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EFFECTS OF TETRACYCLINES ON ALDOSTERONE- AND INSULIN-MEDIATED Na^+ TRANSPORT IN THE TOAD URINARY BLADDER

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Summary

The effect of oxytetracycline and demethylchlortetracycline on aldosterone- and insulin-mediated Na^+ transport (short-circuit current) were examined in toad urinary bladders mounted in modified Ussing chambers. Oxytetracycline had little or no effect on either basal or aldosterone-mediated Na^+ transport. In contrast, demethylchlortetracycline markedly inhibited both basal and aldosterone-mediated Na^+ transport. Furthermore, demethylchlortetracycline inhibited the aldosterone response significantly out of proportion to its effects on basal Na^+ transport. Neither of the drugs had an effect on insulin-mediated Na^+ transport. Consequently, the natriuresis observed in certain patients treated with demethylchlortetracyline may be related to drug-induced renal resistance to the effects of aldosterone.

Introduction

Tetracyclines are well known to have distinct effects on antidiuretic hormone-mediated osmotic water flow in the toad urinary bladder; for example, demethylchlortetracycline, but not oxytetracycline, inhibits antidiuretic hormone-mediated water flow [1,2]. More recently, we have shown that neither demethylchlortetracycline nor oxytetracycline inhibits antidiuretic hormone-mediated Na^+ transport in the toad urinary bladder [3]. In addition,

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demethylchlortetracycline markedly and consistently inhibits basal Na^+ transport whereas oxytetracycline has a smaller and more variable effect on basal Na^+ transport [3].

Demethylchlortetracycline has also been shown to inhibit renal Na^+ transport in patients with cirrhosis or congestive heart failure [4—10] but not in normal subjects or in patients with the syndrome of inappropriate antidiuretic hormone secretion [11—13]. In order to further elucidate the mechanism(s) by which tetracyclines affect renal Na^+ transport we have examined the effects of demethylchlortetracycline and oxytetracycline on aldosterone and insulin-mediated Na^+ transport in the toad urinary bladder, a classical model of the mammalian distal nephron.

Methods

All experiments were performed with the bladders of female toads (*Bufo marinus*) from the Dominican Republic (obtained from National Reagents, Bridgeport, CT) maintained in the unfed state in distilled water for at least 48 h prior to use. The animals were killed by pithing and the bladders were excised and placed in continuously-aerated, modified Ringer's solution at room temperature (22—24°C) for 10—30 min before mounting. The modified Ringer's solution contained 5 mM tris(hydroxymethyl)aminomethane, 103 mM NaCl, 3.4 mM Na_2HPO_4, 3.0 mM KCl, 0.6 mM KH_2PO_4, 0.75 mM $CaCl_2$ and 5 mM glucose (pH 7.95 ± 0.05; osmolality = 225 ± 5 mosM/kg).

The bladders were mounted in modified Ussing chambers so that a single hemibladder provided two electrically isolated quarter-bladders, each of 2.3 cm^2 cross sectional area. The two hemibladders from a single toad thus provided four quarter-bladders for each experiment. The short-circuit current across each quarter-bladder was used to measure net Na^+ transport. Previous studies have shown that tetracyclines do not affect the equivalence between short-circuit current and net Na^+ transport (measured by isotopic techniques) in the toad urinary bladder [3]. Agar-isotonic NaCl bridges were employed in all experiments and current was provided by a battery source through Ag/AgCl electrodes. The potential difference across each quarter-bladder was measured with calomel reference electrodes.

The tetracyclines were dissolved in dimethylsulfoxide (250 mg/ml), stored at 3—5°C and freshly prepared every 2—3 weeks. Aliquots of these stock solutions were added to the modified Ringer's solution (final concentration = 0.5 mg/ml) and the latter was then titrated with 1 M NaOH to a pH of 7.95 ± 0.05 prior to use. Control solutions consisting of an equal volume percentage of dimethylsulfoxide were prepared in an identical fashion. Stock solutions of d-aldosterone (Sigma) ($2.8 \cdot 10^{-5}$ M) and purified porcine insulin (Lilly, lot 615-D63; 25.4 U/mg) (100 U/ml) were prepared in methanol and 0.1 M HCl, respectively. These solutions were stored at 3—5°C and freshly prepared every 1—2 weeks.

The following experimental protocols were employed. For the aldosterone experiments, paired hemibladders were mounted and then incubated under open-circuit conditions in modified Ringer's solution containing penicillin (50 U/ml) and streptomycin (50 μg/ml) overnight (16—18 h). Prolonged in

vitro incubation of toad urinary bladders makes their subsequent response to aldosterone more consistent [14]: in our experiments the response rate was essentially 100%. The following morning the bladders were rinsed several times with fresh modified Ringer's solution and then continuously short-circuited for the remainder of the experiment. After the short-circuit current had stabilized (usually 30—90 min), aldosterone was added to the serosal medium (final concentration $1.4 \cdot 10^{-7}$ M) of two quarter-bladders (No. 2 and No. 4) and an equal amount of methanol carrier was added to the other two quarter-bladders (No. 1 and No. 3). The time of addition of aldosterone was defined as time zero. 2 h later (when the aldosterone response was just becoming evident) the serosal chamber of each quarter-bladder was drained, washed twice with the test solution (quarter-bladder Nos. 3 and 4: modified Ringer's solution plus tetracycline, 0.5 mg/ml final concentration; quarter-bladder Nos. 1 and 2: modified Ringer's solution plus dimethylsulfoxide), and then refilled with the same solution. Aldosterone (final concentration $1.4 \cdot 10^{-7}$ M) was added to the serosal medium of quarter-bladder Nos. 2 and 4 following the drainage procedure. After the addition of the tetracycline (or dimethylsulfoxide) short-circuit current was monitored for 3 h.

A similar protocol was employed for the insulin experiments but the overnight incubation step was excluded. After mounting and stabilization of short-circuit current, the serosal chamber of each quarter-bladder was drained, washed twice with the test solution and then refilled with the same solution (quarter-bladder Nos. 3 and 4: modified Ringer's solution plus tetracycline; quarter-bladder Nos. 1 and 2: modified Ringer's solution plus dimethylsulfoxide). After a 12 min incubation period, insulin was added to the serosal medium (final concentration 100 mU/ml) of quarter-bladder Nos. 2 and 4 and an equal amount of 0.1 M HCl was added to quarter-bladder Nos. 1 and 3. The time of addition of insulin was defined as time zero. Following insulin addition, short-circuit current was monitored for 1—2 h. Since the insulin response is known to be quite variable [15], when the control hemibladders did not demonstrate a response to insulin (with a 15% increase over baseline at 2 h), both the control and paired experimental hemibladders were discarded. There was no difference in the response rate of the bladders in the oxytetracycline and demethylchlortetracycline series of experiments (~70% in each case).

Statistical analyses were performed with the Student's *t*-test for paired comparisons and P values >0.05 were considered non-significant (N.S.).

Results

After 3 h incubation with demethylchlortetracycline, both basal and aldosterone-mediated short-circuit current were markedly inhibited (Table I: basal short-circuit current inhibition, quarter-bladder No. 1 versus quarter-bladder No. 3, $P < 0.005$; aldosterone-mediated short-circuit current inhibition, quarter-bladder No. 2 versus quarter-bladder No. 4, $P < 0.005$). However, when the effect of demethylchlortetracycline on basal short-circuit current is 'removed' by directly comparing the absolute short-circuit current values of quarter-bladders No. 3 (demethylchlortetracycline) and No. 4 (aldosterone plus demethylchlortetracycline), it becomes evident that demethylchlortetracycline

TABLE I

EFFECTS OF TETRACYCLINES ON BASAL AND ALDOSTERONE-MEDIATED SHORT-CIRCUIT CURRENT

Quarter-bladder No.	Additions	Short-circuit current ($\mu A \cdot cm^{-2}$) *			Aldosterone-mediated short-circuit current (%) **
		T_1	T_2	T_3	
1	methanol + dimethylsulfoxide	11.8 ± 2.4	11.7 ± 2.3	10.7 ± 1.7	141.1 ± 29.1
2	aldosterone + dimethylsulfoxide	11.7 ± 2.2	12.8 ± 2.1	24.7 ± 3.7	
3	methanol + oxytetracycline	11.9 ± 2.2	11.4 ± 2.2	8.8 ± 1.0	124.7 ± 24.1
4	aldosterone + oxytetracycline	11.8 ± 2.1	12.8 ± 1.8	19.4 ± 2.7	
1	methanol + dimethylsulfoxide	14.9 ± 2.4	14.7 ± 2.1	13.3 ± 2.0	179.3 ± 25.4
2	aldosterone + dimethylsulfoxide	14.9 ± 3.0	17.2 ± 2.7	36.1 ± 6.3	
3	methanol + demethylchlortetracycline	17.5 ± 2.5	16.1 ± 2.5	4.5 ± 0.6	70.1 ± 16.4
4	aldosterone + demethylchlortetracycline	17.1 ± 3.8	18.9 ± 3.6	7.0 ± 1.3	

* Short-ciruit current (mean ± S.E.) at T_1 (just prior to the addition of methanol or aldosterone), T_2 (after 2 h incubation with methanol or aldosterone and just prior to the addition of dimethylsulfoxide or the tetracycline) and T_3 (after 3 h incubation with dimethylsulfoxide or the tetracycline). In the oxytetracycline series at T_3: quarter-bladder Nos. 1—2 ($P < 0.005$), quarter-bladder Nos. 1—3 (n.s.), quarter-bladder Nos. 2—4 (n.s.) and quarter-bladder Nos. 3—4 ($P < 0.005$) ($n = 6$). In the demethylchlortetracycline series at T_3: quarter-bladder Nos. 1—2 ($P < 0.005$), quarter-bladder Nos. 1—3 ($P < 0.005$), quarter-bladder Nos. 2—4 ($P < 0.005$), and quarter-bladder Nos. 3—4 (n.s.) ($n = 6$).

** Percent increase (mean ± S.E.) in aldosterone-mediated short-circuit current at T_3 in the presence of either dimethylsulfoxide (quarter-bladder No. 2/quarter-bladder No. 1 at T_3 divided by quarter-bladder No. 2/quarter-bladder No. 1 at T_1) or tetracycline (quarter-bladder No. 4/quarter-bladder No. 3 at T_3 divided by quarter-bladder No. 4/quarter-bladder No. 3 at T_1): dimethylsulfoxide versus oxytetracycline (n.s.), dimethylsulfoxide versus demethylchlortetracycline ($P < 0.01$). (The resistance ($k\Omega \cdot cm^2$) of the bladders at T_1 in the oxytetracycline series were: 1.75 ± 0.11 (quarter-bladder No. 1), 1.73 ± 0.13 (quarter-bladder No. 2), 1.76 ± 0.14 (quarter-bladder No. 3) and 1.68 ± 0.15 (quarter-bladder No. 4); the corresponding values in the demethylchlortetracycline series were 1.97 ± 0.12, 1.90 ± 0.10, 1.96 ± 0.25 and 1.72 ± 0.22, respectively.)

TABLE II

EFFECTS OF TETRACYCLINES ON BASAL AND INSULIN-MEDIATED SHORT-CIRCUIT CURRENT

Quarter-bladder No.	Additions	Short-circuit current ($\mu A \cdot cm^{-2}$) *			Insulin-mediated short-circuit current (%) **
		T_1	T_2	T_3	
1	dimethylsulfoxide + HCl	21.1 ± 4.1	25.5 ± 4.9	34.5 ± 6.0	43.5 ± 16.6
2	dimethylsulfoxide + insulin	21.3 ± 3.7	24.7 ± 3.9	43.7 ± 6.0	
3	oxytetracycline + HCl	19.8 ± 4.0	23.7 ± 4.7	26.3 ± 4.1	52.5 ± 20.6
4	oxytetracycline + insulin	18.6 ± 3.3	22.5 ± 4.0	35.2 ± 4.2	
1	dimethylsulfoxide + HCl	23.5 ± 3.3	26.2 ± 3.9	23.8 ± 3.7	74.7 ± 11.6
2	dimethylsulfoxide + insulin	21.5 ± 3.7	23.6 ± 4.0	35.5 ± 4.8	
3	demethylchlortetracycline + HCl	22.3 ± 2.7	22.2 ± 3.5	10.7 ± 2.2	149.7 ± 50.6
4	demethylchlortetracycline + insulin	22.3 ± 3.3	22.5 ± 4.2	23.7 ± 5.6	

* Short-circuit (mean ± S.E.) at T_1 (just prior to the addition of dimethylsulfoxide or the tetracycline), T_2 (after 12 min incubation with dimethylsulfoxide or the tetracycline and just prior to the addition of insulin or HCl carrier) and T_3 (after 1 h incubation with insulin or HCl). In the oxytetracycline series at T_3: quarter-bladder Nos. 1—2 ($P < 0.05$), quarter-bladder Nos. 1—3 ($P < 0.02$), quarter-bladder Nos. 2—4 (n.s.) and quarter-bladder Nos. 3—4 ($P < 0.001$) ($n = 7$). In the demethylchlortetracycline series at T_3: quarter-bladder Nos. 1—2 ($P < 0.005$), quarter-bladder Nos. 1—3 ($P < 0.01$), quarter-bladder Nos. 2—4 ($P < 0.01$) and quarter-bladder Nos. 3—4 ($P < 0.02$) ($n = 12$).

** Percent increase (mean ± S.E.) in insulin-mediated short-circuit current at T_3 in the presence of either dimethylsulfoxide (quarter-bladder No. 2/quarter-bladder No. 1 at T_3 divided by quarter-bladder No. 2/quarter-bladder No. 1 at T_2) or tetracycline (quarter-bladder No. 4/quarter-bladder No. 3 at T_3 divided by quarter-bladder No. 4/quarter-bladder No. 3 and T_2): dimethylsulfoxide versus oxytetracycline (n.s.), dimethylsulfoxide versus demethylchlortetracycline (n.s.). The resistances ($k\Omega \cdot cm^2$) of the bladders at T_1 in the oxytetracycline series were: 2.06 ± 0.26 (quarter-bladder No. 1), 2.33 ± 0.41 (quarter-bladder No. 2), 2.14 ± 0.26 (quarter-bladder No. 3) and 2.00 ± 0.28 (quarter-bladder No. 4); the corresponding values in the demethylchlortetracycline series were 1.57 ± 0.16, 1.68 ± 0.19, 1.85 ± 0.15 and 1.72 ± 0.15, respectively).

inhibits aldosterone-mediated short-circuit current out of proportion to its effect on basal short-circuit current: there is no statistically significant aldosterone response in the presence of demethylchlortetracycline (Table I). The same conclusion is reached by comparing the aldosterone response in the absence (179.3 ± 25.4%) and presence (70.1 ± 16.4%) of demethylchlortetracycline, respectively ($P < 0.01$; Table I). In contrast to these results, oxytetracycline did not affect either basal or aldosterone-mediated short-circuit current (Table I). Thus demethylchlortetracycline (but not oxytetracycline) markedly inhibits aldosterone-mediated short-circuit current in the toad urinary bladder and this effect cannot be entirely accounted for by a demethylchlortetracycline-related inhibition of basal short-circuit current.

The situation with respect to insulin-mediated short-circuit current is quite different, however. Although both demethylchlortetracycline and oxytetracycline inhibited basal short-circuit current in these experiments, neither had any effect on insulin-mediated short-circuit current (Table II). (The slightly different effects of oxytetracycline on basal short-circuit current in the two series of experiments are probably attributable to the different protocols employed.)

Discussion

In addition to confirming the effects of demethylchlortetracycline and oxytetracycline on basal Na^+ transport (that component of transport not attributable to a known natriferic hormone) [3], the present studies have shown that demethylchlortetracycline, but no oxytetracycline, inhibits aldosterone-mediated Na^+ transport in the toad urinary bladder. In addition, neither drug effects insulin-mediated Na^+ transport. Thus, demethylchlortetracycline has remarkably selective and specific effects on trans-epithelial Na^+ transport. Selectivity is shown by the fact that, despite the potent inhibition of both basal and aldosterone-mediated Na^+ transport (and an effect on aldosterone-mediated Na^+ transport that is independent of its effect on basal Na^+ transport), demethylchlortetracycline has no effect on either antidiuretic hormone- or insulin-mediated Na^+ transport in the toad urinary bladder under the conditions employed in the present and our previous [3] experiments. Specificity is shown by the fact that oxytetracycline, despite being a potent antibiotic tetracycline, has no effect on antidiuretic hormone-, aldosterone- or insulin-mediated Na^+ transport in the toad urinary bladder. Thus, demethylchlortetracycline may prove to be a valuable probe of the intracellular events involved in aldosterone-mediated Na^+ transport in renal epithelia.

The effects of aldosterone on Na^+ transport are usually not manifest for 30—60 min and lags of up to 2 h have been reported [16,17]. Aldosterone-mediated Na^+ transport can be abolished by a variety of inhibitors of protein synthesis [18,19] and therefore the lag period is thought to represent the time required for the synthesis of aldosterone-induced protein(s) [20—24]. Since in our experiments demethylchlortetracycline was added 2 h after the addition of aldosterone (when the aldosterone response was just becoming evident, and therefore, after aldosterone-induced protein synthesis had been initiated), our results are compatible with a post-synthetic site of action of the drug. In addi-

tion, since oxytetracycline had no effect on aldosterone-mediated Na^+ transport, it is unlikely that the inhibitory action of demethylchlortetracycline is due to a non-specific effect on protein synthesis. However, biochemical studies will be necessary to confirm this hypothesis.

If the predominant effect of demethylchlortetracycline in humans is to inhibit basal Na^+ transport in the distal nephron (analogous to its effect in the toad bladder), a natriuresis would be expected no matter what the clinical condition might be. On the other hand, it is possible that inhibition of the sodium-retaining effect of high circulating aldosterone levels is responsible for the production of a natriuresis with demethylchlortetracycline. If such is the case, our results may explain why a demethylchlortetracycline-related natriuresis is seen in patients with pathologic edema-forming states [4—10], which are often associated with secondary hyperaldosteronism, but is not prominent in normal subjects on a normal sodium intake [11] or in patients with the syndrome of inappropriate antidiuretic hormone secretion [12,13], when the much lower aldosterone levels are likely to be less important determinants of sodium excretion.

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