Microelectrode Study of Insulin Effect on Apical and Basolateral Cell Membrane of Frog Skin: Comparison with the Effect of 1-Deamino-8-D-Arginine-Vasopressin (dDAVP)

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Abstract. The standard microelectrode technique was used to electrophysiologically characterize the effect of insulin on both apical and basolateral cell membranes of the frog skin, a model Na-transporting epithelium. Insulin applied to the basolateral side of the epithelium stimulated the sodium transport as shown by both increased short-circuit current, I_{sc}, ($P < 0.02$) and transepithelial potential difference, V_{oc}, ($P < 0.002$). Potential difference across the apical cell membrane, V_o, decreased ($P < 0.002$) as did the apical cell membrane resistance, R_o, ($P < 0.05$). The driving force of sodium ions, E_{Na}, increased after insulin ($P < 0.005$). These findings confirm that insulin acts both to increase the apical cell membrane permeability for ions and to stimulate the sodium pump in the basolateral membrane. The effects of insulin were compared with those of a vasopressin analog (dDAVP), known to stimulate transepithelial sodium transport by increasing the permeability of the apical cell membrane for sodium ions. dDAVP applied at the height of insulin effect further stimulated transepithelial transport, but insulin applied at the height of dDAVP action did not. It is concluded that the direct stimulation of the sodium pump by insulin may not represent a decisive component in the stimulation of transepithelial transport across the frog skin. A more potent stimulus for sodium transport is obviously the increased permeability of the apical membrane for ions.

Key words: Frog skin — Cell membranes — Electrophysiological characterization — Intracellular microelectrode — Insulin — dDAVP

Introduction

In nonexcitable tight epithelia, such as the frog skin, insulin was found to

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stimulate transepithelial transport under conditions of cell depolarization due to increased permeability of the apical membrane for sodium ions (Schoen and Erlij 1987). It was therefore suggested that insulin influences sodium transport in these epithelia by stimulating Na,K-ATPase, the sodium pump, as well as by enhancing the permeability of the apical cell membrane for ions, but no measure of the pump stimulation by insulin has been presented in experiments using the intracellular microelectrode technique.

The present experiments were aimed at studying electrophysiologically the insulin action on sodium transport across the frog skin. The standard microelectrode technique was used to characterize the changes in cell membranes. The effect of insulin was found to be similar to that of 1-deamino-8-D-arginine-vasopressin (dDAVP), a synthetic analog of vasopressin. This analog is a known potent stimulator of sodium transport which acts in the same manner as does arginine- or lysine-vasopressin, i.e. it increases the apical cell membrane permeability (Bakoš et al. 1988).

It has therefore been suggested that the decisive component in the stimulation of transepithelial sodium transport across the frog skin by insulin is an increase of apical cell membrane permeability. The simultaneous stimulation of the sodium pump creates the necessary condition for sodium ions to move out of the cells. Interesting, yet not understood, is the increase in shunt pathway resistance observed during the action of insulin on the frog skin.

V_t, transepithelial potential difference
V_{oc}, open-circuit transepithelial potential (V_t at $I_{sc} = 0$)
I_{sc}, short-circuit current at zero transepithelial potential ($V_t = 0$)
V_o, potential across the apical (outer) cell membrane at $V_t = 0$
V_i, potential across the basolateral (inner) cell membrane at $V_o = 0$ ($V_t = V_i$ at $V_o = 0$ since $V_o + V_i = V_t$)
E_{Na}, driving force of the transepithelial sodium transport
R_s, ohmic resistance of the shunt pathways
R_t, transepithelial ohmic resistance
R_{Na}, ohmic resistance of the activite sodium transport pathway
R_o ohmic resistance of the apical (outer) cell membrane
R_i, ohmic resistance of the basolateral (inner) cell membrane
fR_o, fraction resistance of the apical (outer) cell membrane expressed as parcentage of R_{Na} ($R_{Na} = R_o + R_i$)

Materials and Methods

Animals and experimental setup: Experiments were performed within March—May on abdominal skins of European frogs (*Rana temporaria*) of both sexes. The frogs were kept at +5 °C until the day of the experiment. The animals were pithed and the skins were stripped and mounted between

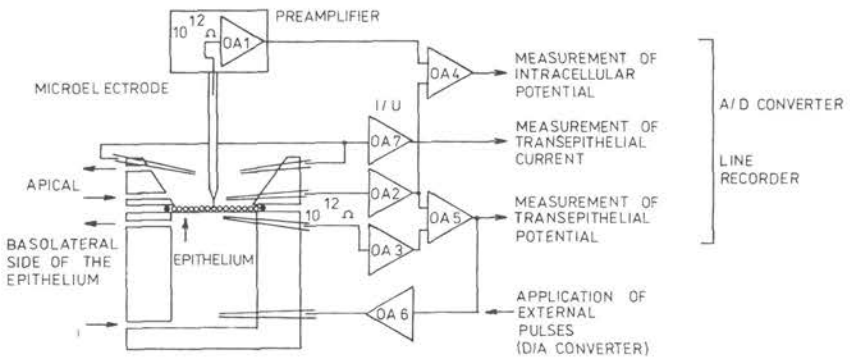

Fig. 1. Schematic representation of the intracellular microelectrode technique based of four-electrode voltage clamp. The apical side bathing solution, measured by OA2, is the electrical reference for the intracellular microelectrode signals. OA = operational amplifiers; those measuring potential have high input impedance (OA1, OA2, OA3); I/U = current to voltage converter for measuring the short-circuit current as voltage; EPITHELIUM = entire untreated frog skin used in the present experiments, represented, for the sake of convenience, as a single layer; other details are explained in Materials and Methods. (Redrawn after Ponec et al. 1989, with permission.)

two parts of a chamber perfused with aerated recirculating Ringer solution (Bentley 1958). The experiments were performed at room temperature (20—22 °C). The perfusion chamber and the electronics were modified (Ponec et al. 1989) as described elsewhere (Nagel 1976; Higgins et al. 1977; Helman and Fisher 1977). In brief, the skin was oriented horizontally and based on a supportive grid. The edges of the epithelial preparation in contact with the parts of the perfusion chamber (as well as edges of the supportive grid) were embedded in high-grade silicone grease to prevent electrical leaks and to cover up the possibly damaged edges.

Standard glass microelectrodes of borosilicate glass tubes with two inner filaments (Glass Factory, Bratislava, Czechoslovakia) were pulled using a vertical puller (Ukrainian Academy of Sciences Kiev, USSR). Microelectrodes with a tip diameter of approx. 1 μm were used. They were impaled perpendicularly from the apical (outer) side of the skin with the aid of a mechanical micromanipulator (Leitz, FRG). The quality of the microelectrode impalements was evaluated according to the criteria published by Tang et al. (1985).

The electronics of the experimental layout was based on four-electrode voltage clamp with the fifth electrode measuring the intracellular potential (=potential across the apical [outer] cell membrane) (Fig. 1). The voltage clamp electronics was on-line connected with a microcomputer through D/A and A/D converters allowing the application of trains of transepithelial pulses and simultaneous sampling of the responses of the epithelium. Transepithelial pulses between -180 and $+200$ mV were used in 20 mV steps. Duration of the pulses until data sampling was 600 ms to avoid polarization effects on the basolateral membrane at the onset of the pulse (Schoen and Erlij 1985, 1987). Standard formulae were employed for data evaluation and for calculation of electrophysiological characteristics of the epithelium from current-voltage relationships (Ussing and Zerahn 1951; Helman 1975; Issacson 1977; Nagel 1978). For statistical analysis Student's *t*-test for paired data and the Wilcoxon matched-pairs signed-ranks test were used.

Experimental protocol: After mounting the skin in the chamber time was allowed until basal

Table 1. Effect of insulin on I_{sc}, V_{oc}, V_o, V_i and E_{Na}. Values are means ± SEM (ranges are shown). Statistical significance was calculated by means of Student's *t*-test for paired data. For symbols see Introduction. Number of experiments $n = 9$.

		Control (Range)	Insulin (Range)	Significance level
I_{sc}	[$\mu A \cdot cm^{-2}$]	-40.8 ± 6.3 (-68.1 to -4.8)	-51.6 ± 6.5 (-74.1 to -8.7)	$P < 0.02$
V_{oc}	[mV]	44.9 ± 6.7 (7.1 to 70.8)	-58.7 ± 7.1 (13.2 to 82.3)	$P < 0.002$
V_o	[mV]	-52.7 ± 2.6 (-70.0 to -45.3)	-43.7 ± 2.0 (-52.9 to -36.1)	$P < 0.002$
V_i	[mV]	82.6 ± 2.3 (75.3 to 95.7)	81.3 ± 4.2 (57.1 to 100.9)	n.s.
E_{Na}	[mV]	109.9 ± 6.6 (92.1 to 156.6)	122.1 ± 6.1 (92.9 to 161.7)	$P < 0.005$

Table 2. Effect of insulin on R_s, R_t, R_{Na}, R_i and fR_o. Data presentation as in Table 1. For symbols see Introduction. Number of experiments $n = 9$.

		Control (Range)	Insulin (Range)	Significance level
R_s	[ohm . cm^2]	2160 ± 306 (979 to 3532)	2487 ± 346 (1207 to 4607)	$P < 0.02$
R_t	[ohm . cm^2]	1193 ± 128 (568 to 1600)	1196 ± 112 (695 to 1560)	n.s.
R_{Na}	[ohm . cm^2]	2619 ± 291 (1352 to 3714)	2238 ± 234 (1252 to 3117)	$P < 0.05$
R_o	[ohm . cm^2]	1692 ± 216 (815 to 2526)	1229 ± 144 (674 to 1779)	$P < 0.05$
R_i	[ohm . cm^2]	927 ± 127 (507 to 1449)	1008 ± 115 (578 to 1512)	n.s.
fR_o	[%]	63.8 ± 2.6 (58.2 to 83.3)	54.3 ± 2.2 (45.9 to 69.0)	$P < 0.01$

parameters stabilized. Then several impalements and control measurements were done. Insulin (Calbiochem, in some experiments SPOFA, of identical characteristics) was added to the solution bathing basolateral (inner) side of the skin. The final concentration of insulin was 2×10^{-5} mol . l^{-1} ($n = 9$ experiments). As soon as insulin reached its maximum effect on the short-circuit current, 1-deamino-8-D-arginine-vasopressin (dDAVP, SPOFA) was added in some experiments to the basolateral (inner) side of the epithelium ($n = 5$ experiments), to a final concentration of 10^{-5} mol . l^{-1}. In another series of experiments ($n = 6$) the agents were added in an inverse sequence: dDAVP (10^{-5} mol . l^{-1}) was applied first and after reaching maximal stimulation, insulin (2×10^{-5} mol . l^{-1}) was added to the solution at the inner side. The cells were impaled and current-voltage data were

Table 3. Effect of dDAVP applied at the height of insulin action on I_{sc}, V_{oc}, R_{Na}, V_o and R_o. Values are means ± SEM. Statistical significance was calculated by means of the Wilcoxon matched-pairs signed-ranks test. For symbols see Introduction. Number of experiments $n = 5$.

		1 Control	2 Insulin	3 Insulin + dDAVP	Signif. ($P < 0.05$)
I_{sc}	[μA . cm^{-2}]	−34.6 ± 9.7	−50.1 ± 12.5	−60.1 ± 10.9	1 : 2 1 : 3 2 : 3
V_{oc}	[mV]	30.5 ± 7.1	43.8 ± 8.2	45.0 ± 5.9	1 : 2 1 : 3
R_{Na}	[ohm . cm^2]	2251 ± 400	1603 ± 273	1488 ± 255	1 : 2 1 : 3 2 : 3
V_o	[mV]	−57.7 ± 4.8	−48.8 ± 3.2	−41.7 ± 2.6	1 : 2 1 : 3 2 : 3
R_o	[ohm . cm^2]	1587 ± 350	967 ± 192	792 ± 112	1 : 2 1 : 3 2 : 3

sampled predominantly during the phase of maximal effects of the hormones. The concentrations of both hormones were chosen to cause maximal stimulation of the sodium transport across the frog skin.

Results

The effect of insulin

Insulin applied to the basolateral (inner) side of the frog skin caused a significant increase in short-circuit current (I_{sc}), ($P < 0.02$), and raised the transepithelial potential difference, measured under open-circuit conditions (V_{oc}), ($P < 0.002$). The potential across the apical (outer) cell membrane (V_o) decreased significantly ($P < 0.002$), but that across the basolateral (inner) cell membrane remained unchanged. The driving force of transepitelial sodium transport (E_{Na}) increased ($P < 0.005$) after the application of insulin (Table 1).

A significant increase of shunt pathway resistance (R_t) did not change due to the proportional increases in both the short-circuit current (I_{sc}) and the transepithelial potential (V_{oc}). The significant decrease in active sodium transport pathway (R_{Na}), ($P < 0.05$), correlated with the increased transepithelial

Table 4. Effect of insulin applied at the height of dDAVP action on I_{sc}, V_{oc}, R_{Na}, V_o and R_o. Data presentation as in Table 3. For symbols see Introduction. Number of experiments $n = 6$.

		1 Control	2 dDAVP	3 dDAVP + Insulin	Signif. ($P < 0.05$)
I_{sc}	[μA . cm^{-2}]	-35.3 ± 9.4	-61.4 ± 13.9	-63.6 ± 9.9	1 : 2 1 : 3
V_{oc}	[mV]	43.2 ± 7.6	57.0 ± 6.8	57.7 ± 6.8	1 : 2 1 : 3
R_{Na}	[ohm . cm^2]	3527 ± 972	1899 ± 385	1908 ± 427	1 : 2 1 : 3
V_o	[mV]	-61.5 ± 3.5	-43.4 ± 2.2	-44.2 ± 3.9	1 : 2 1 : 3
R_o	[ohm . cm^2]	2321 ± 643	995 ± 202	913 ± 205	1 : 2 1 : 3

sodium transport measured as an increased short-circuit current (I_{sc}). The resistance of the apical (outer) cell membrane (R_o) decreased ($P < 0.05$) as did its percentual share (fR_o) in the total transcellular resistance ($P < 0.01$) (Table 2).

The effect of additive dDAVP application

The addition of 1-deamino-8-D-arginine-vasopressin (dDAVP) at the height of the insulin effect further increased the short-circuit current (I_{sc}), but not the open-circut transepithelial potential difference (V_{oc}). (For statistical significance of the additive dDAVP effect see the 2 : 3 significance in Table 3; group 2 = insulin, group 3 = insulin + dDAVP). The active sodium transport pathway resistance (R_{Na}) further decreased after the application of dDAVP. The potential across the aplical (outer) cell membrane (V_o) was also further decreased by dDAVP as well as the resistance across the apical (outer) cell membrane (R_o). Other electrophysiological parameters (see Introduction) were not significantly changed by dDAVP and they have thus been omitted from Table 3.

The effect of additive insulin application at maximal dDAVP effect

In contrast to the effect of dDAVP applied after insulin, the inverse application sequence did not significantly change any of the measured or computed pa-

rameters. The differences between group 2 (dDAVP) and group 3 (dDAVP + insulin) in the parameters shown in Table 4 were insignificant as were those in other parameters between group 2 and group 3 (not shown).

Discussion

Previous studies characterized insulin as an agent which primarily stimulates the Na-extrusion from the cells into the fluid at the basolateral side of the toad bladder (Herrera et al. 1965; Siegel and Civan 1976). Recent data have shown that insulin also increases apical cell membranes permeability for sodium (Cobb et al. 1986) and depolarizes the cells of the frog skin (Civan et al. 1987; Schoen and Erlij 1987). To reevaluate both these modes of action of insulin on sodium transport across the frog skin, the cumulative effects of supramaximal doses of insulin and a vasopressin analog (dDAVP) on the stimulation of sodium ion transport were analyzed. This procedure was chosen as it is known that if two hormones share a common mechanism of action, a supramaximal dose of either hormone prevents the functional expression of the other one. If they act by independent mechanisms, an additive effect might be expected (Cox and Singer 1977). Thus, the cumulative effect of the vasopressin analog added to the frog skin after insulin, observed in our experiments, would suggest at least partly different mechanisms of action. dDAVP acting through increasing permeability for sodium ions in the apical cell membrane with no direct effect on Na,K--ATPase in the basolateral cell membrane, was found to further decrease resistance of the apical cell membrane (R_o). This action of dDAVP induces further depolarization of the epithelial cells (V_o). These findings suggest a further increase of apical cell membrane permeability for sodium ions thus creating conditions for a further stimulation of transepithelial transport (increased short-circuit current, I_{sc}, decreased resistance of the active sodium transport pathway, R_{Na}) (Table 3). On the other hand, insulin applied at the height of dDAVP effect did not further change any of the parameters of the ion transport characteristics (Table 4).

These findings suggest that supramaximal stimulation of sodium transport by insulin involves both an increase in apical cell membrane permeability and stimulatin of the sodium pump in the basolateral membrane. However, the same degree of transepithelial transport induced by dDAVP was obtained only by primarily increasing the apical cell membrane permeability. Although a direct stimulation of the sodium pump may contribute to the sodium transport increase, it needs not represent a decisive component in the stimulation of the transport across the frog skin. A more potent stimulus for the transepithelial sodium transport in the frog skin is the increase in the apical cell membrane

permeability for sodium ions. This might be the reason why dDAVP applied at the height of the insulin action further stimulated the transepithelial transport but insulin applied after dDAVP was ineffective in further increasing the apical cell membrane permeability leaving the transepithelial ion transport unchanged.

Increased driving force of sodium ions (E_{Na}) is considered as evidencing a direct stimulation of sodium pump (Siegel and Civan 1976). A recent paper by Schoen and Erlij (1987) based on results obtained with the microelectrode technique had not brought a similar evidence concerning direct pump stimulation by insulin in the frog skin. This evidence is being provided in the present work using the intracellular microelectrode technique: E_{Na} was observed to increase after insulin application to the frog skin (Table 1).

The stimulation of sodium pump activity in the basolateral membrane, observed in our experiments, was not accompanied with changes of basolateral membrane resistance (R_t, Table 2). No changes of the resistance of the basolateral membrane were observed during the stimulation of Na-K-ATPase by aldosterone in toad bladder (Palmer and Speez 1986). However, the conductance of the basolateral membrane increased in parallel with E_{Na} when aldosterone was applied to toad skin (Nagel and Crabbé 1980). These facts taken together may indicate that the relationship between the sodium pump stimulation and either basolateral membrane resistance or conductance might be of a more complex nature. Hence also E_{Na} is a complex parameter depending on energy and kinetic (permeability) factors of the transport system (Concha et al. 1987). Proportional changes in short-circuit current (I_{sc}) and tissue conductance yield unchanged E_{Na} (Yonath and Civan 1971). In the present experiments the short-circuit current increased by 26.5 per cent whereas the transepithelial resistance (R_t, the reciprocal value of conductance) changed by less that 1 per cent. If this approach different from origin calculations is employed, it also yields an increased value of E_{Na}.

Inhanced biosynthesis of Na-K-ATPase was observed but after several (5—6) hours after toad bladder treatment with aldosterone (Geering et al. 1982; Park and Edelman 1984). It could be inferred that also the first phase of insulin action involves stimulation of the existing pump sites rather than interference with the biosynthesis of enzyme protein.

It is believed that insulin is biologically active in its monomeric form (Ramesh and Bradbury 1987). On the other hand, insulin readily forms dimers when in higher concentrations (Pekar and Frank 1972). Other possible interactions of insulin can occur with various materials during handling and application of insulin solutions (Hefford et al. 1984). Bearing the above in mind, the concentration of insulin in our experiments was chosen so that it maximally stimulated the short-circuit current and increasing its concentration had no additional effect.

Insulin was found to increase the shunt pathway resistance (R_s) (Table 2). The shunt pathway resistance in the frog skin, and also in our experimental layout, is principially represented by resistances of both the paracellular junctions and glandular cells (Lindemann and Voûte 1976). The junction resistance could be influenced by a variety of experimental maneuvers such as changing composition of solutions (Sansom et al. 1984; Hunter et al. 1987), changing transepithelial potential (DiBona 1985), or drug applications (O'Neil and Helman 1976; Neyton and Trautmann 1986; Concha et al. 1987; Bentzel et al. 1987; Fidelman and Watlington 1987). As the ionic and osmotic composition in our experiments did not change, the raised resistance of the shunt pathway could be ascribed to the effect of insulin. However, the functional significance of the increased shunt pathway resistance may not be as simple to explain as our model does not allow distinguishing the different nature of the shunts.

As far as other tissues are concerned (muscle, adipose tissue etc.), insulin causes hyperpolarization in most of them, except for liver (for a review see Zierler 1985). Cell hyperpolarization is secondary to Na,K-ATPase stimulation (for a review see Resh 1985). In the frog skin this occurs at the basolateral cell membrane. However, the result of the insulin action is cell depolarization secondary to the simultaneous increase of the apical cell membrane permeability for ions. By its functional properties the frog skin evidently resembles the renal tubule (Hierholzer 1985) rather than any other tissue.

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