

# S-Carbocysteine-lysine salt monohydrate and cAMP cause non-additive activation of the cystic fibrosis transmembrane regulator channel in human respiratory epithelium

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**Abstract** S-Carbocysteine-lysine salt monohydrate (S-CMC-Lys) has been shown to open a Cl<sup>−</sup> channel in the trachea, thus aiding fluid secretion. The aim of this study was to characterize the channel and the action mechanism on a culture line of human respiratory epithelial cells. The patch-clamp technique (in cell-attached or inside-out configuration) and conventional micro-electrodes were used. The activity and density of a cAMP-dependent Cl<sup>−</sup> channel, identical to the cystic fibrosis transmembrane regulator (CFTR) channel, proved to be maximally stimulated by 100 μM S-CMC-Lys present in the cAMP-free cell incubation medium for 240–290 min (cell-attached configuration). Subsequent addition of cAMP to the medium did not determine any further activation. S-CMC-Lys acted mostly indirectly as, when placed in direct contact with a membrane patch, activation of the CFTR channel was nil (cytoplasmic side) or limited (external side).

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**Key words:** S-Carbocysteine-lysine salt monohydrate; Cl<sup>−</sup> channel; Respiratory epithelium

## 1. Introduction

S-CMC-Lys is a mucoactive drug used in the treatment of different respiratory diseases characterized by abnormal mucus secretion. S-CMC-Lys re-establishes a correct balance between sialo- and fuco-mucins, thus increasing fluidity and removal of the mucus [1], and its ability to modulate Cl<sup>−</sup> transport through rabbit trachea, and thus water secretion, has also been shown [2]. The extent of secretion, which regulates the level of periciliary liquid influencing mucociliary clearance, depends on several factors, including the opening of Cl<sup>−</sup> conductance, at least partially attributable to the CFTR channel (whose inability to function causes cystic fibrosis, CF) [3–5]. Other Cl<sup>−</sup> channels, including ORCC, may be involved in the secretion [3–8]. The involvement of S-CMC-Lys in activating Cl<sup>−</sup> conductance has recently been confirmed by patch-clamp data on respiratory cells, although they were only obtained in whole-cell configuration [9]. These

findings suggest CFTR involvement and a rapid S-CMC-Lys action, presumably addressed directly to the channel. This does not agree with the lengthy activation time observed in rabbit trachea [2].

As already suggested [9], in order to examine the direct contribution of CFTR activation to Cl<sup>−</sup> conductance and to verify the possible effects on cell metabolism or second messengers, single-channel analyses in cell-attached or excised-patch configurations are necessary. The purpose of this study was to establish the effect of S-CMC-Lys on Cl<sup>−</sup> channels in human cells by the patch-clamp technique applied in the cell-attached or excised-patch configuration (i.e. by analysis of single ion channels). An additional purpose was to obtain information about the action mechanism.

## 2. Materials and methods

### 2.1. Cell culture

We used a human respiratory cell line (WI-26VA4) supplied by Centro Substrati Cellulari of the Istituto Zooprofilattico Sperimentale (Brescia, Italy). The cells were placed in a small Petri dish and incubated (37°C; 5% CO<sub>2</sub>) in a culture medium (Minimum Essential Medium Eagle, MEM, +2% L-glutamine, Sigma-Aldrich, Milano, Italy, +10% fetal calf serum, Seromed, Berlin, Germany) for 44–48 h so as to attain nearly 70% confluence; we used cells in the middle of the growing zone for experiments. During long-term treatments with S-CMC-Lys the Petri dishes were kept in an incubator, except for the electrophysiological measurements.

### 2.2. Electrophysiological techniques

The patch-clamp technique and data analysis were applied as reported [10,11]. We used microelectrodes with a resistance of 13–16 MΩ, and the seals had a resistance in the 20–60 GΩ range. The signals were filtered at 100–200 Hz with an eight-pole Bessel filter. The bath was grounded with an Ag/AgCl<sub>2</sub> electrode immersed in an agar bridge with the same ion composition as the cell incubation medium, or with 1 M KCl. The junction potentials were taken into account. In the presence of multiple channel openings, the open probability (*P*<sub>o</sub>) was calculated as already reported [12]. In the cell-attached configuration, the potentials were expressed as overall potentials, considering the cell membrane electrical potential difference, *p*<sub>dcm</sub>, and the holding potentials. The micropipette solution and the solution used on the cytoplasmic side in the inside-out configuration contained (mM): 145 NMGC1, 10 TES/NMG (pH 7.4), 3 EGTA and CaCl<sub>2</sub> to achieve a free [Ca<sup>2+</sup>] of 2 × 10<sup>−7</sup> M [13,14].

The conventional microelectrodes, measurement set-up and acceptance criteria for the impalements were similar to those previously described [15,16]. The microelectrodes had a resistance of 40–70 MΩ, and were connected to a very high input resistance (2 × 10<sup>14</sup> Ω) electrometer (Kethley 617, Cleveland, OH) and a strip chart recorder (Linear 1200, Reno, Nev).

During the experiment the cultured cells were kept in MEM with or without 8-Br-cAMP or S-CMC-Lys.

### 2.3. Drugs and chemicals

The following drugs and chemicals were used (sources are given in

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**Abbreviations:** 8-Br-cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane regulator; db-cAMP, dibutyl adenosine 3':5'-cyclic monophosphate; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid; DPC, diphenylaminecarboxylic acid; NMG, *N*-methylglucamine; NMGC1, *N*-methylglucamine-Cl; ORCC, outwardly rectifying Cl<sup>−</sup> channel; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; S-CMC-Lys, S-carbocysteine-lysine salt monohydrate; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid.

parentheses): 8-Br-cAMP and EGTA (Sigma, St Louis, MO), DIDS and DPC (Aldrich Chemical, Milwaukee), NMG (Fluka, Buchs, Switzerland), and S-CMC-Lys (Dompè SpA, Milano, Italy).

#### 2.4. Statistics

Unless otherwise specified, the experimental values are expressed as means  $\pm$  S.E.M. of  $n$  experiments. Student's  $t$ -test was used (unpaired data unless otherwise specified) for the statistical analysis.

### 3. Results

The  $pd_{cm}$  measured in the absence of 8-Br-cAMP in the incubation medium is reported in Fig. 1. In the cell-attached configuration, a channel in which the current/voltage ( $I/V$ ) relation presented a reversal potential ( $-29.3 \pm 4.2$  mV,  $n=9$ ) compatible with a  $Cl^-$  channel was observed in about 30% of cases ( $n=27$ ). However, both  $P_o$  and active channel density (ratio between number of channels observed and number of patches,  $N_c/p$ ) were low, and  $P_o$  was not significantly different from 0 (Table 1). After switching to the inside-out configuration, in the presence of NMGCl symmetrical solutions, no channel activity was observed. When the cell incubation medium was treated with 8-Br-cAMP ( $250 \mu M$ )<sup>1</sup>, the channel observed presented high  $P_o$  and  $N_c/p$  values (Fig. 2). The  $pd_{cm}$  is shown in Fig. 1. The  $P_o$  tended to remain constant, regardless of the voltage applied (the average conductance was  $6.9 \pm 0.4$  pS,  $n=13$ ). After switching to the inside-out configuration from cells pre-treated with 8-Br-cAMP (NMGCl symmetrical solutions) in the presence of theophylline (3 mM) on the cytoplasmic side, the  $P_o$  levels remained high over a period of time (at least 8–12 min). The  $I/V$  relation was linear, with a reversal potential of  $0.4 \pm 0.4$  mV ( $n=16$ ), as was to be expected for a  $Cl^-$  channel under these conditions. The average conductance was  $6.8 \pm 0.5$  pS,  $n=16$ . In the absence of theophylline, channel activity fell rapidly to zero (3–5 min). In the presence of DPC ( $100 \mu M$ ) and theophylline (3 mM) on the cytoplasmic side, a roughly 50% reduction in  $P_o$  (at all the tested voltages) and conductance was observed. For example, at  $-70$  mV  $P_o$  fell from  $0.387 \pm 0.052$  to  $0.184 \pm 0.03$ , ( $n=4$ ,  $p < 0.01$ , paired data), and conductance fell from  $7.0 \pm 0.86$  to  $3.7 \pm 1.0$ , ( $n=4$ ,  $p < 0.01$ , paired data). However, DIDS ( $100 \mu M + 3$  mM theophylline), if present on the cytoplasmic side, was ineffective.

In the presence of 8-Br-cAMP ( $250 \mu M$ ) and in the cell-attached configuration a channel, presumably a  $Cl^-$  channel, showing conductance higher than that observed previously, was noted in 23% of cases ( $n=13$ ). This channel was never observed when 8-Br-cAMP was not present in the incubation medium ( $n=27$ ). After changing to the inside-out configuration (from cells pre-treated with 8-Br-cAMP and in the presence of theophylline on the cytoplasmic side), these channels appeared after strong depolarizing stimuli. When symmetrical solutions containing  $Cl^-$  were used, the  $I/V$  relation presented an outward rectification (conductance was  $33.4 \pm 2.5/16.5 \pm 1.1$  pS ( $n=8$ ) for positive/negative voltages). DIDS ( $100 \mu M$ , cy-

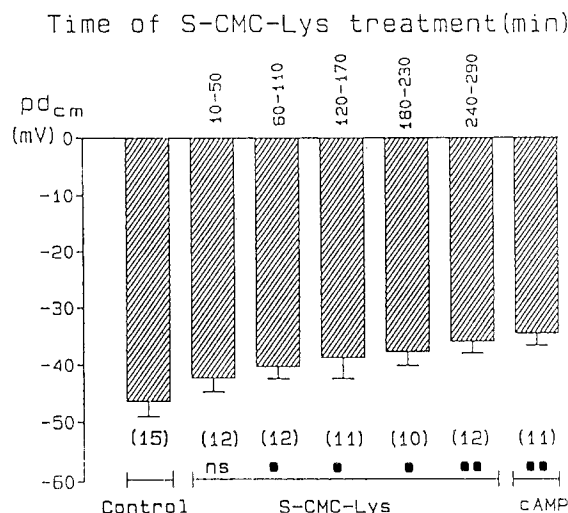


Fig. 1. Cell membrane electrical potential differences ( $pd_{cm}$ ) measured by impalements with conventional microelectrodes under different experimental conditions. Means  $\pm$  S.E.M., number of data in parentheses. •  $p < 0.05$ , ■  $p < 0.01$ , compared to control.

toplasmic side) decreased the  $P_o$  of this channel from  $0.67 \pm 0.051$  to  $0.047 \pm 0.05$ ,  $n=4$ , at  $-40$  mV ( $p < 0.01$ , paired data).

We then measured  $pd_{cm}$  and performed patch-clamp experiments in cell-attached configuration using cells incubated in the presence of S-CMC-Lys ( $100 \mu M$ ). The  $pd_{cm}$  obtained in the treatment periods 10–50, 60–110, 120–170, 180–230 and 240–290 min decreased gradually from the control conditions (Fig. 1).

The data obtained in the cell-attached configuration with S-CMC-Lys ( $100 \mu M$ ) in both the incubation medium and the microelectrode solution (Fig. 2) demonstrated that the values of  $P_o$  and  $N_c/p$  rose as treatment time increased. After treatment lasting 240–290 min, these parameters were not significantly different from those obtained in the presence of 8-Br-cAMP alone. No significant variations of single channel conductance were observed. However, in the inside-out configuration (symmetrical solutions containing only  $Cl^-$  as permeable ion), no effects on the low-conductance  $Cl^-$  channel were seen if S-CMC-Lys ( $100 \mu M$ ) was applied only on the cytoplasmic side, using cells pre-treated or not pre-treated with 8-Br-cAMP. Conversely, when S-CMC-Lys was present in the microelectrode solution, a significant increase in  $P_o$  and a tendency of  $N_c/p$  to increase (2–10 min after the seal) were observed in cell-attached configuration (cells not pre-treated with 8-Br-cAMP) (Table 1). Even after switching to the inside-out configuration (NMGCl symmetrical solutions), both  $P_o$  and  $N_c/p$  were significantly higher than the values obtained when S-CMC-Lys was not present in the microelectrode (Table 1). In the cell-attached configuration, when S-CMC-Lys was present in the incubation medium (but not in the microelectrode) for periods of 10–50 and 240–290 min,  $P_o$  and  $N_c/p$  increased with time (Fig. 3). When 8-Br-cAMP was added to the medium 240–290 min after S-CMC-Lys treatment,  $P_o$  and  $N_c/p$  did not display any further increase (Fig. 3).

The outwardly rectifying  $Cl^-$  channel was noted in 22% of cases ( $n=18$ ) in the cell-attached configuration and in the presence of S-CMC-Lys in both media for a period of 180–290 min.  $P_o$  and  $N_c/p$ , at  $-60$  mV, were  $0.024 \pm 0.012$  and

<sup>1</sup>To stimulate  $Cl^-$  secretion in intact epithelial cells 8-Br-cAMP or db-cAMP was usually used at a concentration from 0.2 to 1 mM when present in the incubation medium [17–21]. Under these conditions the effects were mimicked by some secretagogues (theophylline, forskolin,  $PGE_1$ , acting via intracellular cAMP [12,17,22]). In some experiments we used  $100 \mu M$  8-Br-cAMP obtaining results similar to those observed using  $250 \mu M$  8-Br-cAMP. This suggests that at a lower concentration ( $100 \mu M$ ), 8-Br-cAMP already exerted the maximal effect.

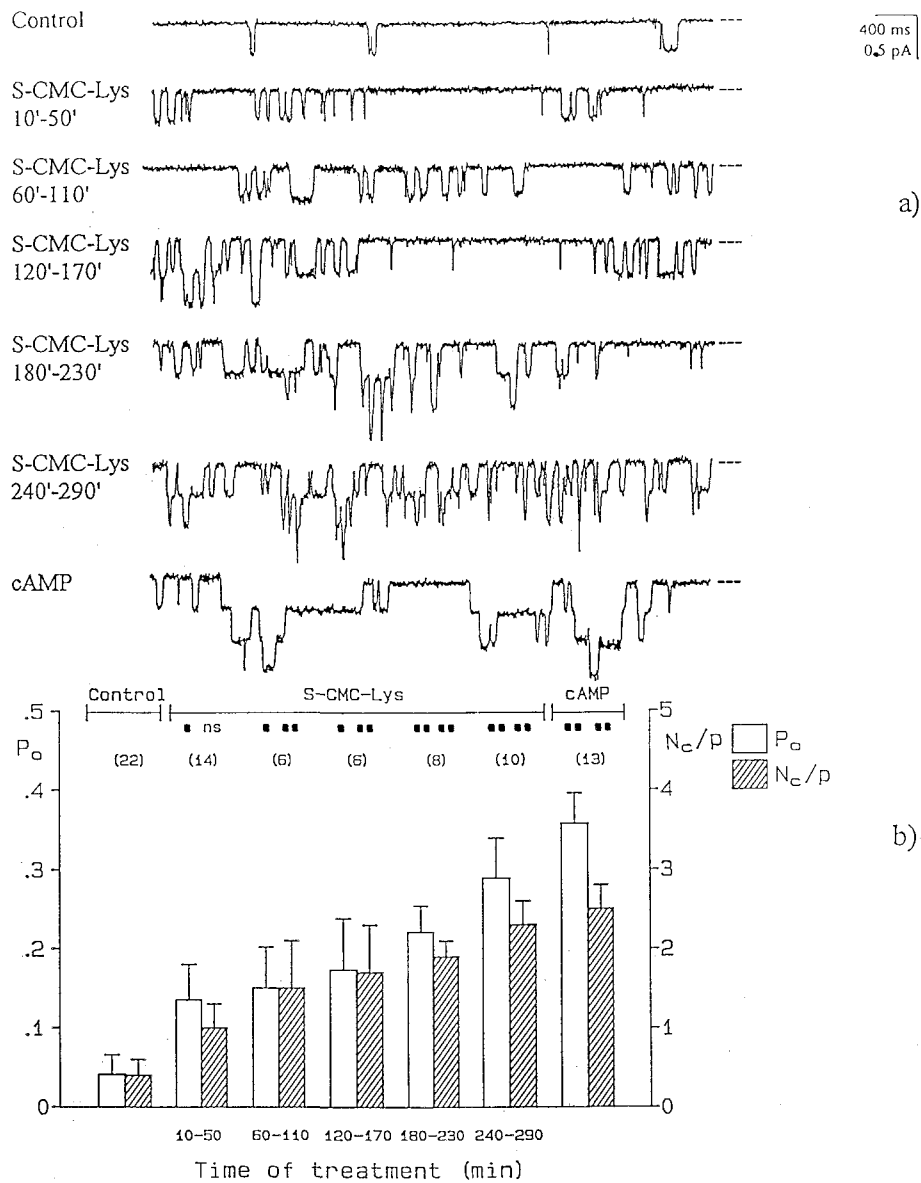


Fig. 2. Effect of S-CMC-Lys or 8-Br-cAMP treatments on small conductance  $\text{Cl}^-$  channel observed in cell-attached configuration. S-CMC-Lys (100  $\mu\text{M}$ ) or 8-Br-cAMP (250  $\mu\text{M}$ ) were present in both the incubation medium and the microelectrode filling solution. The voltage was  $-80$  mV. (a) Tracings obtained under indicated conditions. (b) Effects on open probability ( $P_o$ ) and the number of the channels observed/number of patches ( $N_c/p$ ). Means  $\pm$  S.E.M., number of experiments in parentheses. •  $p < 0.05$ , ••  $p < 0.01$ , compared to control. Data refer to experiments performed on the same days.

Table 1  
Effects of the presence of S-CMC-Lys in the microelectrode filling solution using cells not treated with 8-Br-cAMP

Configuration	Conditions	$P_o$	$N_c/p$
Cell-attached	Control	$0.036 \pm 0.021$ (27)	$0.5 \pm 0.2^b$ (27)
	+S-CMC-Lys (100 $\mu\text{M}$ ) in the microelectrode	$0.095 \pm 0.027^a$ (14)	$0.9 \pm 0.3$ (14)
Inside-out	NMGCl symetric solutions	$0 \pm 0$ (27)	$0 \pm 0$ (27)
	+S-CMC-Lys (100 $\mu\text{M}$ ) in the microelectrode	$0.078 \pm 0.042^a$ (5)	$0.8 \pm 0.2^a$ (5)

The voltage was  $-80$  mV. Means  $\pm$  S.E.M., number of data between parentheses. NMGCl, *N*-methylglucamine  $\text{Cl}^-$ .  
<sup>a</sup> $p < 0.05$ , compared to control; <sup>b</sup> $p < 0.05$ , compared to zero.

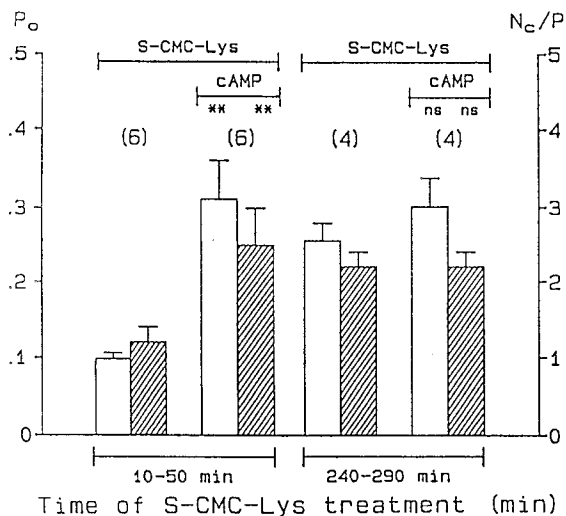


Fig. 3. Effects of 8-Br-cAMP (250  $\mu$ M) treatment after addition of S-CMC-Lys (100  $\mu$ M) to the incubation medium on open probability ( $P_o$ ) of the low-conductance  $Cl^-$  channel (cell-attached configuration). The voltage was  $-80$  mV. Means  $\pm$  S.E.M., number of experiments in parentheses. \* $p < 0.05$ , \*\* $p < 0.01$  (paired data analysis; the comparison is between data obtained in the presence or absence of 8-Br-cAMP at the same time as S-CMC-Lys treatment).

$0.2 \pm 0.1$  ( $n = 18$ ) respectively, and differed significantly ( $p < 0.05$ ) from the condition without S-CMC-Lys ( $0 \pm 0$ , for each parameter,  $n = 27$ ). When this channel was considered in the inside-out configuration (symmetrical  $[Cl^-]$ , activation obtained with marked depolarization), addition of S-CMC-Lys on the cytoplasmic side had no effect on the parameters examined.

#### 4. Discussion

The low-conductance  $Cl^-$  channel has characteristics similar to CFTR [3–5]. Its conductance is low, the  $I/V$  relation is linear, and the channel can be inhibited by DPC but not by DIDS. In addition, channel activity increases in the presence of 8-Br-cAMP and, similarly to CFTR, channel activity in the inside-out configuration tends to remain constant for longer times in the presence of theophylline (which tends to inhibit dephosphorylation of the channel) on the cytoplasmic side [23].

Considering the low-conductance channel in the cell-attached configuration, S-CMC-Lys determined (after 240–290 min of exposure) an increase in  $P_o$  and  $N_c/p$  values (6–7 times higher in both cases) which was not significantly different from that obtained in the presence of 8-Br-cAMP. The action of S-CMC-Lys is complex. The first action seems to be a direct effect on the channel on the external side of the cell (S-CMC-Lys in the microelectrode solution). The second effect is much greater, and occurs under conditions (cell-attached and presence of S-CMC-Lys only in the medium; long action time) in which a direct effect on the channel cannot be involved. In fact, a slow effect due to entry into the cell of S-CMC-Lys or its direct action on the  $Cl^-$  channel on the cytoplasmic side can be excluded from inside-out configuration experiments in which S-CMC-Lys on the cytoplasmic side has no effect. Rather, the slowness of the effect suggests that the action mechanism mainly involves modification of the level of an intracellular mediator, or metabolic action. The

fact that 8-Br-cAMP has no effect on cells pre-treated for a lengthy period with S-CMC-Lys suggests an action of S-CMC-Lys on the 8-Br-cAMP final target or pathway or production. The ability of S-CMC-Lys to activate CFTR channels may be still more important if, as suggested [9], it has also been observed in CF cells.

The larger conductance  $Cl^-$  channel possesses characteristics similar to ORCC [3–5,7]. The effects of S-CMC-Lys on it are difficult to evaluate, since the activity of this channel is not easily observed in the cell-attached configuration [5]. In any event, the data obtained in the inside-out configuration tend to exclude a possible direct effect of S-CMC-Lys on the cytoplasmic side of the channel. Moreover, the data obtained in rabbit trachea [2] indicate a low inhibitory effect of SITS and a marked effect of the DPC S-CMC-Lys-dependent component (characteristics corresponding to those of CFTR), so that this larger  $Cl^-$  channel seems to be of secondary importance.

Taken as a whole, the data obtained in this study demonstrate the ability of S-CMC-Lys to modulate  $Cl^-$  conductance through  $Cl^-$  channels with characteristics corresponding to CFTR, confirming the previous findings [2,9]. This effect might account, at least in part, for the favorable effect of S-CMC-Lys on mucus viscosity and removal.

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