Influence of Salmon Melanin Concentrating Hormone on Vasopressin Analogue (dDAVP) Activity and Sodium Transport in Frog Skin

M. SMRIGA, P. BAKOŠ and D. JEŽOVÁ

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia

Abstract. Salmon melanin-concentrating hormone (sMCH) is a peptide known to regulate skin pigmentation both in fish and tetrapod (frog and lizard). To evaluate the influence of sMCH on ionic transport in frog skin, standard voltage-clamp technique for the measurement of transepithelial short-circuit current (I_{sc}) reflecting net sodium transport was used. It was found that sMCH alone applied at concentrations of 0.5; 5 or 10 μmol/l failed to influence I_{sc}. The application of 5 μmol/l of sMCH, however, inhibited I_{sc} across the skin stimulated by a synthetic analogue of vasopressin (dDAVP), whereas no influence on natriferic effect of 1 μmol/l forskolin by the studied peptide was observed. The results indicate that cAMP was presumably not involved in the mediation of sMCH action in frog skin. We assume that the interaction of sMCH with the basolateral membrane could lead either (1) to changes of membrane structure including organization of its lipid surrounding or (2) to modification of AVP/dDAVP receptor activity and binding capacity. The nature of these interactions and change(s) in cell membrane and signal(s) which trigger processes responsible for the inhibitory effect of sMCH on dDAVP-stimulated frog skin sodium transport remains to be elucidated.

Key words: Melanin-concentrating hormone — Short-circuit current — Arginine-vasopressin analogue — Forskolin — Frog skin

Introduction

Salmon melanin-concentrating hormone (sMCH) is a cyclic heptadecapeptide, first isolated from salmon pituitary glands (Kawauchi et al. 1983), which is known to stimulate melanosome (melanin granule) aggregation to a perinuclear position within integumental melanocytes, resulting in lightening of the fish skin. On the

Correspondence to: Daniela Ježová, Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, 833 06 Bratislava, Slovakia

other hand, sMCH stimulates melanosome dispersion in frogs and lizards (Matsunaga et al. 1992). Different activities of sMCH in two different epithelia (fish and frog) appear to be attributable to distinct regions of the hormone (Lebl et al. 1989). In addition, sMCH was reported to inhibit ACTH and cortisol secretion in teleost fish (Baker and Rance 1981), while a related peptide, rat MCH, had an opposite action in rats (Ježová et al. 1992).

Cellular pathways and mechanisms responsible for the action of sMCH are far from being understood. Visconti et al. (1989) investigated ionic requirements for receptor activation by sMCH in fish. They found that calcium ions, but not extracellular sodium or tetrodotoxin-sensitive sodium channels, play an important role in sMCH activated melanosome movement.

The influence of sMCH on colour changes in frog epithelia is different from its effect in fish, probably requiring other ionic conditions, and is identical with the role of α-melanocyte stimulating hormone found in frog skin (Hruby et al. 1987, Matsunaga et al. 1992). We therefore decided to study the influence of sMCH on electrical parameters and the ionic transport in frog skin (predominantly represented by sodium). In accordance to a pilot work of Ussing and Zerahn (1951) and the hypothesis of Koefoed-Johnsen and Ussing (1958) considering the frog skin epithelium as a two-membrane system, the unidirectional sodium transport is realized in two stages. When sodium influx is increased through the amiloride-sensitive sodium channels in the apical site, sodium is accumulated in the skin interior, thus starting its outflow through Na/K-ATPase in the basolateral membrane.

There is a wide range of factors influencing the function of sodium channels (e.g. protein kinase C, Ca^{2+}, intracellular sodium). Some physiologically important substances, among others vasopressin, act in frog skin via accumulation of cAMP content. Increase in cAMP level causes rise in permeability for sodium ions, probably via phosphorylation of gating sites for sodium in the apical membrane. Svensson et al. (1991) showed that pigment changes in fish stimulated by sMCH were associated with a significant reduction in cAMP content. Hence we concentrated on the study of the effects of sMCH in combination with two substances acting in frog skin through activation of cAMP. Both 1-deamino-8-D-arginine-vasopressin (dDAVP) and forskolin are known stimulators of sodium transport (Bakoš et al. 1984, Castillo et al. 1990). While dDAVP operates through membrane receptors, forskolin bypasses receptors.

Materials and Methods

Experiments were performed on the skin of common frogs (*Rana temporaria*) of both sexes, which were kept in tap water at 4 °C. Abdominal skins were stripped and mounted between two halves of an Ussing type chamber (area = 2 cm^2), perfused with Ringer solution (Bentley 1958) and continuously bubbled with air to provide stirring and oxy-

genation. To prevent electrical leaks, the space between the skin and chamber was sealed by a thin layer of high-grade silicon gel. The volume of each halfchamber was 2 ml. A voltage clamp was used continuously to maintain the skins at a transepithelial voltage of zero, thereby permitting to monitor the short-circuit current (I_{sc}) and open-circuit transepithelial potential difference (V_{oc}). Transepithelial ohmic resistance of skin (R_t) was estimated according to the Ohm's equation. Electrophysiological characteristics of epithelium were determined using a system of Ag-AgCl electrodes (Ponec and Bakoš, unpublished data) and recorded every 10 minutes, plus 5 minutes before and after the addition of the agent tested.

Each experimental procedure started with a 20–30 min period during which all electrical parameters were allowed to stabilize. After this period the agent under study was added to the basolateral side of one halfskin. The second halfskin from the same animal was used for the control of electrical parameters stability and solvent effects. Volumes of 20–60 μl of the tested substances were applied reaching the required final concentrations. Experiments were done at room temperature. Salmon MCH was synthesized by Dr. J. Rivier from Salk Institute for Biological Studies, USA and dissolved in saline. Solution (0.1 mg/ml) of dDAVP (Adiuretin) was purchased from SPOFA (Czechoslovakia). Forskolin obtained from SIGMA was dissolved in 40% ethanol. The volume of ethanol never exceeded 0.4% (v/v) of the bath volume. Though this concentration of the solvent failed to influence the electrical parameters measured, the same amount of ethanol was added to the control halfskins in all experiments involving forskolin administration. The results are expressed as means ± S.E.M. The data were statistically evaluated using one way analysis of variance and pairwise comparisons according to Dunnett (1955) and Dunn (1976) as appropriate.

Table 1. Effect of salmon melanin-concentrating hormone (sMCH) on absolute values of short-circuit current (I_{sc}), open-circuit transepithelial potential difference (V_{oc}) and transepithelial ohmic resistance (R_t) determined 60 min after addition of the hormone. Abdominal frog skins were stripped and mounted between two Ussing type chambers, perfused with Ringer solution and continuously bubbled with air. Each experiment started with a control-period (20–30 min) during which all electrical parameters were allowed to stabilize. After this period sMCH was added to the basolateral bathing solution. Both I_{sc} and V_{oc} were recorded every 10 min using a system of Ag-AgCl electrodes. Transepithelial ohmic resistance (R_t) was estimated according to the Ohm's equation. Means of 6 values ± S.E.M.

	I_{sc} [μA.cm^{-2}]	V_{oc} [mV]	R_t [Ohm.cm^2]
control	49.2 ± 9.4	86.7 ± 13.0	1661 ± 245.0
sMCH (0.5 μmol/l)	47.5 ± 13.5	72.1 ± 12.8	1410 ± 220.5
control	44.8 ± 11.5	72.7 ± 15.0	1674 ± 260.0
sMCH (5 μmol/l)	40.0 ± 10.8	58.9 ± 16.1	1447 ± 233.0
control	42.3 ± 14.3	72.0 ± 13.7	1639 ± 310.0
sMCH (10 μmol/l)	39.1 ± 14.6	59.8 ± 15.8	1329 ± 313.0

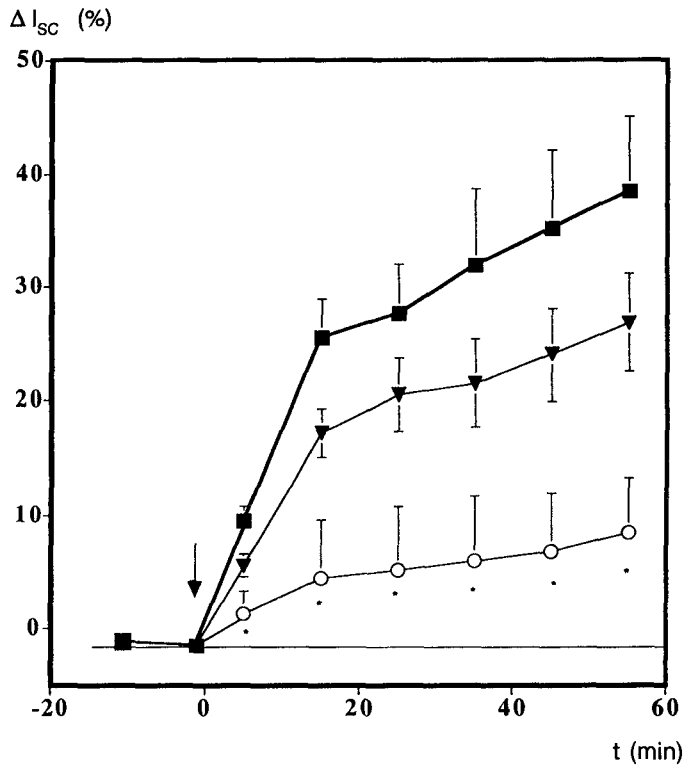

Figure 1. Comparison of the effect of 1-deamino-8-D-arginine-vasopressin (dDAVP) and cumulative effect of dDAVP + salmon melanin-concentrating hormone (sMCH) on short-circuit current (I_{sc}) across the frog skin, expressed as percentage difference between the basal and stimulated levels (Δ %). Arrow indicates the time of addition of hormones. Asterisk denotes statistically significant differences vs. dDAVP group (■) . Significance level: * – $P < 0.05$; ■ – 10 μmol/l dDAVP; ▼ – dDAVP + 0.5 μmol/l sMCH; ○ dDAVP + 5 μmol/l sMCH. Means of 6 values ± S.E.M.

Results

1.Effect of sMCH

Sensitivity of epithelia was tested at the end of each experiment using ouabain (1 mmol/l). Only data obtained in epithelia with adequate inhibitory response to ouabain were considered and used for evaluation of results. The effects of three different concentrations of sMCH (0.5–10 μmol/l) added to the basolateral bathing solution on short-circuit current (I_{sc}), transepithelial potential difference (V_{oc}) and open-circuit transepithelial ohmic resistance (R_t) were evaluated. Sixty minutes after addition of sMCH a small decrease in all electrical parameters was detected (Table 1), however these changes failed to be significant.

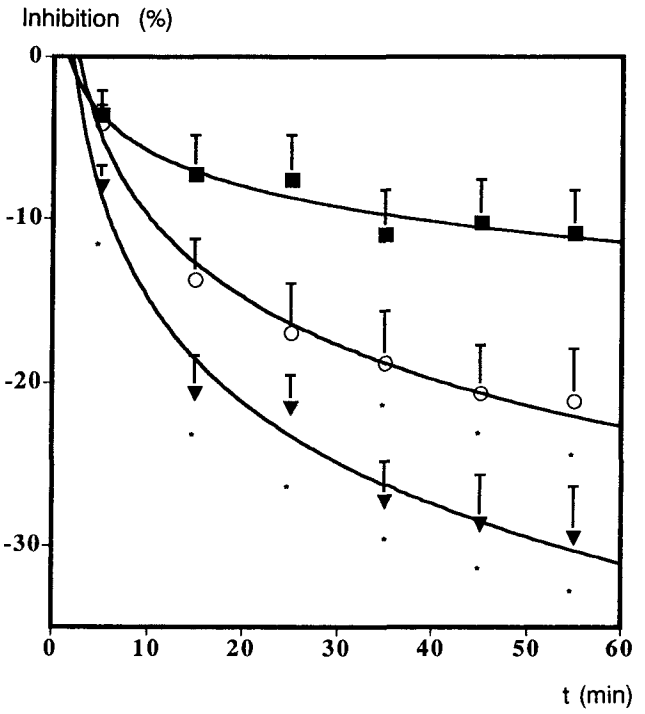

Figure 2. Time dependence of the inhibition of 1-deamino-8-D-arginine-vasopressin (dDAVP) effect induced by three various concentrations of salmon melanin-concentrating hormone (sMCH); ■ – 0.5 μmol/l; ○ – 10 μmol/l; ▼ – 5 μmol/l. Asterisk denotes statistically significant differences vs. dDAVP group (zero line). Significance level * – $P < 0.05$. Means of 6 values ± S.E.M.

2. Simultaneous effect of sMCH and dDAVP

The time course of I_{sc} changes after treatment with dDAVP (10 μmol/l) is shown in Fig. 1. Results are expressed as the ratio between the initial value of I_{sc} (before addition of dDAVP) and the values of I_{sc} at subsequent times studied. The known stimulatory effect of dDAVP on sodium transport was confirmed in these experiments. A corresponding increase in V_{oc} (Fig. 3) and a decrease in R_t (Fig. 4) was also detected. The obtained effects were compared to the results from the same skin to which sMCH was added shortly (within 5 s) after dDAVP. Salmon MCH attenuated the dDAVP-induced increase in both experimentally measured electrical parameters (Figs. 1 and 3), while the inhibition of transepithelial potential difference (V_{oc}) was significant only at the beginning of sMCH action. Whereas sMCH added at the lowest final concentration used (0.5 μmol/l) failed to have a significant effect, treatment with 5 μmol/l sMCH caused rapid decrease in dDAVP stimulated

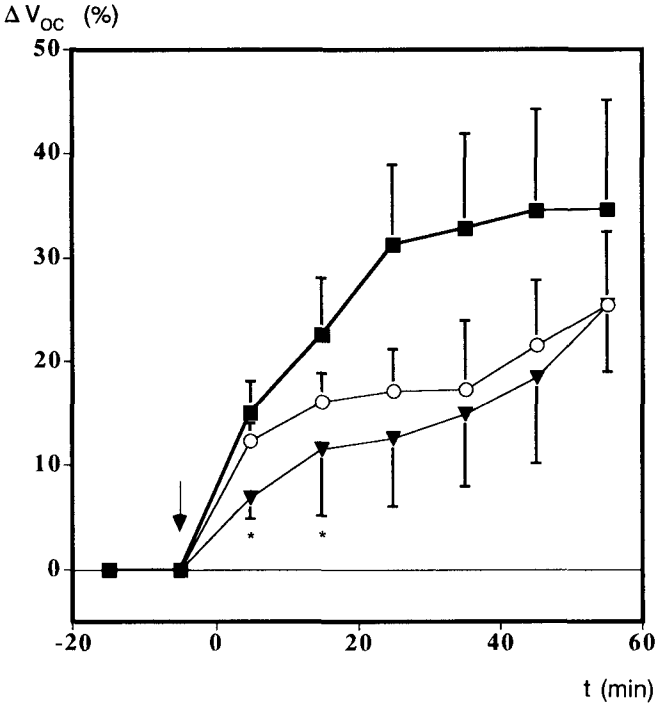

Figure 3. Comparison of the effect of 1-deamino-8-D-arginine-vasopressin (dDAVP) and cumulative effect of dDAVP + salmon melanin-concentrating hormone (sMCH) on transepithelial potential difference (V_{oc}) of the skin, expressed as percentage difference between the basal and stimulated levels (Δ %). Arrow indicates the time of addition of hormones. ■ – 10 μmol/l dDAVP; ○ – dDAVP + 5 μmol/l sMCH; ▼ – dDAVP + 0.5 μmol/l sMCH. *Means of 6 values* ± S.E.M.

I_{sc}, the difference being statistically significant at all time intervals studied. Compared to I_{sc} values in intact epithelia (baseline), significant increase ($P < 0.05$) of I_{sc} in epithelia treated with dDAVP plus 5 μmol/l sMCH was found only at 45 and 55 min. Further increase in sMCH concentration (10 μmol/l) had no additional inhibitory influence on natriferic effect of dDAVP. To better demonstrate the inhibition of I_{sc} by three various concentrations of sMCH used, the differences between I_{sc} values in dDAVP and those in dDAVP/sMCH administered epithelia are depicted in Fig. 2. On the other hand, the inhibitory effects of sMCH (0.5 or 5 μmol/l) on transepithelial potential difference, V_{oc}, failed to be significant. A small, also non-significant increase in transepithelial ohmic resistance was observed in skins treated with sMCH plus dDAVP as compared to those treated with dDAVP only (Fig. 4).

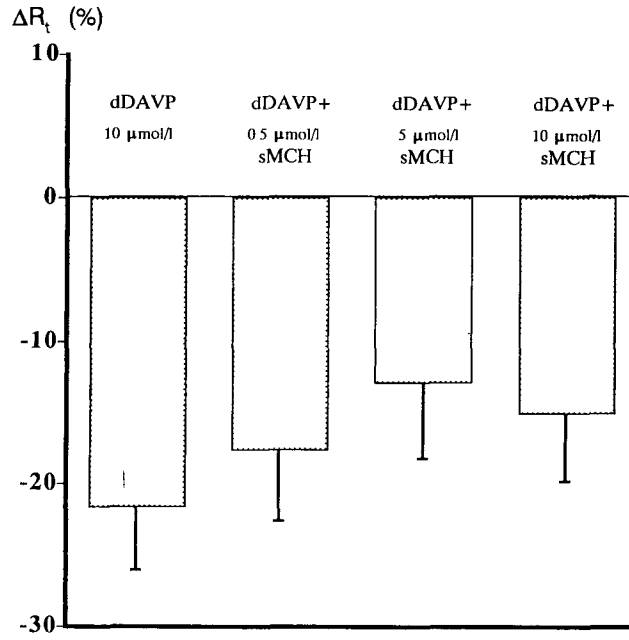

Figure 4. Comparison of the effect of 1-deamino-8-D-arginine-vasopressin (dDAVP) and cumulative effect of dDAVP + salmon melanin-concentrating hormone (sMCH) on transepithelial ohmic difference (R_t) of the skin, expressed as percentage difference between the basal and stimulated levels (Δ %) determined 60 min after addition of hormones. Means of 6 values ± S.E.M.

3.Simultaneous effect of sMCH and forskolin

The stimulatory effect of forskolin (1 μmol/l) on the level of I_{sc} is shown in Fig. 5. This effect was compared to the measurements in which forskolin was used simultaneously with sMCH (5 μmol/l) to modify transepithelial sodium transport. In contrast to its effect on dDAVP, sMCH had no inhibitory influence on the natriferic effect generated by forskolin.

Discussion

Under the presented experimental conditions I_{sc} reliably reflected net sodium transport, which was not significantly changed by the addition of sMCH to the basolateral side, although a small decline was detected in all experiments. These data indicate that sMCH did not change the function of Na/K-ATPase and amiloride-sensitive Na-channels in the apical membrane. Direct effects of sMCH from the apical site were not measured because hormones are suggested to act predominantly

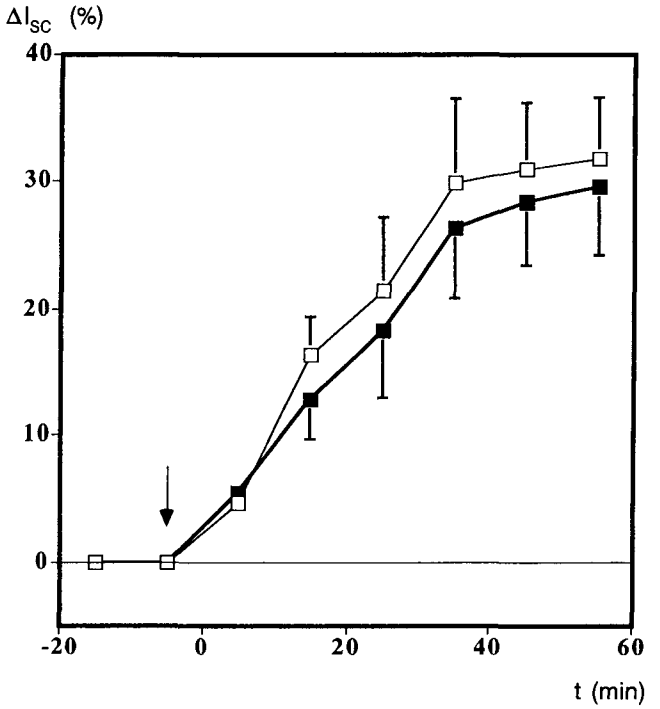

Figure 5. Comparison of the stimulatory effect of forskolin and forskolin + salmon melanin-concentrating hormone (sMCH) on short-circuit current (I_{sc}). Values are expressed as percentage difference between the basal and stimulated levels (Δ %). ■ – 1 μmol/l forskolin; □ – forskolin + 5 μmol/l sMCH. Means of 5 values ± S.E.M.

through the basolateral side. The influence of sMCH on Na/K-ATPase cannot be entirely excluded. Changes in Na/K-ATPase can be expected even within several hours (Collins et al. 1987, Geering et al. 1982). Our measurements of sMCH influence lasted however not more than one hour.

The frog skin is a hormonally sensitive organ extensively used for the evaluation of the activity of various hormones. Aldosterone is known to induce an increase in active sodium transport in frog skin epithelia (Crabbé 1964) and some glucocorticoids have a similar action (Porter and Edelman 1964). Insulin was shown to serve as a physiological trigger of the protein kinase C stimulated sodium transport (Civan et al. 1991).

On the other hand, both oxytocin and vasopressin cause a raise in Na transport through the accumulation of cAMP (e.g. Schoen et al. 1988, Els and Helman 1981). The synthetic analogue of vasopressin, 1-deamino-8-D-arginine-vasopressin (dDAVP), is also a known potent stimulator of sodium transport, which acts in

the same manner as does arginine- or lysine-vasopressin (Bakoš et al. 1984) yet with a more protracted effect (Bakoš et al. 1990). The principal second messenger for dDAVP effect is cAMP. Cyclic AMP increases the permeability for sodium ions in the apical cell membrane, probably via phosphorylation of gating sites. We have confirmed the stimulatory action of dDAVP. Moreover, we found that although sMCH did not change significantly sodium movement across the frog skin, at concentrations of 0.5–10 μmol/l it effectively inhibited the natriferic effect of the synthetic analogue of vasopressin. The latter effect appears to be specific rather than morphologically destructive at the level of membrane or dDAVP binding sites, since no major changes in transepithelial resistance occurred. Should sMCH exert a strong toxic effect, the electrical resistance would display a further decrease exceeding that induced by dDAVP.

Little information is presently available to evaluate mechanisms of sMCH action both in frog and fish. Abrao et al. (1991) investigated the diacylglycerol/inositol triphosphate pathway in the sMCH effect upon teleost melanocytes. They proposed that protein kinase C mediated the pigment aggregating activity of sMCH. Svensson et al. (1991) observed that pigment changes elicited through sMCH were associated with a significant reduction in the cAMP content and that sMCH response was effectively blocked by the adenylate cyclase stimulator forskolin. Forskolin is a diterpene, which in frog skin activates cAMP, but unlike dDAVP, it does so directly, bypassing any membrane receptors (Heisler and Reisine 1984). Forskolin is also a potent stimulator of I_{sc} (Els and Mahlagu 1987). Castillo et al. (1990) found that stimulation of I_{sc} by forskolin at concentrations of up to 4.4 μmol/l reflected predominantly active transepithelial sodium transport. We have studied the influence of sMCH on forskolin effect in frog epithelia. In contrast to its action on dDAVP induced increase in I_{sc}, the investigated peptide did not inhibit forskolin stimulation of short-circuit current.

With regard to the inhibitory influence of sMCH on dDAVP natriferic effect, but not on the effect of forskolin, it is suggested that cAMP may not be involved in the mediation of sMCH influence in frog skin. Thus alternative mechanisms of sMCH inhibitory action have to be considered. We assume that one of the possible mechanisms included may be the effect of sMCH on the basolateral membrane. A possible incorporation of the small sMCH molecule (17 aminoacids) into the structure of the basolateral membrane could lead to changes in its organization, whereby wide lipidic surroundings might be affected. Structural changes may consequently cause modification in dDAVP receptor effectivity, reflected through the drop in I_{sc} level. On the other hand sMCH may influence directly the binding of dDAVP to its V_2-receptor or the function of G-proteins regulating cAMP production. However, neither the function of G-proteins nor the catalytic unit of cAMP seem to have been affected since the inhibition of dDAVP action was very rapid. Changes generated by modification of the catalytic unit of cAMP or by G-proteins would

most probably start more slowly and with a longer delay. As to the primary structure of sMCH compared to arginine-vasopressin and dDAVP, there is an identical sequence of three amino acids (Cys-Pro-Arg) and a disulphide bridge in the structure of all three hormones. Several vasopressin analogues were synthesized which present potent V_2-antagonism (e.g. Manning et al. 1987). Some of them inhibit vasopressin with high efficacy but fail to affect forskolin stimulated water and ionic changes (Kinter et al. 1988, Mann et al. 1986). In spite of the apparent tolerance of these antagonists to changes in their C-terminals, it is not known how long or bulky the C-terminal structure could be so as to still allow binding to receptors. Since there are pronounced differences in the length and structure of both C- and N-terminals between sMCH and arginine-vasopressin or dDAVP, the answer of a possible sMCH V_2-antagonistic potency remains to be elucidated.

Hardy (1985) indicated that calcium binding sites at a basolateral membrane may be identical with the arginine-vasopressin receptors. An influence of extracellular calcium ions on sMCH binding, found in our preliminary experiments (unpublished data), may further support the idea that sMCH regulates directly the binding of dDAVP to its receptors, which are identical with receptors for arginine-vasopressin. Nevertheless, much more information about the possible existence of sMCH binding sites and the effects of sMCH is needed to reveal the mechanisms of sMCH and sMCH/dDAVP action, including also a possible role of calcium ions and the activation of diacylglycerol/inositol pathway proposed by Abrao et al. (1991). In conclusion, salmon melanin-concentrating hormone significantly reduced the natriferic effect of the vasopressin analogue dDAVP, whereas sMCH alone had no pronounced effect on the net sodium transport in frog skin. It seems that sMCH does not change the content of intracellular cAMP. The possible involvement of change(s) in cell membrane(s), in signal(s) which trigger these processes or other mechanisms responsible for the inhibitory effect of sMCH on dDAVP-stimulated frog skin sodium transport remain to be elucidated.

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