free buffer: K^+ replaced by 25 mM Na^+. Ca^{2+}-free buffer: Ca^{2+} was omitted from standard buffer. At start of incubation, the flasks were closed with rubber stoppers perforated with polyethylene tubings through which a gas phase

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consisting of oxygen–carbon dioxide (95:5) was bubbled into incubation medium (pH adjusted to 7.4). The flasks were shaken gently (60 oscillations/min) in a water bath (37°). Unless otherwise stated, the tissue was preincubated (15 min) before addition of ^{14}C-tetraethylammonium (TEA). The external concentration of ^{14}C-TEA was kept constant during the entire incubation period owing to the relatively large volume of buffer. Incubations were terminated by separating tissue from medium on nylon nets. If necessary slices were carefully transferred to other media by means of a forceps. Otherwise, the tissue was placed in preweighed counting vials and weighed (wet wt).

Measurement of radioactivity. Tissue samples digested (48 hr at room temperature) in 1 ml tissue solubilizer 'Soluene-350' (Packard Instrument) and medium samples of 1 ml were completed for β-liquid scintillation counting by addition of 15 ml scintillation fluid 'Dimilume' (Packard Instruments). Both types of samples were counted with identical efficiencies (internal standardization) in a liquid scintillation spectrometer (Beckman 250) using a combined ^{14}C[^{3}H] window. Tissue concentrations of ^{14}C-TEA were calculated from c.m.p./g tissue (post incubation wet wt) using counting rates in the medium as reference. Addition of ^{14}C-TEA to blank tissue and medium samples gave counting rates similar to those obtained in absence of tissue or medium.

Wash out procedure. Exit of ^{14}C-TEA was studied as previously described [10]. Tissue from each animal was pre-equilibrated (30 min) with 1 μM ^{14}C-TEA in a standard medium. Part of the tissue was removed for ^{14}C-measurements while the remainder was divided into a control group (transferred to a standard medium) and an experimental group (transferred to a different type of medium). The concentration of unlabeled TEA in both wash out solutions was 1 μM. After 5 min wash out tissue slices were removed and ^{14}C-activity determined. ^{14}C-TEA exit was expressed as a fraction of the initial ^{14}C-TEA content per mass unit tissue according to the formula: $C_o - C_t/C_o$, where C_o represents the initial ^{14}C-TEA content after pre-equilibration and C_t the ^{14}C-TEA content after wash out. Due to the large volume of wash out medium (100 ml) back flux of isotope into the tissue was negligible (the isotope concentration of the wash out solution did never exceed background counting). This means that any observed effect on ^{14}C-TEA exit cannot be ascribed to changes in a parallel re-uptake of ^{14}C-TEA from the wash out medium. Differences between control and experimental groups were expressed as percentage of control values (paired comparisons).

Determinations of the potassium content of the tissue. Tissue (100 mg) was immersed in 2 ml 9 N HCl and shaken vigorously at room temperature (48 hr). The tissue extract was filtered on paper and its K^+ concentration determined by flame photometry (IL flame photometer). Known amounts of K^+ added to tissue extract could be completely recovered by this method.

RESULTS

TEA transport in metabolically blocked slices. Accumulation of ^{14}C-TEA in mouse kidney cortex slices is almost completely depressed when the metabolic inhibitor cyanide (1 mM) is added to the medium at start of incubation (Fig. 1). Addition of cyanide after the tissue has accumulated ^{14}C-TEA evokes a rapid decrease in the tissue concentration of TEA which seems to fall to the same level as that of slices exposed to cyanide from the very start of the incubation (Fig. 1). This effect of cyanide was not only due to a diminished influx of ^{14}C-TEA but also to a stimulation of ^{14}C-TEA efflux. Exit (5 min) of ^{14}C-TEA (cf. methods) was thus increased by 77 ± 15 per cent ($N = 6$, $P < 0.005$) when cyanide was added to the wash out solutions.

TEA uptake in presence and absence of Na^+ as a function of the external TEA concentration. The steady state* distribution ratio of ^{14}C-TEA in the tissue was studied as a function of the external TEA concentration. These experiments were performed in the presence as well as in the absence of extracellular Na^+. Figure 2 shows that steady state distribution ratio of ^{14}C-TEA decreases with increasing concentrations of external TEA regardless of whether Na^+ is present or not. The steady state distribution ratios are, however, consistently lower in the absence of Na^+. The Na^+ dependent TEA uptake, i.e. the difference between steady state distribution ratios determined in the presence and absence of Na^+ constitutes a decreasing fraction of the total uptake with increasing TEA concentrations in the medium (Fig. 2). Furthermore, according to

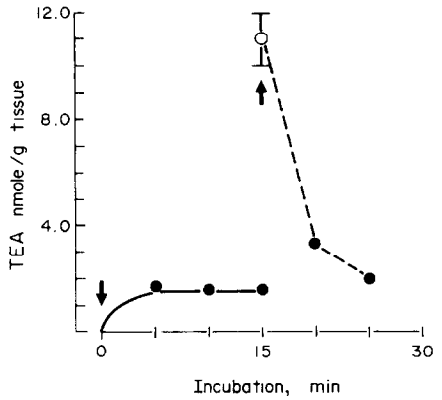

Fig. 1. Effect of cyanide on time course of ^{14}C-TEA uptake by mouse kidney cortex slices. Concentration of TEA in external medium was 1 μM. Arrows indicate addition of 1 mM cyanide. Lower curve: cyanide was added to the incubation medium just before TEA (●–). Upper curve: TEA was allowed to accumulate in the tissue in absence of cyanide (○). At the indicated time, cyanide was added to some of the media and incubation was continued (●– – –). Each symbol represents the mean with S.E.M. (vertical bars indicate values higher than 0.3 nmole/g tissue) of five to six experimental values.

* *Steady state* describes a situation where the ^{14}C-TEA concentration of the tissue after incubation at a given external ^{14}C-TEA concentration has reached a final maximum level and remains constant upon further incubation (net transport of ^{14}C-TEA into the tissue does not occur any more since efflux balances influx).

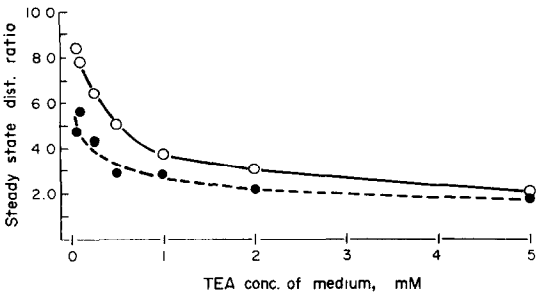

Fig. 2. Steady state distribution ratio of ^{14}C-TEA in mouse kidney cortex slices plotted as a function of the TEA concentration in the medium. Tissue was incubated (30 min) in standard media (○–) or Na^+-free media (●– –). Preliminary studies on the time course of TEA uptake at three external TEA concentrations (1 μM, 0.5 mM and 5 mM) showed that steady state distribution ratios were attained after 30 min incubation in the presence or absence of external Na^+. Each symbol represents the mean of six experimental values (S.E.M. within circles).

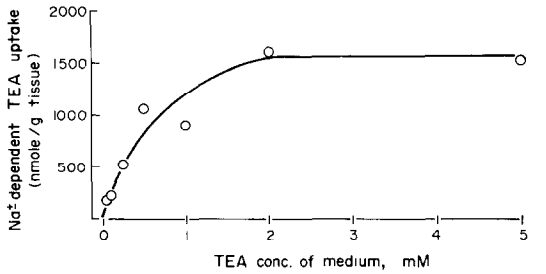

Fig. 3. Na^+ dependent uptake of ^{14}C-TEA in mouse kidney cortex slices plotted as a function of the external TEA concentration. Na^+ dependent uptake was calculated from the difference between the steady state distribution ratios determined in the presence and absence of Na^+ (data from Fig. 2): Na^+ dependent uptake = total uptake – uptake in absence of Na^+.

Fig. 3 the Na^+ dependent uptake seems to reach a maximum level at external TEA concentrations above 2 mM.

Effect of ouabain and ethacrynic acid on TEA uptake. Table 1 shows that two inhibitors of active Na^+ extrusion in kidney slices ouabain [6], and the diuretic ethacrynic acid [11], depress TEA uptake, the effect, however, being more pronounced with ethacrynic acid.

From the data in Table 2 it can be inferred that ouabain (1 mM) has no inhibitory effect on Na^+ independent TEA uptake, i.e. the uptake occurring in absence of Na^+. This part of the uptake is, however, almost completely inhibited in the presence of equimolar concentrations of ethacrynic acid or cyanide.

Onset and reversibility of inhibition of TEA uptake following removal of extracellular Na^+ *or impairment of active* Na^+ *extrusion.* Experiments were performed to investigate onset of inhibition of TEA uptake after omission of external Na^+ or depression of active Na^+ transport. Table 3 shows that initial TEA uptake is inhibited in absence of Na^+ regardless of whether the tissue has been preincubated in the Na^+-free medium or not prior to addition of ^{14}C-TEA. However, no inhibition of TEA uptake occurs in the absence of K^+ or in the presence of 1 mM ouabain unless the tissue is pre-exposed to

Table 1. Effect of ouabain and ethacrynic acid on ^{14}C-TEA uptake by mouse kidney cortex slices

Drug	Incubation period, min	^{14}C-TEA tissue-medium distribution ratio (c.p.m. g^{-1} tissue/c.p.m. ml^{-1} medium) Control	With drug	Difference (%)
Ouabain (1 mM)	5	7.4 ± 0.2	4.9 ± 0.2	−34 ± 2, $P < 0.001$
Ouabain (1 mM)	30	12.8 ± 0.3	9.3 ± 0.3	−27 ± 3, $P < 0.001$
Ethacrynic acid (1 mM)	5	7.0 ± 0.4	3.2 ± 0.2	−55 ± 2, $P < 0.001$
Ethacrynic acid (1 mM)	30	12.0 ± 0.5	2.6 ± 0.2	−78 ± 1, $P < 0.001$

Kidney tissue from each animal was divided into two groups incubated in standard media with or without (control) addition of drug (ouabain or ethacrynic acid). Tissue was stored and incubated in the same medium. ^{14}C-TEA concentration was 1 μM. Results are mean values ± S.E.M. ($N = 6$).

Student's 't'-test on paired differences.

Table 2. Effect of ouabain, ethacrynic acid and cyanide on ^{14}C-TEA uptake by mouse kidney cortex slices in absence of external Na^+

Drug	^{14}C-TEA tissue-medium distribution ratio after 30 min Control	With drug	Difference (%)
Ouabain (1 mM)	7.6 ± 0.4	7.2 ± 0.4	−4 ± 7
Ethacrynic acid (1 mM)	7.5 ± 0.2	2.4 ± 0.3	−70 ± 3, $P < 0.001$
Cyanide (1 mM)	7.6 ± 0.7	1.6 ± 0.1	−79 ± 2, $P < 0.001$

Kidney tissue from each animal was divided into two groups incubated in Na^+-free media with or without (control) addition of drug. Tissue was stored and incubated in the same medium. Concentration of ^{14}C-TEA in the external medium was 1 μM. Results are mean values ± S.E.M. ($N = 6$).

Student's 't'-test on paired differences.

Table 3. Onset of inhibition of initial ^{14}C-TEA uptake after omission of Na^+ or impairment of active Na^+ extrusion

Experimental medium	Incubation (min) before addition of TEA	^{14}C-TEA tissue-medium distribution ratio after 5 min Control	Experimental	Difference (%)
Na^+-free	0	4.5 ± 0.1	3.6 ± 0.1	−20 ± 2, P < 0.001
Na^+-free	30	4.7 ± 0.2	3.5 ± 0.2	−25 ± 5, P < 0.005
K^+-free	0	4.9 ± 0.2	5.1 ± 0.6	+7 ± 13
K^+-free	30	4.5 ± 0.2	3.7 ± 0.1	−17 ± 3, P < 0.005
Ouabain (1 mM)	0	5.2 ± 0.4	5.1 ± 0.3	−2 ± 4
Ouabain (1 mM)	30	4.7 ± 0.5	3.6 ± 0.2	−21 ± 4, P < 0.005

Kidney tissue from each animal was stored and incubated in standard media. The tissue was then divided into a control group (transferred to another standard medium) and an experimental group (transferred to an experimental medium). ^{14}C-TEA (1 μM) was added to the second medium either at start of incubation or after 30 min. Results are mean values ± S.E.M. ($N = 6$).

Student's 't'-test on paired differences.

these experimental conditions before addition of ^{14}C-TEA.

The data in Fig. 4 represent another experimental approach to the problem. Tissue pre-equilibrated to steady state (efflux balances influx) with 1 μM ^{14}C-TEA under standard conditions was transferred to and re-incubated in other media also containing 1 μM ^{14}C-TEA. The steady state distribution ratio of ^{14}C-TEA was significantly depressed after 5 min re-incubation in a Na^+-free medium as compared to control experiments where tissue was just transferred to another standard medium (Fig. 4). However, transfer of tissue to K^+-free media or media with 1 mM ouabain had no statistically significant effect on the steady state distribution ratio (Fig. 4). The diminution of the steady state distribution ratio seen after exposure to Na^+-free media (Fig. 4) must be due to a decreased influx of TEA in absence of Na^+ since ^{14}C-TEA exit is unaffected by the absence of external Na^+ (3 ± 7 per cent, $N = 6$).

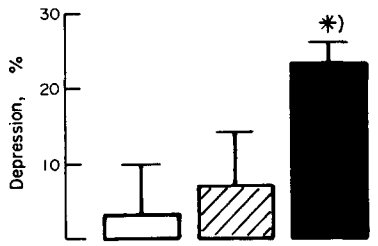

Fig. 4. Changes in steady state distribution of ^{14}C-TEA in mouse kidney cortex slices after exposure to Na^+-free media (black bars), K^+-free media (white bars) or media containing 1 mM ouabain (cross-hatched bars). Tissue pre-equilibrated (30 min) in standard medium to steady state with 1 μM ^{14}C-TEA was divided into a control group (transferred to another standard medium) and an experimental group (transferred to one of the above media). ^{14}C-activities were measured after 5 min incubation in the second medium which also contained 1 μM ^{14}C-TEA. Changes in the steady state distribution ratio were calculated from the difference between control and experimental values (per cent of control value). Transfer of tissue from one standard medium to the other (control experiments) had no effect on steady state distribution ratio. Results are given as the mean with S.E.M. of six values. * P < 0.001.

The results in Table 4 illustrate the reversibility of the inhibition of TEA uptake seen after omission of Na^+ or impairment of active Na^+ transport. Pre-incubation of the tissue for 30 min in the absence of Na^+ has no effect on the subsequent initial uptake of TEA in standard media. However, pre-exposure of the tissue for the same period of time to K^+-free media as well as media containing ouabain or ethacrynic acid depresses the subsequent uptake of ^{14}C-TEA.

The K^+ concentration of the tissue was determined in a few experiments. The K^+ concentration (m-moles kg^{-1} wet wt) was 60 ± 5 after incubation (30 min) in standard media, but fell to 29 ± 3 and

Table 4. Reversibility of inhibition of ^{14}C-TEA uptake by mouse kidney cortex slices after omission of Na^+ or impairment of active Na^+ extrusion

Experimental preincubation conditions	^{14}C-TEA tissue-medium distribution ratio after 5 min Control	Experimental	Difference (%)
Na^+-free medium	5.8 ± 0.3	6.0 ± 0.3	+7 ± 7
K^+-free medium	5.6 ± 0.2	4.1 ± 0.2	−27 ± 4, P < 0.001
Ouabain (1 mM)	5.4 ± 0.3	4.2 ± 0.3	−23 ± 4, P < 0.001
Ethacrynic acid (1 mM)	5.3 ± 0.3	1.8 ± 0.1	−66 ± 3, P < 0.001

Kidney tissue from each animal stored in standard buffer until use was divided into a control group (transferred to a standard medium for preincubation) and an experimental group (transferred to an experimental medium for preincubation). After 30 min preincubation tissue was transferred to and incubated in standard media with 1 μM ^{14}C-TEA. Results are mean values ± S.E.M. ($N = 8$–9).

Student's 't'-test on paired differences.

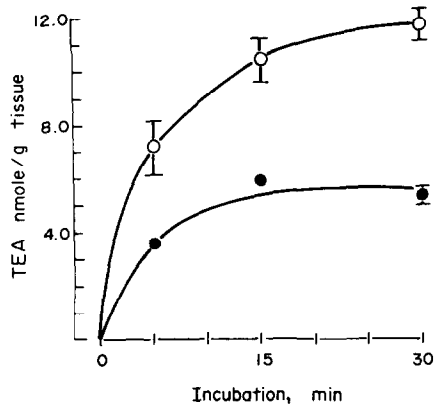

Fig. 5. Time course of ^{14}C-TEA uptake in mouse kidney cortex slices incubated in standard media (○) and Ca^{2+}-free media (●). Concentration of TEA in the external medium was 1 μM. Each symbol is the mean with S.E.M. (vertical bars indicate values higher than 0.3 nmole/g tissue) of four to six experimental values.

8 ± 2 after incubation in K^+-free media for 5 and 30 min, respectively (mean with S.E.M. of four values). Incubation of tissue in the presence of ouabain (1 mM) gave results very similar to those found in the absence of K^+.

TEA uptake in Ca^{2+}-free media. The possibility that the divalent inorganic cation Ca^{2+} may influence TEA uptake was also tested. Figure 5 shows a depression of TEA uptake in Ca^{2+}-free media. This inhibition could also be demonstrated in absence of K^+ (control media and Ca^{2+}-free media were K^+-free): 40 ± 5 per cent inhibition of 30 min TEA uptake; mean ± S.E.M. of six paired comparisons. $P < 0.001$. Similary, omission of K^+ inhibited TEA uptake in absence of Ca^{2+} (control media and K^+-free media were Ca^{2+}-free): 33 ± 5 per cent inhibition of 30 min TEA uptake; mean ± S.E.M. of six paired comparisons, $P < 0.005$.

DISCUSSION

The present data confirm previous observations showing that TEA uptake is influenced by cell metabolism and the ionic environment [2, 4]. Slices deprived of their supply of metabolic energy by cyanide poisoning did not only loose the ability to accumulate TEA (Fig. 1) but also to retain TEA already accumulated in the tissue (stimulation of TEA exit in the presence of cyanide).

Steady state distribution ratio of TEA in the tissue decreased with increasing external concentrations of TEA (Fig. 2). This saturation type phenomenon could be demonstrated whether or not Na^+ was present in the external solution. With increasing external TEA concentrations Na^+-dependent TEA uptake seemed to become saturated (Fig. 3) and to constitute a decreasing fraction of the total uptake (Fig. 2). Inhibition of TEA uptake after omission of external Na^+ is characterized by its rapid onset and reversibility (Tables 3 and 4). The effect is apparently due to a diminished influx of TEA in the absence of extracellular Na^+ ions since TEA exit was unaffected (Fig. 4).

Two inhibitors of active Na^+ extrusion of kidney slices, ouabain [6], and the diuretic ethacrynic acid [11], depress TEA uptake (Table 1). The greater inhibitory potency of the latter drug may be ascribed to its unspecific depression of respiration in kidney slices [11]. The lack of any effect of ouabain on Na^+-independent TEA uptake (Table 2) suggests that this drug specifically inhibits the Na^+-dependent fraction of TEA uptake without having any non-specific toxic effects on the tissue. However, the finding that ethacrynic acid just like cyanide acts as a potent inhibitor of TEA uptake even in the absence of extracellular Na^+ (Table 2) suggests that inhibition with ethacrynic acid at least to some extent is secondary to non-specific effects on cell metabolism.

Inhibition of TEA uptake after exposure to K^+-free media or ouabain has a slow onset and is difficult to reverse (Tables 3 and 4). This argues against the possibility that depression of active Na^+-extrusion has direct inhibitory effects on TEA uptake, i.e. the uptake should derive energy directly from ATP hydrolysis via the Na K ATPase system involved in active Na^+–K^+ exchange. The findings are rather consistent with an indirect effect on TEA uptake secondary to changes in the intracellular electrolyte concentrations in time brought about by the impairment of active Na^+ transport, i.e. increase of Na^+ and a concomitent decrease of K^+. Data in this study showing that exposure to K^+-free media (or media containing ouabain) for some time is required not only for inhibition of TEA uptake to develop but apparently also for tissue K^+ to fall to a low level are thus in accordance with the latter suggestion. Optimal intracellular K^+ concentrations are necessary for the function of many enzymic reactions involved in energy-yielding metabolic processes [7], and a decrease in the intracellular level of K^+ due to inhibition of active Na^+–K^+ exchange may therefore have non-specific effects on an energy-requiring transport process such as TEA uptake. One might, however, suggest the alternative possibility that the parallel rise of the intracellular Na^+ level by analogy with the effect of extracellular Na^+ causes a stimulation of TEA efflux resulting in a diminished accumulation of TEA in the tissue.

The Na^+-gradient hypothesis postulates that Na^+ acts as cosubstrate in transport of organic solutes and thereby increases the affinity between substrate and transport site [7]. Due to the asymmetric distribution of Na^+ across the cell membrane (a high extracellular and a low intracellular concentration) influx of solute would then be stimulated to a greater extent than efflux resulting in intracellular accumulation of solute. The present data are consistent with the concept that such a mechanism may be partially responsible for accumulation of TEA in the tissue. Nevertheless the data only provide indirect evidence and may be compatible with other interpretations. Future studies on the exact quantitative relationship between the bidirectional fluxes of TEA and varying intra- and extracellular Na^+ concentrations are necessary to provide more direct and compelling evidence in favour of the Na^+-gradient model.

The presence of a ouabain-insensitive ATPase which was specifically activated by external Ca^{2+}

ions could be demonstrated in membranes isolated from kidney cortex tubules [12]. This enzyme was shown to play an important role in the regulation of concentrations of intracellular ions i.e. Na^+ and K^+ [12]. Hence the possibility exists that inhibition of TEA uptake in absence of external Ca^{2+} ions (Fig. 5) may be secondary to changes in the intracellular electrolyte concentrations (increase of Na^+ and decrease of K^+) caused by an impaired activity of Ca^{2+} ATPase. The ouabain-sensitive Na-K ATPase system involved in active Na^+ extrusion requires in contrast to Ca^{2+} ATPase the presence of external K^+ ions. The finding that inhibition of TEA uptake in absence of Ca^{2+} occurs in K+-free media (*vice versa* inhibition in absence of K^+ occurs in Ca^{2+}-free media) thus seems to be consistent with the assumption that inhibition in absence of Ca^{2+} is unrelated to changes in the activity of Na-K ATPase.

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