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Electrophysiological Responses to Oxytocin and ATP in Monolayers of a Human Sweat Gland Cell Line

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It was shown that oxytocin (OT) elicits electrophysiological responses in cultured monolayers of NCL-SG3, a human immortalized sweat gland cell line. The response to OT was greater for basal applications. It was also found that monolayers respond to ATP with a transient transepithelial-potential change, with a more pronounced response to apical than to basal applications. The IC_{50} for the response to OT was 180 nM at room temperature. The response to OT was not due to effects of OT on vasopressin (AVP) receptors as evidenced by three tests: (a) The response was completely blocked by the selective OT-receptor antagonist [Mpa^1,D-Tyr(Et)^2,Thr^4,Orn^8]-OT (CAP) applied at equal concentrations (100 - 1000 nM) to that of OT. (b) The response to OT was similar to that of ionomycin (2 μM) or ATP (150 μM). In contrast, the response to AVP (500 nM) or cAMP (2 mM) were smaller and of a different time course. (c) OT increased but AVP had no effect on the intracellular free calcium. It is suggested that OT may have a role in the regulation of salt balance in sweating.

Sodium excretion in humans (1 - 500 mEq/day) is controlled by aldosterone and other adrenocortical hormones as well as neuronal mechanisms. Regulation of the sodium chloride level is important in the long term determination of the extracellular fluid volume and of blood pressure. In the male rat it has been shown that a salt load induces release of oxytocin (OT) (1) and that OT produces sodium loss (natriuresis) in the kidney (2). OT receptors couple to intracellular calcium ($[Ca^{2+}]_i$) mobilization in the rat kidney collecting duct. Selective blocking of OT receptors inhibits natriuresis induced by a salt load, whereas blocking of OT receptors has no effect on natriuresis induced by volume expansion (3).

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Sweat gland cells have been cultured (4-9) and immortalized cell lines derived from sweat gland have been established (10, 11). Interest has focused on the mechanisms of chloride transport. Human cell models are of a particular interest, since these enable investigation of defective mechanisms of chloride transport seen in the disease cystic fibrosis (5, 8, 12-14).

NCL-SG3 is an immortalized cell line obtained by Simian virus 40 infection of human sweat gland cells (11). NCL-SG3 cells were shown to transport chloride and to respond to β-adrenergic agonists (11) and to ATP (15). ATP may have multiple roles in sweat gland function since ATP has been found to be directly involved in the regulation of chloride channel activity (12, 16). $[Ca^{2+}]_i$ dependent chloride channels in NCL-SG3 were characterized (15, 17). Preliminary results showed that NCL-SG3 cells lose chloride on stimulation with cAMP (18) but Wilson *et al.* (15) found no cAMP-dependent chloride channels. Nevertheless, we have recently described a cAMP-dependent activation of chloride permeability (19).

In the present study it is shown that NCL-SG3 cells respond to OT and the response is compared with that to ATP. It is found that OT also raises $[Ca^{2+}]_i$ in NCL-SG3 cells. The response results in the loss of chloride and potassium from the cells. These findings are of interest in the investigation of the mechanisms of dehydration induced natriuresis and are relevant also to the long term regulation of the extracellular fluid volume. A summary of these results (20) has been presented.

MATERIAL AND METHODS

Cell culture. The NCL-SG3 cells were a kind gift from Dr. C.M. Lee. The cells were cultured in William's E medium (Gibco BRL, Life Technologies Ltd., Paisley, UK) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone (5 ng/ml), epidermal growth factor (10 ng/ml) and sodium selenite (10 ng/ml) (11).

Transmission electron microscopy. The procedure for the preparation of the cells was similar to that described previously (18).

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Ussing chamber. Solutions were administered by gravity feed and the replaced liquid was discarded using an overflow arrangement. In this way, in contrast to the traditional method of using recirculating solutions with the Ussing chamber (21) the agonists were here administrated directly and in the final concentrations. Due to the technical difficulties the experiments were therefore performed at room temperature, with the exception of two experiments which were carried out at 37 °C for comparison. The control solution was always flushed through the chamber as a control prior to the solutions containing the agonist.

The responses were amplified, electrically filtered with a 4-pole 10 Hz low-pass filter and digitised using a home made computerised system. Cells were grown on permeable membranes of a 1 cm^2 area (Snapwell, Costar, Corning Costar Corporation Cambridge, MA, USA) and mounted in an Ussing chamber (WPI Inc., Sarasota, FL, USA). The membrane potential was measured using silver chloride electrodes (WPI) and isotonic NaCl-agar bridges. The transepithelial resistance was measured using pulses of current (1.22 μA) from a second pair of electrodes. The resistance of the membrane itself (pore size 0.4 μm) was insignificant compared to the resistance of the solution between the electrodes (73 Ω). The resistance of a confluent monolayer of cells was 200-400 Ω. The transepithelial-potential responses to agonists were seen to increase with the resistance of the monolayer, as would be expected given the effects of paracellular leakage on the electrical properties of the membrane. The short-circuit current (Isc) was not measured so as not to influence the ion pumps of the monolayer. The Isc may be estimated from the measured values of the membrane potential and membrane resistance since the low values of the membrane potential measured here are consistent with a linear behaviour of the I-V curve for our experimental conditions. The current pulses were only applied for short time intervals during an experiment. No significant change of transepithelial resistance was observed during the course of the experiment, except during the peaks of response to ATP and calcium ionophores. At the peaks of response, slightly reduced resistance values were noted, as could be expected, given the presumed activation of calcium-dependent chloride and potassium channels.

X-ray microanalysis. The procedure was similar to that described previously (17-19). For X-ray microanalysis, the cells were seeded out on titanium grids (75 mesh, Agar Scientific, Stanstedt, UK). The grids were coated with a Formvar (Merck, Darmstadt, FRG) film and a thin carbon layer. Cells were allowed to attach and spread for 2 days. The cells were stimulated with 1 μM OT for 3 minutes at room temperature. The incubation was stopped by a quick rinse in distilled water at 4 °C. After rinsing, the grids were blotted on filter paper and freeze dried. One spectrum was acquired from each cell and Student's t-test was used to evaluate statistical significance.

Fluorescence measurements. $[Ca^{2+}]_i$ was measured using a spectrophotometer and a modified cuvette system as described previously (17) and, also, with the aid of a conventional microscope/ photomultiplyer technique described elsewhere (22).

Cells were grown to confluence (5-7 days) on 13 mm glass coverslips and loaded with fura-2 tetraoxymethylester (Molecular Probes Inc., Eugene, OR, USA) at 37 °C using 2.5-5 μM fura-2 ester in the growth medium. For spectrophotometer measurements the coverslip was mounted vertically in a non-fluorescent plastic cuvette using hollow stainless steel tubes. Excitation was switched manually between 340 nm and 380 nm. In both the fluorescence techniques used here, the chambers were perfused continuously and the agonists were administered by changing the solution in the input vessel.

Chemicals and solutions. 8-Bromo-cAMP (8Br-cAMP), a permeable cAMP analogue was from Sigma (St. Louis, MO., USA). The standard bath solution consisted of 140 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1.2 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES. Bovine serum albumin was added at 0.01% for the OT experiments (and in these cases, also to the control solutions). The pH was adjusted to 7.35 using NaOH. Ionomycin and A23187 were stock solutions of 4 mM and 1 mM in DMSO, respectively. OT, [Mpa^1,D-Tyr-$(Et)^2$,Thr^4,Orn^8]-OT (CAP) (both gifts from Ferring, Malmö, Sweden) were 100 μM in H_2O with 0.01% BSA. ATP was 15 mM in H_2O, AVP (Arg-vasopressin, Sigma) was 30 μM in H_2O, SKF 9365 (Calbiochem, USA) was 5 mM in H_2O. Stock solutions were kept at −20 °C except for AVP which was stored at 4 °C.

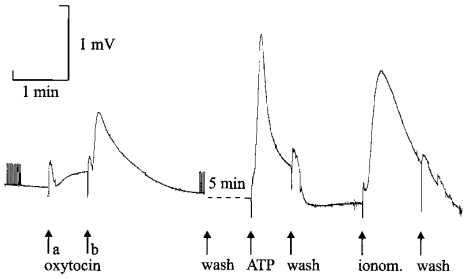

FIG. 1. Response to OT. 1 μM OT applied at the apical side (arrow a) and then at the basal side (arrow b). The response was also larger for applications at the apical side when the order of applications was reversed (not shown). As a reference control, the response to ATP (150 μM) and then to ionomycin (1 μM) were measured. ATP and ionomycin were applied at both sides simultaneously. Vertical dashes in the beginning and before the washout are the effects of the applied current pulses during resistance checks.

RESULTS

The NCL-SG3 cells exhibited tight junctions and microvilli only at the upper side of the cells (i.e. the side not facing the support). The cell morphology was similar to that reported by us previously for NCL-SG3 cells grown on a similar type of permeable membrane (17-19). We concluded that the cells were polar with the apical side upwards.

The transepithelial potential was of the order of 1 mV and comparable to that reported previously (11), but its precise value was not determined because of limitations in slow electrode drifts on mounting the preparation. The reproducibility of measurement of the potential offset was ± 0.5 mV, as assessed by disassembling the chamber and then remounting the same preparation.

Figure 1 shows a typical response (18 experiments) to OT (1 μM). The small upward deflections seen on application of the agonists are caused by electrical disturbances. The registration method was subsequently improved to eliminate such artifacts. Also, a series of vertical traces can be seen before and 2 minutes after the application of OT. These narrow lines are the responses to current pulses that were applied to measure resistance. In preliminary experiments the resistance was measured throughout the experiment but since only small changes were seen also during the peak of the response, the resistance was measured only intermittently to ascertain that the passive properties of the monolayer did not change significantly during the course of the experiment. It may be noted that using

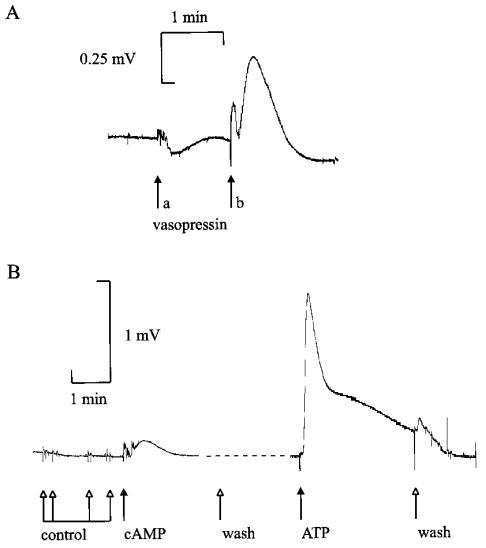

FIG. 2. Response to Arg-vasopressin (AVP) and to cAMP. (A) 500 nM AVP. The response to AVP is greater on application at the basal side (arrow b) than at the apical side (arrow a). (B) 2 mM 8Br-cAMP applied at both sides has a small transient effect on the transmembrane potential. ATP (150 μM) is applied simultaneously at both sides as a control.

the resistance to calculate Isc is generally not possible due to the uncertainty of the membrane potential offset. Three minutes after OT application 150 μM ATP was applied at both sides to obtain a control response for each experiment. The response to 150 μM ATP was always greater than the response to OT (1 nM -2 μM).

To exclude the possibility that the OT response was a "cross-talk" effect due to binding to AVP type V2 receptors, the specific OT-receptor antagonist CAP was used. The epithelium was incubated for 1 minute with the bathing solution containing CAP and then OT was applied together with CAP at the same concentration (100 nM , 500 nM or 1 μM). The response to OT was completely blocked by CAP (5 experiments). 150 μM ATP was used as a control at the end of each experiment.

The response to AVP (500 nM, Fig. 2A) was not systematically studied but the response was much smaller (5 experiments) than that to OT. Similarly, 8Br-cAMP (Fig. 2B, 3 experiments) produced a smaller response.

The response of the transepithelial potential to any agonist depends on the transepithelial resistance since transepithelial shunts will diminish the measured response. Therefore, a normalized response was calculated at each concentration of OT by dividing the peak value of the response with the peak value of the control response (i.e. the response to 150 μM ATP). The response to ATP was obtained after the response to OT. The dose-response curve for OT is shown in figure 3. In this figure only experiments with OT applied at both sides simultaneously are included. The IC_{50} was about 180 nM.

The effect of 500 nM OT on $[Ca^{2+}]_i$ is shown in figure

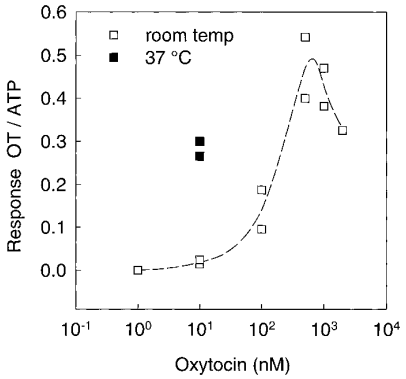

FIG. 3. OT dose-response curve at room temperature (1 nM, 10 nM, 100 nM, 500 nM, 1000 nM and 2000 nM). The response is calculated by dividing the peak potential response to OT by the peak response to 150 μM ATP.

4. The rise in $[Ca^{2+}]_i$ upon application of OT (8 experiments with the spectrophotometer method, 6 experiments with photomultiplyer technique) was markedly different to the slight decrease in $[Ca^{2+}]_i$ observed for AVP (5 experiments, spectrophotometer method). The $[Ca^{2+}]_i$ response to OT was compared to the $[Ca^{2+}]_i$ response to ATP (insert Fig. 4) and the responses were of similar magnitude and time course.

Figure 5 shows the effect of OT on the total intracellular Na, K and Cl content. The changes were small but significant ($p < 0.001$) for Cl and K which were decreased by application of OT consistent with the assumption of activation of calcium activated chloride and potassium channels in NCL-SG3 cells.

DISCUSSION

Evidence was previously presented (1-3) that indicated a physiological role for OT in the regulation of

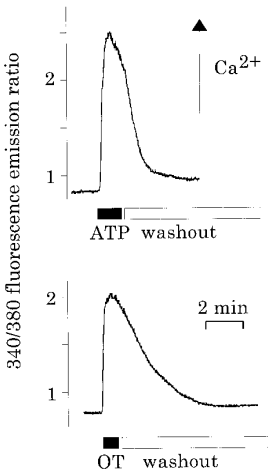

FIG. 4. Effects of OT (500 nM) and ATP (150 μM) on the fluorescence from fura-2 loaded NCL-SG3 cells. The y-axis gives the ratio of the emission at 510 nm for excitation at 340 and 380 nm.

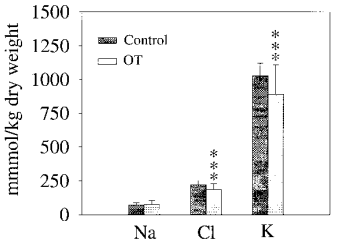

FIG. 5. X-ray microanalysis determination of the total cellular Na, Cl and K content of NCL-SG3 cells. Incubation with OT (1 μM) for 3 minutes significantly reduces the total K and Cl content (***, $p<0.001$). The data are expressed as mmol/kg dry weight and represent the mean of 42 measurements. Thin bars indicate standard deviations.

natriuresis in the kidney. The action of OT in the kidney is mediated by generation of IP_3 and recruitment of $[Ca^{2+}]_i$ but stimulation of guanylate cyclase has also been implicated (23, 24). In the present report we have addressed the question whether OT also may have a role in the regulation of sweat gland secretion. We have shown for the first time that OT elicits electrophysiological responses in NCL-SG3, a human immortalized sweat gland cell line. It has been suggested that NCL-SG3 cells possess purinergic receptors of the P_{2u} type (PY2 with current terminology) which raise $[Ca^{2+}]_i$ by generation of IP_3 (15). The direction and time course of the electrophysiological and $[Ca^{2+}]_i$ responses to OT are similar to those of ATP (Fig. 1 and 4). Calcium ionophores also give a response similar to that to OT or ATP but the response declines more slowly (Fig. 3). In contrast, AVP or incubation with a membrane permeable cAMP analogue produced only smaller responses (Fig. 2). Application of AVP from the apical side gives a response in the opposite direction to that of OT, ATP or ionomycin. ATP or OT raise $[Ca^{2+}]_i$ whereas AVP does not. We have recently shown that cAMP does not raise $[Ca^{2+}]_i$ in NCL-SG3 cells (19). The response to OT was completely blocked by the selective OT antagonist CAP. These results strongly suggest that NCL-SG3 cells express OT-selective receptors and that the electrophysiological response is mediated by recruitment of $[Ca^{2+}]_i$.

The relative magnitude of the responses were ionomycin $\approx$ ATP $>$ OT $>$ AVP $>$ cAMP. The response to OT may decrease for high concentrations of OT (Fig. 3). A similar behavior has also been noted for the OT response in myometrium.

OT elicits a greater response when applied at the basal side than when applied at the apical side. Taken in conjunction with the conclusions based on the ultrustructure of the cells indicates that the cells are polar. It is noteworthy that the response to applications of OT from the basal side is greater although accessibility from the basal side is restricted to the patches of cells protruding into the pores of the membrane.

As noted in the introduction, evidence for a role for OT in the renal regulation of the plasma salt concentration has been presented (1-3). It is therefore interesting that sweat gland cells express OT-receptors.

Contrasting with the effects of aldosterone, regulation of Na^+ reabsorption by OT, could facilitate both a rapid and a slow increase in salt secretion. The possibility of a role for a rapid regulatory mechanism in the sweat gland has been raised previously (14).

Figure 5 shows that NCL-SG3 cells lose chloride and potassium on stimulation with OT. This behavior is consistent with the expected behavior of a secretory cell. We have previously shown (17) that NCL-SG3 express calcium activated chloride channels. A rise in $[Ca^{2+}]_i$ in the cells, in response to OT, is expected to activate calcium activated potassium channels (K(Ca) channels) on the basal side, resulting in a hyperpolarization. This gives an increased outward driving force for chloride and the simultaneous activation of calcium activated chloride channels on the apical side facilitates a net secretion of Cl. This assumption is consistent with the results of figure 5 where it is seen that the chloride content of the cell has decreased in response to stimulation with OT. Tentatively, the effect of OT, and possibly also other natriuretic factors, could be to both increase the rate of sweat production and the NaCl content of the sweat.

It is interesting that menopausal women show increased OT levels (25). This occurs because the endorphin secretion in the CNS is decreased during menopause and endorphins inhibit OT release. It is possible therefore that the present findings also have bearing on involuntary night sweating during menopause. The finding that NCL-SG3 cells express OT-receptors may be relevant to the mechanisms giving rise to dehydration induced natriuresis as well as involuntary excessive sweating

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