THE $Na^+2Cl^-K^+$ COTRANSPORTER IN THE RECTAL GLAND OF *SQUALUS ACANTHIAS* IS ACTIVATED BY CELL SHRINKAGE

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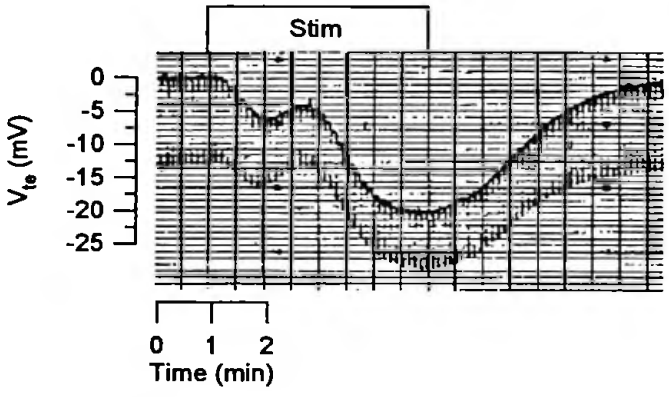

For many years we have known that cAMP dependent stimulation of exocrine secretion starts with some delay and occurs in a biphasic manner. Fig. 1 shows a typical perfusion experiment in an isolated and *in vitro* perfused rectal gland tubule (RGT) of *Squalus acanthias* (V_{te} = transepithelial voltage, pulses correspond to transepithelial resist-ance, Stim = is a stimulation cocktail consisting of 0.5 mmol/l cAMP, 0.5 mmol/l adenosine and 10 µmol/l forskolin). Stim leads to an initial lumen negative deflection of V_{te} and to a decrease in R_{te}. Both changes result in a transient increase in equivalent short circuit current (I_{sc}). The magnitude of this current was 104 ± 9.6 µA/cm^2 (n = 12) and is compatible with the extrusion of some 20% of cytosolic Cl^- content (together with an equivalent amount of K^+) due to the opening of CFTR-type Cl^- channels.

The second phase only occurred with a larger delay and resulted in a much larger steady state I_{sc}: -480 ± 96 µA/cm^2 (n = 12). This second phase, but not the first one, was completely prevented by furosemide ($3x10^{-4}$ mol/l, bath): -30.2 ± 8.4 µA/cm^2 (n = 12).

These data indicate that the delayed second phase is caused by the slow activation of the $Na^+2Cl^-K^+$ cotransporter. This kinetic behavior, i.e. the delayed activation of the $Na^+2Cl^-K^+$ cotransporter, enabled us to examine the mechanisms of its activation in the intact *in vitro* perfused RGT.

Previous data (Evans, R. L. and Turner, R. J.: J Physiol (Lon) 499.2:351-359, 1997, Greger, R. et al. Pfluegers Arch Eur J Physiol 402:376-384, 1984, Warth, R. et al. Pfluegers Arch Eur J Physiol 436:521-528, 1998) suggested that 3 signaling pathways may be be involved: 1. cAMP dependent phosphorylation; 2. a transient fall in cytosolic Cl^-, as it has been reported in our previous studies and 3. transient cell shrinkage (Greger, R. et al. Pfluegers Arch Eur J Physiol 402:376-384, 1984). Possibility 1 appears unlikely because the activation of the $Na^+2Cl^-K^+$ is much slower than that of cAMP-regulated CFTR type Cl^- channels (cf. Fig 1, first phase). Therefore, PKA probably does not activate the $Na^+2Cl^-K^+$ cotransporter directly.

Next we have examined the possible role of a fall in cytosolic Cl^- ($[Cl^-]_i$). We have measured $[Cl^-]_i$ by 6-methoxy-N-(3-sulfopropyl) quinolinium (SPQ) fluorescence. This method works extremely well in RGT, with fairly easy loading by repetitive cell shrinkage and swelling for 30 min and with a rather stable fluorescence for 1 h. The present SPQ data indicate, in confirmation of our previous microelectrode data (Greger, R. et al. Pfluegers Arch Eur J Physiol 402:376-384, 1984), that Stim leads to a transient and marked fall in $[Cl^-]_i$ to 78 ± 1 % (n = 23) of control. Thereafter (ca. 1 - 2 min), $[Cl^-]_i$ returned to control values: 100 ± 3.2 % (n = 13).

Now we asked whether an increase in $[Cl^-]_i$ could prevent the activation of the $Na^+2Cl^-K^+$ cotransporter. Therefore, the RGT were exposed to a depolarizing solution containing 25 mmol/l K^+. With this solution the relative $[Cl^-]_i$ increased to 134 ± 6.2 % (n = 9). To avoid concomitant cell swelling with this solution 50 or 100 mmol/l mannitol were added and it was shown in separate measurements utilizing calcein fluorescence that the cell volume could be kept fairly constant by this maneuver (102 ± 2.9 %, n = 5, addition of 50 mmol/l mannitol). The activation of the $Na^+2Cl^-K^+$ cotransporter by Stim was almost normal in these high K^+ solutions and even the time constant for half maximal activation was very similar to that observed under control conditions: 1.03 ± 0.14 (n =4) versus 0.59 ± 0.06 (n = 19). These data indicate that a fall in $[Cl^-]_i$ cannot be responsible for the activation of the $Na^+2Cl^-K^+$ cotransporter, because the activation occurs even with largely increased $[Cl^-]_i$.

Next we examined whether cell shrinkage could be the signal of $Na^+2Cl^-K^+$ cotransporter activation. Relative cell volume was measured by calcein fluorescence using a 100x oil immersion objective with very shallow focus. Stim initially reduced apparent relative cell volume significantly to 92 ± 3.3 % (n = 5) and then led to a significant cell swelling: 106 ± 1.7 % (n = 5), which we have also noticed in our previous study (Greger, R. et al. Pfluegers Arch Eur J Physiol 436:538-544, 1998).

Now we tested whether increased cell volume caused by the removal of 150 mmol/l NaCl from the normal shark Ringer (ca. 600 versus 900 mosm/l) would interfere with the activation of the $Na^+2Cl^-K^+$ cotransporter. This hypotonic solution led to a relative cell volume increase 115 ± 3.8 % (n = 8). Under these conditions the $Na^+2Cl^-K^+$ cotransporter could not be activated by Stim: under control conditions Stim led to an increase I_{sc} of from -31 ± 7.5 to -374 ± 78 $\mu A/cm^2$ (n = 12), in the presence of the hypotonic solution I_{sc} was only increased to -65 ± 12 $\mu A/cm^2$ (n = 11) and the half time of activation which was 0.61 ± 0.08 (n = 9) min in normotonic solution could not be determined.

These data suggest that the $Na^+2Cl^-K^+$ cotransporter can only be activated by cAMP if cell shrinkage is permitted to occur. If it is prevented by hypotonic solutions only the first ($Na^+2Cl^-K^+$ cotransporter-independent) but not the second phase of activation of secretion can be observed.

Next we searched for the putative effect of several inhibitors of protein kinases in the stimulation of the $Na^+2Cl^-K^+$ cotransporter. Bisindoylmaleimide (1 µmol/l); chelerythrin (10 µmol/l); W7 (10 µmol/l); calphostin (2 µmol/l), all putative blockers of protein kinase C, were without effect (n = 3). Staurosporin (5 µmol/l) had an inhibitory effect, which at this concentration cannot be regarded to be specific (n = 3). The myosin light chain kinase inhibitor ML7 had no significant effect at 10 µmol/l (n = 5). Also the inhibitor KN 62 (25 µmol/l) was without effect (n

= 2). Entirely predicted was the inhibitory effect of the protein kinase A inhibitor H89, which reduced the activation of I_{sc} to 34 ± 7 % (n = 4) at 20 µmol/l. It was also predicted that ocadaic acid and calyculin at 1 µmol/l led to a stim-independent activation (n = 3). These data suggest that the activation of the $Na^+2Cl^-K^+$ cotransporter requires protein kinase A activation. The other tested kinases are probably not involved in the activation mechanism.

We further examined whether volume sensing, and hence $Na^+2Cl^-K^+$ cotransporter activation require an intact actin turnover. Therefore we have preincubated perfused RGT with very high phalloidin and cytochalasin D concentrations (10^{-4} mol/l, 10 - 15 min) and examined the activation of the $Na^+2Cl^-K^+$ cotransporter. We found that both inhibitors reduce $Na^+2Cl^-K^+$ cotransporter activation significantly to less than one third (n = 6 and n = 4, respectively). Also the half time of $Na^+2Cl^-K^+$ cotransporter activation was largely reduced by both compounds. A similar inhibitory effect was observed for nordehydroguaiaretic acid (NDGA, 80 µmol/l, n = 6) and indomethacin (100 µmol/l, n = 2).

The present data suggest that the $Na^+2Cl^-K^+$ cotransporter is activated indirectly during cAMP stimulation. PKA phosphorylation activates the Cl^- conductance. This leads to transient cell shrinkage. This transient cell shrinkage is absolutely required for cotransporter activation. The effective inhibitors of cotransporter activation probably act proximally and interfere with volume sensing (phalloidin, cytochalasin D, NDGA, Indo) or they interfere with PKA activation (H89). The sensing of cytosolic Cl^- activity appears not be causally involved in cotransporter activation. These data is in complete agreement with the physiological role of this cotransporter, namely to upregulate reduced cell volume (Hoffmann, E. K. and Pedersen, S. Contrib Nephrol 123:50-78 (1998).

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