INFLUENCE OF EXTRACELLULAR BICARBONATE ON THE SHORT-CIRCUIT CURRENT AND INTRACELLULAR FREE CALCIUM OF HUMAN CULTURED SWEAT DUCT CELLS

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SUMMARY

Transepithelial short-circuit current ($I_{sc}$) and intracellular free Ca$^{2+}$ ($Ca_{i}^{2+}$) was studied in monolayers of cultured human sweat duct cells (CSDCs) in the presence or absence of $HCO_{3}^{-}$ (and CO$_2$) in the bathing solutions. Addition of $HCO_{3}^{-}$ (and CO$_2$) increased the control $I_{sc}$ by more than 50%. The effect of $HCO_{3}^{-}$ (and CO$_2$) on $I_{sc}$ was confined to the serosal bath. The $HCO_{3}^{-}$ (and CO$_2$) effect was also studied during stimulation with the cholinergic agonist methacholine (MCh), which in CSDC induces a complex response consisting of an initial $I_{sc}$ and Ca$^{2+}$ spike, which is independent of extracellular Ca$^{2+}$, followed by regular $I_{sc}$ and Ca$^{2+}$ oscillations, which are absent during Ca$^{2+}$-free bathing conditions. The sustained $I_{sc}$ and Ca$^{2+}$ oscillations, but not the initial $I_{sc}$ and Ca$^{2+}$ spike, were abolished by the removal of extracellular HCO$_3^{-}$ (and CO$_2$). It is concluded that the Ca$^{2+}$ influx and the $I_{sc}$ in CSDCs are critically influenced by the presence of extracellular HCO$_3^{-}$ (and CO$_2$) in the bathing solutions.

INTRODUCTION

Ion transport properties of human cultured sweat duct cells (CSDCs) are analogous to those of the intact sweat duct (Quinton, 1987), i.e. a barium-sensitive K$^+$ leak and ouabain-sensitive Na$^+$-K$^+$-pumps are situated in the serosal membrane and amiloride-sensitive Na$^+$ channels have been located in the mucosal membrane (Pedersen, 1989, 1990a). Such asymmetrical distribution of channels and pumps leads to an active transepithelial Na$^+$ transport, which can be measured as amiloride-sensitive short-circuit current ($I_{sc}$). When stimulated with cholinergics an additional tetraethylammonium (TEA)-sensitive K$^+$ leak is activated in the serosal membrane, leading to an increased amiloride-sensitive $I_{sc}$, which includes an initial $I_{sc}$ spike followed by a sustained phase of regular $I_{sc}$ oscillations (Pedersen, 1990b, 1991; Larsen, Novak & Pedersen, 1990; Brayden, Pickles & Cuthbert, 1991). Preliminary studies, showing that sustained $I_{sc}$ oscillations following cholinergics were abolished during HCO$_3^{-}$-free conditions, motivated the present study, in which the effect on $I_{sc}$ of extracellular HCO$_3^{-}$ during control and cholinergic stimulation was studied. In addition we measured the effect of HCO$_3^{-}$ on intracellular free Ca$^{2+}$ ($Ca_{i}^{2+}$), which is assumed to be the main intracellular regulator of $I_{sc}$ in these cells (Pedersen, 1990b). The transepithelial bioelectric properties of CSDCs, grown to confluence on a permeable support, were studied by voltage clamp technique, and Ca$^{2+}$ was measured by microfluorescence techniques using the Ca$^{2+}$ probe, fura-2.
METHODS

Skin samples were obtained from young healthy subjects, who underwent plastic surgery. Cell culture procedures have been described elsewhere (Pedersen, 1984, 1989). Briefly, sweat glands were isolated and dissected after a period of collagenase treatment, and the coiled part of the reabsorptive duct was transferred to culture flasks supplied with a hormone-supplemented growth medium. After 3 weeks of exponential growth, the cells were subcultured and stored in liquid nitrogen. For electrophysiological measurements the frozen cells were thawed and subcultured high density on a dialysis membrane which covered a hole in the centre of the bottom of a small plastic cup. When confluence was obtained, measurements were performed at 37 °C in Ussing chambers as previously described (Pedersen, 1989). For Ca\(^{2+}\) measurements cells were subcultured in glass flasks (Tecnucc) at low density, i.e. it was intended in these experiments to produce small clusters of cells, in order to get better fura-2 load and facilitate the action of agonists acting from the serosal membrane facing the glass surface. Fura-2 AM was added to CSDCs, bathed in the same Ringer solution as used after incubation, at a final concentration of 20 \(\mu\)M for 30 min at 37 °C. After loading, cells were washed twice and mounted in a modified Sykes–Moore chamber with a volume of 0.2 ml. This chamber was stationed on the stage of a Zeiss Axiosvert-10 microscope equipped for epifluorescence with a 75 W xenon lamp and a 20 × objective (Plan-Neofluar, Zeiss). A continuous gravity-produced flow with fully aerated and prewarmed Ringer solution was applied to the chamber with a perfusion rate of at least 10 ml/min. In comparison with the small volume of the Sykes–Moore chamber, this continuous high flow prevented accumulation of any fura-2 that leaked from the cells during experiments, thus allowing observations of repeated pulses of stimuli to be made. The temperature in the chamber was maintained at 37 °C by regulation of the flow rate. Glass tubes were used in order to avoid gas diffusion from the flow system. Measurements of intracellular Ca\(^{2+}\) were made using a central processor unit (MSP-21 Zeiss), controlling shutters and filters. Through interference filters, barrier filters and a dichroic mirror (all Zeiss) the fluorescence signal from CSDCs was acquired alternatively at excitation wavelengths 340 and 380 nm using an emission wavelength of 520 nm. A pinhole diaphragm situated before the photomultiplier (R928/PMT Zeiss) was used to pass the fluorescence arising from each of the two excitation wavelengths from the central region, representing a collection of approximately ten cells. Background fluorescence values for each wavelength, obtained before each experiment using unloaded cells, were automatically subtracted from the corresponding signals measured in fura-2-loaded cells before taking the ratio (340/380). Readings of both wavelengths (average of 100 measurements each) were done every 5 s, and the shutter was closed between readings. The ratio, corrected for background values, was converted to Ca\(^{2+}\) concentration values using an external calibration standard and the formula derived by Grynkiewitz, Poenie & Tsien (1985) for dual wavelength measurements:

\[
Ca^{2+} = K(R_b - R_a)/R_s - R_a,
\]

where \(R_s\) and \(R_a\) are the ratios at 0 Ca\(^{2+}\) and saturating Ca\(^{2+}\), respectively. \(R_s\) is the experimental ratio. \(K\) represents \(K_a(F_b/F_s)\), where \(K_a\) (2.25 × 10\(^{-2}\) m at 37 °C) is the effective dissociation constant for fura-2, and \(F_b\) and \(F_s\) are the fluorescence intensities at 380 nm minus and plus Ca\(^{2+}\), respectively. In the present set-up a \(F_b/F_s = 8.8\) was measured.

The ‘standard’ bicarbonate-buffered Ringer solution contained (mm): 140 Na\(^+\), 5.2 K\(^+\), 1.2 Ca\(^{2+}\), 1.2 Mg\(^2+\), 1.198 Cl\(^-\), 25 HCO\(_3^-\), 5.2 phosphate, and 5.6 glucose. The Tris-buffered Ringer solution contained (mm): 137 Na\(^+\), 2.7 K\(^+\), 143.7 Cl\(^-\), 1.2 Ca\(^{2+}\), 0.8 Mg\(^2+\), 10 Tris, and 5.6 glucose. For the experiments with solution change (illustrated in Table 1 and Fig. 1) we used two solutions with high gluconate content, with or without HCO\(_3^-\) (Star, Burg & Knepper, 1985; solution A with HCO\(_3^-\) and solution B without HCO\(_3^-\)). In this way only a fraction of an inert component was replaced with a supposed active component, HCO\(_3^-\), and simple substitution of Cl\(^-\) with HCO\(_3^-\) was avoided. These two solutions contained (mm): 146 Na\(^+\), 5 K\(^+\), 1.2 Mg\(^2+\), 50 Cl\(^-\), 2.5 phosphate, 4 lactate, 1.2 sulphate, 1 citrate, 5.5 glucose, 6 alanine. Solution A additionally contained 25 mm HCO\(_3^-\) and 74 mm gluconate and solution B contained 101 mm gluconate. Because of the gluconate content the ionic Ca\(^{2+}\) activity (measured by an automatic pH and ionized Ca\(^{2+}\) analyser, ICA2, Radiometer, Denmark) was titrated with calcium salts to 1.2 mm. Solutions containing HCO\(_3^-\) were gassed with 5% CO\(_2\) and 95% O\(_2\), and HCO\(_3^-\)-free solutions were gassed with O\(_2\). All (aerated) solutions were titrated to pH = 7.40.

Chemicals were from Sigma Chemical Co. Ltd, USA. Results are presented as means ± s.e.m., and
RESULTS
In the presence of extracellular HCO$_3^-$ a highly significant stimulation of $I_{\text{sec}}$ is seen in CSDC's. Figure A and B illustrates the effect of a bilateral solution change from HCO$_3^-$-free to HCO$_3^-$-buffered Ringer solution. In Fig. 1A amiloride (1 μM) is added to the mucosal side during HCO$_3^-$-buffered conditions, whereas in Fig. 1B amiloride is added after return to HCO$_3^-$-free conditions. It can be seen that the major part of the HCO$_3^-$-induced $I_{\text{sec}}$ increase is amiloride sensitive, i.e. is likely to be carried by Na$^+$. The results from thirty-six experiments are presented in Table 1, showing that bilateral addition of 25 mM HCO$_3^-$ (and 5% CO$_2$) stimulates the $I_{\text{sec}}$ approximately 60%. In order to investigate whether the effect of HCO$_3^-$ on $I_{\text{sec}}$ is a true effect, control experiments were performed with a gluconate Ringer solution, in which gluconate was substituted with Cl$^-$ instead of...
HCO$_3^-$: In this situation only a minor increase of the $I_{sec}$ was seen (from 53.1±6.2 to 55.8±6.3 μA/cm$^2$; n = 8); i.e. less than one-tenth of the increase seen following substitution with HCO$_3^-$: Thus, extracellular HCO$_3^-$ exerts a specific effect on $I_{sec}$, which cannot be assigned the presence of anions in general. The $I_{sec}$ response to sequential addition of 25 mM HCO$_3^-$, first to the mucosal solution and thereafter to the serosal solution, is shown in Fig. 1C. Serosal exposure to HCO$_3^-$ Ringer solution is seen to dominate the final steady-state $I_{sec}$, which is significantly increased. The small inhibition of $I_{sec}$ following mucosal HCO$_3^-$ addition might reflect either a transepithelial mucosa-to-serosa HCO$_3^-$ flux due to the difference in HCO$_3^-$ concentrations between the two sides, or an intracellular acidification due to permeation of CO$_2$, which on the mucosal side is not counteracted by a simultaneous HCO$_3^-$ entry.

The polarized response to HCO$_3^-$ probably reflects the result of HCO$_3^-$ entry across the serosal membrane. The nature of such a HCO$_3^-$ pathway, however, remains unknown, i.e. addition of recognized anion exchange inhibitors, DIDS (4,4′-disothiocyanatostilbene-2,2′-disulphonic acid, 50 μM) or SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid, 100 μM), had no effect on the HCO$_3^-$-induced $I_{sec}$ alterations. This, however, does not rule out the presence of an anion exchanger as the disulphonic stilbenes do not always block such pathways (Binder, Foster, Budinger & Hayslett, 1987).

When CSDCs are stimulated with methacholine (MCh) in HCO$_3^-$-buffered solutions, an abrupt large transient $I_{sec}$ spike is seen followed by a sustained phase of regular $I_{sec}$ oscillations (Fig. 2A; Pedersen, 1987, 1990b, 1991). During HCO$_3^-$-free bathing conditions the CSDCs respond to MCh addition with an apparently unaffected initial $I_{sec}$ spike, whereas the otherwise seen subsequent $I_{sec}$ oscillations are absent (Fig. 2B and C). Addition of HCO$_3^-$ to obtain small concentrations (2–5 mM) in the serosal bath, however, immediately revives regular $I_{sec}$ oscillation (Fig. 2B; representative of more than 45 similar experiments), whereas HCO$_3^-$ addition to the mucosal bath has no effect (5 out of 5 experiments). To see whether the effect of HCO$_3^-$ on $I_{sec}$ oscillations was specific for this ion, or rather related to a HCO$_3^-$-induced increase of intracellular pH (pH$_i$), the effect of NH$_4$Cl addition was studied. One might predict that addition of NH$_4$Cl leads to an initial intracellular alkalization due to non-ionic diffusion of NH$_3$, which subsequently associates with intracellular H$^+$. Addition of 2 mM NH$_4$Cl to the mucosal bath, during HCO$_3^-$-free conditions, also restored missing $I_{sec}$ oscillations (Fig. 2C; representative for 9 out of 9 similar experiments) i.e. producing a response similar to that of HCO$_3^-$ addition.

No revival of oscillations was seen when NH$_4$Cl was added to the serosal bath (seen in 4 out of 4 similar experiments). This NH$_4$Cl sidedness, which is similar to the polarized NH$_3^-$/NH$_4^+$ permeability demonstrated in renal tubular cells (Kikeri, Sun, Zeidel &

### Table 1. Bioelectric response of CSDCs to bilateral solution change from HCO$_3^-$-free to CO$_2^-$HCO$_3^-$-buffered Ringer solutions

|        | $I_{sec}$ (μA/cm$^2$) | PD (mV) | Resistance (Ω cm$^2$) |
|--------|----------------------|---------|---------------------|
| Control | 52.3±3.4             | −19.9±1.9 | 379±24               |
| + CO$_2^-$HCO$_3^-$ | 840±5.3 | −23.7±2.0 | 298±24               |

Values, given as means±S.E.M., represent the results from thirty-six different epithelial preparations, derived from six preparations from each of six donors. ** P < 0.001; * P < 0.01.
Fig. 2. Illustration of the $I_{sec}$ response of CSDC preparations exposed to MCh (20 $\mu$M in panels A and B, and 10 $\mu$M in panel C), when initially bathed in HCO$_3^-$-Ringer solution (panel A) or a HCO$_3^-$-free Tris-buffered Ringer solution (panels B and C). The abortive response in B and C was revived by HCO$_3^-$ (5 mM) added to the serosal bath (S, panel B), or NH$_4$Cl (2 mM) added to the mucosal bath (M, panel C), respectively. Mucosal HCO$_3^-$ (M, panel B) or serosal NH$_4$Cl (S, panel C) had no such effect. Additional NH$_4$Cl in the mucosal bath increased the $I_{sec}$ deflections (panel C).

Herbert, 1989), might indicate that the serosal membrane is permeable to NH$_4^+$, e.g. via the serosal K$^+$ channels (Pedersen, 1990b; Larsen, Novak & Pedersen, 1990). NH$_4^+$ entry would then dominate over NH$_3$ permeation in the serosal membrane, leading to intracellular acidification, whereas the mucosal membrane, which notably has no significant K$^+$ permeability, only allows NH$_3$ entry.

Following the addition of MCh, CSDCs produce an initial Ca$^{2+}$ spike which in the presence of Ca$^{2+}$ in the bathing solutions is followed by sustained Ca$^{2+}$ oscillations (Fig. 3 A); i.e. a response similar to the $I_{sec}$ response in these cells (Pedersen & Poulsen, 1991). The Ca$^{2+}$ response to MCh (25 $\mu$M) during HCO$_3^-$-free bathing conditions (Fig. 3 B) was therefore investigated. It can be seen that the Ca$^{2+}$ perturbations in Fig. 3 B are similar to the $I_{sec}$ response illustrated in Fig. 2 B, in which an identical experimental condition was used. It is likewise seen that Ca$^{2+}$ oscillations are induced by subsequent addition of small amounts of HCO$_3^-$ (5 mM). By analogy with the $I_{sec}$ experiment these oscillations were not influenced by further exposure to CO$_2$ (1–2 %), regulating the pH of the Ringer solution to 7.4 (not shown).

In order to substantiate an assumed effect of HCO$_3^-$ on the Ca$^{2+}$ influx pathway
Fig. 3. Examples of Ca\(^{2+}\) variations in CSDC preparations when stimulated with MCh (25 \(\mu\)M), either during HCO\(_3\)\(^-\)-buffered conditions (panel A), or bathed in HCO\(_3\)\(^-\)-free Tris-buffered Ringer solution (panels B and C). The role of extracellular Ca\(^{2+}\) on the sustained phase is demonstrated in panel A. In panel B the addition of HCO\(_3\)\(^-\) (5 mM) was able to revive oscillations. During additional Ca\(^{2+}\)-free conditions (panel C) addition of HCO\(_3\)\(^-\) (5 mM) failed to evoke oscillatory activity, until extracellular Ca\(^{2+}\) (1-2 mM) was added.

(reflected by the oscillatory phase), cells were stimulated with MCh during both Ca\(^{2+}\)- and HCO\(_3\)\(^-\)-free conditions (Fig. 3 C). During these conditions HCO\(_3\)\(^-\) addition alone did not evoke Ca\(^{2+}\) oscillations, which first appeared when Ca\(^{2+}\) was added.

**DISCUSSION**

The present study shows that the presence of HCO\(_3\)\(^-\) in the serosal bath stimulates both control \(I_{sec}\) and MCh-induced \(I_{sec}\) and Ca\(^{2+}\) oscillations in CSDCs. The \(I_{sec}\) stimulation is evoked from the serosal side only, it requires low HCO\(_3\)\(^-\) concentrations (i.e. 2–5 mM), and it seems to be independent of CO\(_2\). Hence, it seems likely that a HCO\(_3\)\(^-\)-specific pathway is present in the serosal membrane of CSDCs. In this way the response of CSDCs is comparable with that of the intact tissue, in which the Na\(^+\) reabsorption was reduced 30% following removal of HCO\(_3\)\(^-\) from the bath (Sato, 1977).

One of the important events following addition of HCO\(_3\)\(^-\) into the bathing solutions includes increase in pH, (Jentsch, Korbmacher, Janicke, Fischer, Stahl, Helbig, Hollwede, Cragoe, Keller & Wiederholt, 1988; Boyarsky, Ganz, Sterzel & Boron, 1988). Another agent, NH\(_4\)Cl, which unspecifically alkalinizes the cytoplasm, mimicked the HCO\(_3\)\(^-\)
response when added during HCO$_3^-$-free bathing conditions. This comparable effect on $I_{sec}$ in CSDCs of NH$_3$Cl and HCO$_3^-$ addition might indicate that HCO$_3^-$ acts via an increased pH$_i$. In contrast to the effect of HCO$_3^-$ and NH$_4$Cl, only insignificant alterations of the $I_{sec}$ were produced when the CSDCs were exposed to variations of the extracellular pH (Pedersen, 1989). This might indicate that the presented HCO$_3^-$ effects are mediated by sites that are accessible only from the cytoplasm, i.e. the Na$^+$ reabsorption in CSDCs, reflected by $I_{sec}$ (Pedersen, 1989; Larsen Novak & Pedersen, 1991), might be modified by pH$_i$, as demonstrated in other tissues (Funder, Ussing & Wieth, 1967; Goldfarb, Egnor & Charney, 1988; Harvey, Thomas & Ehrenfeld, 1988). Any of the systems involved in net active Na$^+$ transport in the CSDC might be influenced by pH$_i$ variations, i.e. it is known that intracellular acidification (1) reduces Na$^+$–K$^+$ pump activity (Skou & Esmann, 1984; Eaton, Hamilton & Johnson, 1984), (2) decreases Na$^+$ conductance (Palmer, 1985; Harvey et al. 1988), and (3) decreases K$^+$ conductance (Moody, 1984; Keller, Jentsch, Koch & Wiederholt, 1986). However, pH$_i$ might also influence the activity of ion transport-regulating intracellular messengers such as Ca$^{2+}$ (Siffert & Akkerman, 1987; Baker & Honerjager, 1978).

When CSDCs are stimulated with the cholinergic agonist, MCh, an initial $I_{sec}$ spike is seen in parallel with an initial Ca$^{2+}$ spike followed by regular $I_{sec}$ and Ca$^{2+}$ oscillations. The initial response is independent of extracellular Ca$^{2+}$, whereas the subsequent oscillations are absent if extracellular Ca$^{2+}$ is omitted (Pedersen, 1990b; Pedersen & Poulsen, 1991). During MCh stimulation a substantial effect of HCO$_3^-$ removal was seen, presumably caused by an increased metabolism and acid production, which would lead to an increased demand for pH$_i$-regulating processes. Although a normal MCh-induced initial $I_{sec}$ and Ca$^{2+}$ spike was obtained, the normally seen sustained phase of $I_{sec}$ and Ca$^{2+}$ oscillations was abolished or highly reduced during the absence of extracellular HCO$_3^-$. Oscillatory activity was, however, revived by the addition of HCO$_3^-$ or NH$_4$Cl. Thus, it can be theorized that the Ca$^{2+}$ influx pathway in CSDCs is regulated by pH$_i$.

In a variety of other tissues repetitive Ca$^{2+}$ release involves the action of phosphoinositides (Berridge & Gallione, 1988), acting at a specific receptors, which interestingly has been demonstrated to be dependent on pH$_i$ (Ives & Daniel, 1987; Enyedi, Brown & Williams, 1989; Siskind McKoy, Chobanian & Schwartz, 1989; Ferris, Huganir, Supattapone & Snyder, 1989). We have not been able to demonstrate a direct action of phosphoinositides in CSDCs. A significant phosphoinositide hydrolysis was, however, demonstrated in CSDCs following stimulation with MCh (Doughney, Pedersen, McPherson & Dormer, 1989). Thus, in CSDCs phosphoinositides might act via such an intracellular messenger in a pH$_i$-dependent way, as suggested above.

The oscillations in CSDCs are assumed to be the result of a repetitive Ca$^{2+}$ discharge from a plasma membrane-associated intracellular Ca$^{2+}$ pool, incorporated as a link between the extracellular space and the cytoplasm (Pedersen, 1990b, 1991) as also suggested in other tissues (Wakui, Potter & Petersen, 1989). Taken together it therefore seems likely, that pH$_i$ acts via an phosphoinositide-dependent repetitive Ca$^{2+}$ discharge pathway from this membrane-associated Ca$^{2+}$ pool, which, on the other hand, is continuously supplied with Ca$^{2+}$ from the extracellular space.

The close relation between oscillatory activity and the presence of HCO$_3^-$ in the bathing solutions demonstrated in this study might have implications for the investigation of oscillatory phenomena in other tissues. Thus inferences derived from studies in the absence of CO$_2$–HCO$_3^-$ may not reveal the exact nature of this interesting and significant physiological phenomenon.
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