Water and Sodium Transport: Effects of Calcium Channel Blocker and Calmodulin Antagonists on the Apical and Basolateral Membranes of Amphibian Epithelia

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Abstract. Ca^{2+} channel blocker (sensit) and calmodulin antagonists (thioridazine, perphenazine, oxyprothepine) applied to the mucosal side of frog urinary bladder, weakened the response of epithelial cells to vasopressin. Thioridazine (2.7×10^{-5} mol.l^{-1}) and sensit (1.7×10^{-4} mol.l^{-1}) applied to the serosal side rapidly increased the permeability of the epithelia for sodium and potassium ions along the concentration gradient (from serosa to mucosa). The same concentrations of these blockers when applied to the mucosal side of frog urinary bladder selectively decreased vasopressin stimulated water permeability and did not influence ionic permeability. Both thioridazine and sensit decreased the short-circuit current across frog skin. The results show that the Ca^{2+} channel blocker and the calmodulin antagonists tested influenced water and ionic transport across the epithelial cell membranes, and had different effects upon the apical and the basolateral cell membranes.

Key words: Frog skin — Frog urinary bladder — Ionic and water permeabilities — Antidiuretic hormone — Thioridazine — Perphenazine — Oxyprothepine — Sensit

Introduction

In the recent years increasing information has accumulated concerning the importance of calcium as a regulator of intracellular processes, membrane stabilization and cell adhesion (Campbell 1983). Upon removing calcium ions from Ringer solutions at the serosal side of amphibian urinary bladder and

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simultaneous addition of EDTA water permeability rapidly increased (Hays et al. 1965) and the bladder cells became detached (Komissarchik et al. 1978). The presence of calcium has been shown to be unavoidable on the outer surface of epithelial cells of the apical membrane to enable ionic (Natochin 1985) and osmotic permeability changes as well as adequate responses of the cells to vasopressin (Natochin and Shakhmatova 1985). The observation that apical and basolateral membranes of the epithelial cells have different characteristics stimulated studies aimed at elucidating possible effects of Ca^{2+} channel blockers and calmodulin antagonists on both the serosal (Schlondorff et al. 1981; Grosso et al. 1982) and the mucosal membrane. In the present work the suggestion was tested that calcium-dependent processes, functionally important for the response to vasopressin and sensitive to the above inhibitors, exist in the outer side of apical membrane (Natochin 1985). The existence of such processes may be related to Ca^{2+} channels and calmodulin present in the apical border membrane, to the penetration of inhibitors to the cell through the membrane, or to the existence of calcium-dependent reaction in which macromolecules possible inactivated by Ca^{2+} channel or calmodulin inhibitors take place.

If these assumptions are valid, both Ca^{2+} channel blocker and calmodulin antagonists would act from the apical cell outer surface and would influence sodium and water transport as well as vasopressin action.

Materials and Methods

Experiments were performed on isolated urinary bladders and abdominal skin of the frog, *Rana temporaria*, of either sex, during February and March The osmotic permeability of the urinary bladder was determined by the method of weighing (Natochin and Shakhmatova 1966), this method consists of bathing the serosal side of the bladder with aerated Ringer solution (in mmol l^{-1} 111 NaCl, 2 38 $NaHCO_3$, 3 35 KCl, 0 8 $CaCl_2$, glucose 5.5, osmolality 225 mosm kg^{-1} H_2O) The mucosal side was bathed with the same solution, diluted 10-fold Vasopressin preparations (Pituitrin, Kaunas Factory of Endocrine Preparations, Arginine-Vasopressin, Spofa, synthetic Lysine-Vasopressin) were added to the bathing solution at the serosal side in a final concentration of 2 mU ml^{-1} In experiments with the inhibitors applied to the mucosal side, one half of the urinary bladder was filled with hypotonic Ringer solution, containing the drugs tested The other half of the bladder, which served as control, was filled with hypotonic Ringer solution only This protocol eliminated individual differences between experimental animals Isolated frog urinary bladders were put into the aerated Ringer solution, weighed every 30 minutes and the water transport along the osmotic gradient was measured and expressed in μl cm^{-2} min^{-1} The effects of the agents studied upon sodium transport was studied following the application of the drugs to the abdominal skin of the same frogs The potential difference (PD) and the short-circuit current (SCC) were determined by the method of Ussing and Zerahn (1951) in a modification by Ivanov and Natochin (1968) Experiments were performed according to 3 protocols 1) continual monitoring of the short-circuit current, 2) measurements of potential differences only; 3) determination of potential differences and measurement of SCC, lasting 5—7 seconds (once at least every 15 minutes) Calmodulin antagonists (Thioridazine, Spofa, Perphenazine, Chemapol; Oxyprothepine, Research Institute of Pharmacy and Biochemistry, Prague) and a Ca^{2+} channel blocker (Sensit, Chinoin) were dissolved in 96%

ethanol to give a concentration of 20 mg ml $^{-1}$ The drug solutions (0 1 ml) were added to the respective volume of normal (applied to the serosal) or hypotonic Ringer solution (applied to the mucosal side of the bladder) Final concentrations of the compounds used are given in Tables and Figures Control experiments were performed by adding the respective amounts of ethanol solutions to Ringer solution The results were statistically processed using Student's *t*-test, and expressed as mean ± S E M In some cases (indicated in Tables), distribution were compared using paired tests This latter method was used when unidirectional weak responses to the substances added were obtained Differences between initial and experimental values of water and Na^+ transport were marked Δ Results were expressed as $\Delta \pm m_{\bar{d}}$, and the differences were evaluated using Student's *t*-test (Urbakh 1963)

Results

The addition of vasopressin into Ringer solution bathing the serosal side of the frog urinary bladder was followed by an increased osmotic permeability and an increased water flow, the stimulated values being more than 20-times higher (Table 1). The application of a calmodulin antagonist thioridazine in a final concentration of 2.7×10^{-4} mol.l^{-1} to the solution at the mucosal side of the bladder only caused an slight increase in water absorption. At lower concentrations thioridazine, perphenazine, oxyprothepine and the Ca^{2+} channel blocker sensit, did not change the the basal level of water absorption (Table 1). The application of calmodulin antagonists resulted in a rapid decrease of the ability of vasopressin to stimulate water permeability (Table 1). Sensit applied in a concentration of 3.5×10^{-5} mol.l^{-1} to the serosal side decreased the response to vasopressin when applied to the mucosal side of the urinary bladder, sensit in the same concentration did not modify the effect of the hormone. A higher sensit concentration (1.7×10^{-4} mol.l^{-1}) inhibited the effect of vasopressin applied to the serosal side more rapidly. A distinct decrease of vasopressin action was observed after the application of sensit to the apical membranes of the cells (Fig. 1, Table 1). The application of 3.5×10^{-4} mol.l^{-1} of sensit into the urinary bladder completely abolished the response of the epithelium to 2 mU.ml^{-1} of vasopressin (Table 1). In the same experiments, when vasopressin concentration of the serosal side was raised to 20 mU.ml^{-1} during subsequent 30 minutes, a smaller increase in water permeability (from 0.079 ± 0.027 to 0.31 ± 0.08 μl.cm^{-2}.min^{-1}) ($n = 6$, $p < 0.02$) could be observed than after 2 mU.ml^{-1} vasopressin in control experiments (Table 1).

The processes underlying inhibitor-induced changes in the vasopressin-dependent water transport across frog urinary bladder under the influence of the inhibitors studied may include 1) a decreased osmotic permeability of the epithelial cell membranes, or 2) a rapid increase in permeability of the epithelia to sodium and chloride ions, and a resulting decrease of the osmotic gradient across the urinary bladder, resulting in turn in a drop of the driving force for water flow across the bladder. To analyse these possibilities, hypotonic Ringer

Table 1. Effects of calmodulin antagonists (thioridazine, perphenazine, oxyprothepine) and Ca^{2+} channel blocker (sensit) on the vasopressin stimulated water flow (expressed in µl cm ^{2} min^{-1}) Means ± S E M Significance levels to the respective paired control $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$

	Substance applied	Concentration mol l^{-1}	n	Side of application	Water absorption		
					Basal level 0—30 min	Vasopressin action	
						30—60 min	60—90 min
1a	control		9		0 036 ± 0 007	0 88 ± 0 11	1 30 ± 0 19
b	Thioridazine	2.7×10^{-4}	9	mucosal	0 29 ± 0 07^{b}	0 47 ± 0 17	0 47 ± 0 12^{b}
2a	control		10		0 065 ± 0 02	1 18 ± 0 15	0 92 ± 0 16
b	Thioridazine	2.7×10^{-4}	10	serosal	0 059 ± 0 019	0 098 ± 0 027^{c}	0 11 ± 0 028^{c}
3a	control		9		0 09 ± 0 029	0 88 ± 0 15	0 72 ± 0 092
b	Thioridazine	2.7×10^{-5}	8	mucosal	0 068 ± 0 019	0 54 ± 0 11	0 50 ± 0 10
4a	control		6		0 065 ± 0 009	1 08 ± 0 42	0 75 ± 0 19
b	Thioridazine	2.7×10^{-6}	6	mucosal	0 043 ± 0 014	0 94 ± 0 27	1 22 ± 0 27
5a	control		7		0 071 ± 0 02	1 51 ± 0 20	0 91 ± 0 093
b	Perphenazine	2.5 ± 10^{-4}	7	mucosal	0 115 ± 0 026	0 31 ± 0 16^{c}	0 15 ± 0 044^{c}
6a	control		8		0 048 ± 0 015	1 05 ± 0 28	1 16 ± 0 24
b	Perphenazine	2.5×10^{-5}	8	mucosal	0 059 ± 0 11	0 41 ± 0 06^{a}	0 67 ± 0 12
7a	control		7		0 102 ± 0 037	1 38 ± 0 26	1 27 ± 0 20
b	Oxyprothepine	10^{-4}	7	mucosal	0 073 ± 0 009	0 49 ± 0 11^{b}	0 51 ± 0 13^{b}
8a	control		7		0 13 ± 0 04	0 93 ± 0 21	1 0 ± 0 24
b	Oxyprothepine	10^{-4}	8	serosal	0 081 ± 0 034	0 62 ± 0 16	0 74 ± 0 23
9a	control		7		0 035 ± 0 006	1 12 ± 0 3	1 88 ± 0 35
b	Sensit	3.5×10^{-4}	6	mucosal	0 088 ± 0 02^{a}	0 078 ± 0 036^{b}	0 18 ± 0 065^{b}
10a	control		6		0 065 ± 0 014	1 10 ± 0 17	2 46 ± 0 33
b	Sensit	1.7×10^{-4}	7	mucosal	0 10 ± 0 02	0 30 ± 0 08^{b}	0 54 ± 0 14^{c}
11a	control		10		0 039 ± 0 008	1 45 ± 0 22	1 17 ± 0 15
b	Sensit	1.7×10^{-4}	10	serosal	0 052 ± 0 017	0 33 ± 0 07^{c}	0 33 ± 0 04^{c}
12a	control		6		0 14 ± 0 04	0 92 ± 0 21	0 86 ± 0 19
b	Sensit	3.5×10^{-5}	5	mucosal	0 079 ± 0 033	0 71 ± 0 18	0 81 ± 0 21
13a	control		10		0 071 ± 0 14	1 86 ± 0 25	1 22 ± 0 14
b	Sensit	3.5×10^{-5}	9	serosal	0 067 ± 0 012	0 55 ± 0 10^{c}	0 48 ± 0 07^{c}

solution containing thioridazine or sensit was given first to the urinary bladder for 60 minutes. The addition of vasopressin to the solution at the serosal side of the bladder was followed by a decreased response to hormone during the following 30 minutes (Table 2). The concentrations of sodium and potassium ions in the solution applied to the urinary bladder in experiments with thioridazine and sensit were identical with those in the solution applied to the control pair of bladders. These results suggest that the blockers used selectively decreased the osmotic permeability of the membrane from its mucosal side.

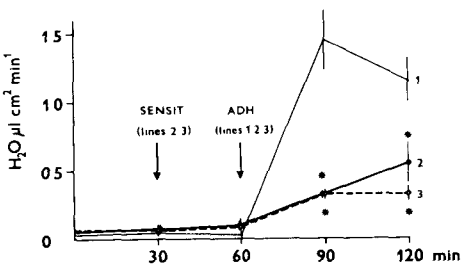

Fig. 1. Inhibition by sensit (1.7×10^{-4} mol l^{-1}) of the vasopressin (ADH) effect (2 mU ml^{-1}) on water permeability in frog urinary bladder Asterisks denote statistical significance ($p < 0.01$, line 2, $p < 0.001$, line 3) as compared to control experiments (line 1) Figures below the arrows indicate the addition of sensit to the solution at the mucosal (2) and serosal (3) side Vasopressin (ADH) was added to the serosal solution only 1-controls, 2-mucosal side, 3-serosal side

Different results were obtained after the application the same concentrations of the inhibitors to the serosal side of the bladder. Calmodulin antagonists induced a rapid increase in ionic permeability of the mucosal membrane of the urinary bladder, so that the concentrations of sodium and potassium on both sides were practically equilibrated within 30 minutes. When thioridazine concentration was decreased to 2.7×10^{-6} mol.l^{-1}, the response to vasopressin remained unchanged, and the ionic permeability did not increase (Table 1, 2). The Ca^{2+} channel blocker sensit showed an incomparably more intensive effect at the serosal side of the bladder as compared with sensit applied at the mucosal side, inducing a rapid and very high increase in ionic permeability as compared to the basal level (Table 2). After the application of sensit to the solution at the serosal side of the bladder, the concentrations of sodium and potassium on both sides of the bladder equilibrated within 30 min, due to an increase in ionic permeability. Under these conditions vasopressin obviously could not develop its effect due to a decreased osmotic gradient (Table 2). The Ca^{2+} channel blocker (sensit) and the calmodulin antagonists (thioridazine, perphenazine) also influenced the sodium transport across the frog skin cells. A gradual decrease of the potential difference and the short-circuit current was observed after the application of thioridazine (5.4×10^{-4} mol.l^{-1}) to the outer surface of the skin, where the apical membranes of cells with sodium channels are localized (Table 3). Sensit (3.5×10^{-4} mol.l^{-1}) applied to the inner side of the skin decreased both the potential difference and the short-circuit current (Table 3).

Table 2. Effects of calmodulin antagonist (thioridazine) and Ca^{2+} channel blocker (sensit) on water absorption and ionic concentration at the mucosal side of the frog urinary bladder (ADH — antidiuretic hormone, vasopressin) Means ± S E M

	Substance applied	Concentration mol l^{-1}	n	Side of application	Water absorption		Ionic concentration at the mucosal side	
					Basal 0—30 min	ADH 30—60 min	Na^+ mmol l^{-1}	K^+ mmol l^{-1}
1a	ADH		7		0 028 ± 0 005	1 69 ± 0 22	27 1 ± 2 5	0 96 ± 0 10
b	Thioridazine + ADH	$2\,7 \times 10^{-5}$	8	serosal	0 11 ± 0 02[b]	0 89 ± 0 13[b]	93 0 ± 9 4[c]	3 10 ± 0 2[c]
2a	ADH		7		0 065 ± 0 008	1 30 ± 0 15	29 0 ± 2 4	1 03 ± 0 10
b	Thioridazine + ADH	$2\,7 \times 10^{-6}$	7	serosal	0 24 ± 0 07[a]	1 51 ± 0 21	32 6 ± 2 1	1 10 ± 0 10
3a	Thioridazine + ADH	$2\,7 \times 10^{-5}$	8	mucosal	0 057 ± 0 011	0 50 ± 0 10	20 1 ± 1 12	0 61 ± 0 10
b	Thioridazine + ADH	$2\,7 \times 10^{-5}$	10	mucosal	0 088 ± 0 028	0 47 ± 0 10	22 7 ± 2 2	0 91 ± 0 10
4a	Sensit	$1\,7 \times 10^{-4}$	7	serosal	0 27 ± 0 04	0 36 ± 0 039	99 3 ± 1 0	3 3 ± 0 30
b	Sensit + ADH	$1\,7 \times 10^{-4}$	8	serosal	0 28 ± 0 05	0 35 ± 0 046	101 8 ± 1 0	3 2 ± 0 40
5a	Sensit	$1\,7 \times 10^{-4}$	6	mucosal	0 22 ± 0 036	0 19 ± 0 043	23 5 ± 2 7	0 63 ± 0 15
b	Sensit + ADH	$1\,7 \times 10^{-4}$	7	mucosal	0 09 ± 0 015[b]	0 23 ± 0 056	22 3 ± 3 3	0 65 ± 0 08

Basal concentrations in the solution at the mucosal side sodium 12 mmol l^{-1}, potassium 0 4 mmol l^{-1} Significance of differences to the respective paired control [a] $p < 0\,05$, [b] $p < 0\,01$, [c] $p < 0\,001$

Table 3. Effects of Ca^{2+} channel blocker (sensit) and calmodulin antagonists (thioridazine, perphenazine) on the potential difference (mV) and the short-circuit current (μA cm $^{-2}$) in frog skin Means ± S E M Significance levels [a] $p < 0\,05$, [b] $p < 0\,01$, [c] $p < 0\,001$

Substance applied	n	Side of application	Concentration mol l^{-1}	Time min	Potential difference		Short-circuit current	
					before	after	before	after
Sensit	10	mucosal	$3\,5 \times 10^{-4}$	32	64 0 ± 7 8	51 7 ± 7 5	21 9 ± 4 0	20 4 ± 3 1
	10	serosal	$3\,5 \times 10^{-4}$	60	62 5 ± 10	8 0 ± 1 8[c]	25 6 ± 3 4	13 4 ± 3 5[a]
	10	mucosal	7×10^{-4}	8—11	68 7 ± 8 2	51 0 ± 7 8	26 0 ± 3 2	15 2 ± 2 4[a]
					$\bar{\Delta} \pm m_{\bar{\Delta}}$	17 6 ± 3 0[b]	$\Delta \pm m_{\bar{\Delta}}$	10 1 ± 1 8[c]
Thioridazine	14	mucosal	$5\,4 \times 10^{-4}$	30	90 9 ± 7 9	59 5 ± 8 8[a]	31 9 ± 3 5	17 4 ± 2 2[b]
Perphenazine	5	mucosal	$2\,5 \times 10^{-4}$	30	62 1 ± 9 1	90 0 ± 9 6	30 8 ± 2 8	26 5 ± 3 1

Following the application of the substances the parameters studied were monitored until a stabilization reached

The same concentration of sensit had no effect from the outer side of the skin. When applied to the outer side, sensit (7×10^{-4} mol.l^{-1}) decreased the short-circuit current. The decrease of the potential difference was significant (paired test, see Methods).

The effect of perphenazine on the short-circuit current was not significant when the drug was applied from the outer side of the skin. The potential showed a suggested increasing tendency. Our results showed that the calcium channel blocker (sensit) and the calmodulin antagonist (thioridazine) were able to inhibit sodium transport measured in terms of the short-circuit current level. These effects were due to the actions of the drugs studied on the sodium pump (when the blocker acted from the inner side of the frog skin only) or on the macromolecule of the sodium channel (when the blocker acted from the outer side of the epithelium).

In both cases, these effects may results from a modification of the calcium--dependent parts of the sodium transporting system.

Discussion

Much convincing evidence has accumulated concerning the role of calcium ions in different steps of vasopressin action and in the control of osmotic permeability of the urinary bladder. The removal of calcium ions from the solution at the serosal side was accompanied by a high increase in the permeability of the bladder for water (Hays et al. 1965). At the same time, the application of a calcium free solution to the mucosal side, or even removal of calcium ions from the premembrane layers using EDTA, did not induce any increase in osmotic permeability (Natochin and Shakhmatova 1985). These findings suggest different roles of calcium ions in the extracellular environment on both sides of the cell. Penetration of calcium ions into the cell is considered as an important process. Experiments with the application of Ca^{2+} ionophore, A23187, showed that the accelerated penetration of calcium ions through the basolateral membrane side to the cytoplasm was accompanied by an increase in osmotic permeability (Hardy 1978), and that the passage of calcium ions across the apical membrane into the cell was accompanied by a block of the vasopressin action (Natochin and Shakhmatova 1981). The process of Na/Ca exchange (Taylor 1981) and changes in the conformation of the calcium-dependent proteins (Ausiello et al. 1984) are considered to be of importance for the regulation of osmotic permeability and for vasopressin activity in amphibian bladder cells. The inhibition of the vasopressin effect by trifluoperazine (a calmodulin antagonist) applied from the serosal side of the bladder (Levine et al. 1981; Svelto and Casavola 1984) confirmed this assumption, suggesting a role of calmodulin in the reaction cascade of the cell to hormone after cAMP production (Barber

and Taylor 1984) In the present work the possible effects of a Ca^{2+} channel blocker and calmodulin antagonists after their application to the outer surface of the apical membrane on water permeability and sodium transport were investigated Results of previous experiments showed that the removal of calcium ions or the addition of verapamil to the outer surface of the urinary bladder, decreased the response of cells to vasopressin and inhibited the hydroosmotic effect of the hormone (Natochin and Shakhmatova 1985) It was shown that frog skin cells responded to the application from the outer side of the apical membrane of Ca^{2+} channel blockers of inorganic origin (cobalt ions, nickel ions) by increasing the SCC levels and the potential difference, while D-600 decreased SCC and PD (Natochin 1985) A new calcium blocker, nisolidipine, stimulated SCC (Wiederholt et al 1984), while verapamil inhibited it (Bentley 1974) It can be supposed that calcium ions participate in the regulation of osmotic permeability and of sodium transport when applied to the outer side of the apical membrane of epithelial cells

Our results have shown that the sensitivity of cells to the Ca^{2+} channel blocker and calmodulin antagonists studied is essentially different depending on whether the inhibitor is applied to the solution bathing the serosal or the mucosal side of the bladder The application of sensit to the solution at the serosal side of the membrane induced a decrease of the hydroosmotic effect of vasopressin A similar effect was observed after the application of calmodulin antagonists thioridazine, perphenazine and oxyprothepine

Two possible explanations of the mechanism of action of these substances seem to be possible 1) Due to their hydrophobicity, these drugs penetrate the apical membrane, where they can inactivate the Ca^{2+} channels and calmodulin present at the inner surface of this membrane As a result, calcium-dependent processes of water permeability of the apical membrane become changed, 2) The inhibitors used have the ability to inactivate calcium-dependent components in the outer side of apical membrane in sites characterized by a similarity of molecular organization with calmodulin and a high affinity to calcium Calcium ions may thus be involved in biochemical reactions taking place in the apical membrane and making water permeability to increase

Difference in concentrations of inhibitors used in the solutions bathing the serosal and mucosal surface of the membranes respectively and the qualitative differences in their effects obtained on the respective side also support the above considerations Both thioridazine and sensit in lower concentrations inhibited the vasopressin-induced stimulation of water flow when applied to the serosal side Experimental analysis of these results suggested that the changing ionic permeability might be the driving force for the movement of sodium and potassium ions from the serosal side of the epithelium to the solution at the mucosal side, resulting in the equilibraiton of the ionic concentrations The

effects of the blockers studied depend on changes induced in the cell This conclusion is based on our previous results indicating that the effect of vasopressin applied from the serosal side is inhibited only by lipophilic derivates of phenothiazine containing tertiary amine groups (Natochin and Magazanik 1964) Thioridazine and sensit are suggested to destroy the ionic permeability of membranes, affecting the cascade of intracellular calcium-dependent reactions The other effect of these inhibitors is due to their ability to decrease the response to vasopressin and to inhibit water flow

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