(Dys)regulation of epithelial chloride channels

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Chloride (Cl\textsuperscript{−})-conducting channel proteins in plasma and intracellular membranes subserve a diversity of cellular functions, including neurotransmission, osmoregulation, pH regulation in organelles, acid secretion by parietal cells in the stomach, and salt absorption or secretion in exocrine glands (pancreas, sweat gland) and transport epithelia (trachea, intestine). With the notable exception of the \gamma-amino butyric acid (GABA)- and glycine-gated neuronal Cl\textsuperscript{−} channels [1], none of the anion-selective channel proteins have so far been purified, cloned and sequenced, hampering a structural comparison and classification of the various types of epithelial and non-epithelial Cl\textsuperscript{−} channels. However, the partial purification of epithelial Cl\textsuperscript{−}-channel proteins has been achieved recently by affinity chromatography of trachea and kidney membrane extracts on an immobilized indanyloxyacetic acid (GABA)- and glycine-gated neuronal Cl\textsuperscript{−} channels [1], (pancreas, sweat gland) and transport epithelia (trachea, intestine). With the notable exception of the \gamma-aminobutyric acid (GABA)- and glycine-gated neuronal Cl\textsuperscript{−} channels [1], none of the anion-selective channel proteins have so far been purified, cloned and sequenced, hampering a structural comparison and classification of the various types of epithelial and non-epithelial Cl\textsuperscript{−} channels. However, the partial purification of epithelial Cl\textsuperscript{−}-channel proteins has been achieved recently by affinity chromatography of trachea and kidney membrane extracts on an immobilized indanyloxyacetic acid derivative belonging to a new class of potent and selective Cl\textsuperscript{−} channel inhibitors [2].

In contrast, rapid progress has been made in the characterization and classification of anion-selective channels on the basis of biophysical criteria, following the introduction of patch-clamp and planar lipid bilayer techniques [3–5]. The major anion conductance occurring in the apical membrane of all salt-secreting epithelial cell types analysed so far is a 30–50 pS outwardly rectifying channel characterized by the selectivity sequence Cl\textsuperscript{−} > NO\textsubscript{3}\textsuperscript{−} > I\textsuperscript{−} > Br\textsuperscript{−} > Cl\textsuperscript{−} > acetate > gluconate [3, 6]. In intact cells, the gating of this apical Cl\textsuperscript{−} channel is triggered by a variety of intracellular signals including cyclic AMP, cyclic GMP (acting exclusively in intestinal epithelial cells), and Ca\textsuperscript{2+} [7, 8]. In addition, the same Cl\textsuperscript{−} channel can be activated non-physiologically and irreversibly by other manoeuvres, such as a patch excision at depolarizing voltages and cell perfusion during whole-cell patch-clamp recording, possibly resulting in the removal of an endogenous Cl\textsuperscript{−}-channel inhibitor [3, 4, 9]. The selective loss of Ca\textsuperscript{2+}, cyclic GMP-, but not of cyclic AMP-, responsive Cl\textsuperscript{−} currents observed during maturation of Caco-2 colon carcinoma cells in culture [10] and the recent isolation of a T-84 colon carcinoma subclone displaying Ca\textsuperscript{2+}-activable, but cyclic nucleotide-insensitive, Cl\textsuperscript{−} currents (H. R. De Jonge, unpublished work) support a regulatory model in which each signal is capable of activating the Cl\textsuperscript{−} channel independently of the other messengers through a common or different regulatory component.

Hypothetically, cyclic nucleotide and Ca\textsuperscript{2+} regulation of ion channels may occur through: (i) a direct interaction with an allosteric modifier site on the channel protein; examples of this mechanism include the cyclic GMP-gated cation channel in rod and cone photoreceptors [11] and cyclic GMP inhibition of an amiloride-sensitive Na\textsuperscript{+} channel in renal inner medullary collecting duct cells [12]; (ii) phosphorylation of the channel protein or regulatory subunit by cyclic AMP-, cyclic GMP- or Ca\textsuperscript{2+}-dependent protein kinases; (iii) cyclic nucleotide- or Ca\textsuperscript{2+}-provoked fusion of channel-containing vesicles with the apical membrane ('channel recruitment'). Direct evidence in favour of the second mechanism has been obtained recently in patch-clamp studies of excised membrane patches from human tracheal cells [9, 13], lymphocytes [14], and colon carcinoma cells

(Fig. 1). Exposure of the inside-out patch to the catalytic subunit of cyclic AMP-dependent protein kinase and ATP under conditions preventing voltage activation resulted in phosphorylation-induced activation of the Cl\textsuperscript{−} channel that could be reversed by removal of the kinase from the bath. In contrast, the addition of cyclic nucleotides alone or a large variation in Ca\textsuperscript{2+} levels (10⁻⁸–10⁻³ m) was unable to promote the opening of intestinal Cl\textsuperscript{−} channels under similar conditions, arguing against a role of mechanism (i) in Cl\textsuperscript{−} channel activation (J. Bijman, unpublished work). Further evidence pointing to a role of membrane protein phosphorylation in cyclic GMP-provoked Cl\textsuperscript{−}-channel activation is inferred from the following findings.

(a) Using 8-N3-³²P cyclic IMP as a photolabeling probe, we have identified an 86 kDa protein (p86) in the brush border membrane of intestinal epithelial cells serving as the sole receptor site for cyclic GMP in rat, pig and human enterocytes [7, 8, 15]. After its purification by salt/detergent extraction and cyclic AMP affinity chromatography, p86 was characterized further as a membrane cyclic GMP-dependent protein kinase (type II) displaying major structural, immunological and functional differences with the soluble isoenzyme found in other tissues (type I). As demonstrated by Western blotting, non-intestinal epithelial cells including trachea and sweat gland cells failed to express detectable levels of the membrane isoenzyme, which could possibly explain the

Abbreviations used: CF, cystic fibrosis; CaM, calmodulin.

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Fig. 1. Cl\textsuperscript{−}-channel activation by the catalytic subunit of cyclic AMP-dependent protein kinase in a membrane patch excised from HT-29 cl.19A colonocytes

Recordings were obtained from the upward-facing membranes of confluent island of 15-40 cells. The pipette solution was (mM): 95, NaCl; 50, KCl; 1.5, CaCl\textsubscript{2}; 1, MgCl\textsubscript{2}; 5 Hepes, pH 7.2; the bath contained 140, NaCl; 5, KCl; 1, MgCl\textsubscript{2}; 1.5, CaCl\textsubscript{2}; 5 Hepes, pH 7.2. Current records were obtained at a filter bandwidth of 1 kHz. Membrane voltage is expressed with respect to the pipette as reference. Top trace: the excised patch was electrically silent at -60 mV for 4 min. Trace 2: 1 mM-MgATP + 100 nm-purified catalytic (C) subunit (kindly donated by Dr U. Walter, Univ. Klinik, Würzburg) were added to the bath; inward currents were evoked by the catalytic subunit ~2 min after its addition. Lower traces: outward Cl\textsuperscript{−} currents recorded after stepwise depolarization to +40 and +70 mV. The HT-29 subclone was kindly made available by Dr Laboisse, Unite 239 INSERM, Paris.
The apparent insensitivity of the apical Cl⁻ channels to cyclic GMP signals in these tissues [16].

The isoquinolinesulphonamide, H-8, a potent and selective inhibitor of the type II cyclic GMP-dependent protein kinase in vitro, was able to block Cl⁻ secretion in rat proximal colon provoked by 8-bromoguanosine 3′:5′-cyclic monophosphate and heat-stable Escherichia coli toxin (a specific activator of intestinal guanylate cyclase; [17]) at concentrations which did not affect cyclic AMP- or Ca²⁺-triggered Cl⁻ secretion [18].

The p86 kinase is different from the cyclic AMP-dependent protein kinases in that: (i) it is anchored firmly into the intestinal brush border membrane; (ii) it does not dissociate into separate cyclic nucleotide and catalytic subunits following cyclic GMP activation, but remains immobilized in the membrane; (iii) it displays a very narrow substrate specificity; and (iv) it is involved mainly or exclusively in ion transport regulation. A search for endogenous substrate proteins for this kinase could therefore be used as a strategy to identify putative Cl⁻-channel regulators or the Cl⁻ channel itself. So far, the sole substrate protein detected by phosphorylation of intestinal brush border membranes in vitro is a 25 kDa acidic proteolipid (p25) serving as a cosubstrate for endogenous and exogenous cyclic AMP-dependent protein kinase [7, 8]. In contrast, protein kinase C or endogenous Ca²⁺-calmodulin (CaM)-dependent protein kinase failed to phosphorylate this minor membrane component. Intriguingly, the phosphorylating protein is recognized on Western blots by a specific antibody directed against the cytoplasmic tail of the band 3 protein from erythrocytes [16]. The tissue distribution of p25 and its possible role as a Cl⁻-channel regulator is under present investigation.

The putative role of p25 and other aspects of intestinal Cl⁻-channel regulation are visualized in the hypothetical model depicted in Fig. 2. As indicated in this model, the coupling mechanism between Ca²⁺ signals and the Cl⁻ channel is not yet elucidated, but may involve a high-affinity Ca²⁺-binding protein serving as a dissociable subunit of the channel or a Ca²⁺-CaM-dependent protein kinase catalysing channel phosphorylation. In contrast, the activation of protein kinase C by phorbol esters in human ileal mucosa and T-84 colonocytes leads to a rapid closure (dephosphorylation?) of cyclic nucleotide-activated Cl⁻ channels, suggesting a physiological role of protein kinase C as an inhibitory modulator [H. R. De Jonge, unpublished work].

Our recent discovery of an enrichment of GTP-binding proteins (G-proteins), including G₁, G₂, G₃ (the putative activator of phosphoinositide-specific phospholipase C) and p21 ras in the apical membrane of intestinal epithelial cells, in spite of the apparent lack of G-protein-coupled hormone receptors in this subcellular region ([19]; N. van den Berghe, unpublished work), has also prompted us to explore their role as potential regulators of intestinal Cl⁻ channels. Measurements of membrane-potential-driven ¹²⁵I⁻ overflow in apical membrane vesicles isolated from a Cl⁻-secreting subclone of the HT-29 colon carcinoma cell line have provided preliminary evidence for the occurrence of a G-protein-sensitive anion conductance that was activated by intravesicular entrapment of GTPyS and inhibited by GDPβS (Table I). Activation by GTPyS appeared independent of intravesicular Ca²⁺ levels, arguing against a role of Ca²⁺-dependent and G-protein-regulated phospholipase C or A₁ in the channel-gating mechanism (results not shown). In analogy to similar studies of G-protein activation of the cardiac muscarinic K⁺ channel [20, 21], patch-clamp analysis and reconstitution of the channel protein in planar lipid bilayers is clearly needed to identify the G-protein involved and to assess the relationship between the G-protein-activated anion conductance and the Ca²⁺- and cyclic nucleotide-sensitive Cl⁻ channel in the HT-29 colonocytes. Moreover, further research is needed to identify potential physiological activators of the G-protein in the apical membrane, e.g. arachidonic acid metabolites [21] or apical receptor proteins.

Abnormalities of Cl⁻-channel regulation in cystic fibrosis

Cystic fibrosis (CF), the most common lethal genetic disease in Caucasians, is a generalized exocrinopathy characterized by an impairment of conductive Cl⁻ transport. Electrophysiological studies in trachea [9, 13], sweat gland [22, 23], intestinal mucosa [10, 24, 25] and lymphocytes [24] have provided evidence for (i) a defect in cyclic AMP-activation of the Cl⁻ channel at a site distal to cyclic AMP-depen-

![Fig. 2. Hypothetical model of Cl⁻-channel regulation in intestinal epithelial cells](image_url)

The p25 proteolipid is depicted as a putative regulatory component associated with the channel. PK-C, protein kinase C; PK-G, cyclic GMP-dependent protein kinase (type II); PK-A, cyclic AMP-dependent protein kinase (type II); C, catalytic subunit; R₉0, regulatory subunit; molecular masses are indicated in kDa; DAG, diacylglycerol; TPA, 12-O-tetradecanoylphorbol 13-acetate; CaBP₁, a Ca²⁺-binding protein conferring Ca²⁺ sensitivity to the Cl⁻ channel; CaM, calmodulin; CFAG, CF antigen, a Ca²⁺-binding protein accumulating in the serum of CF patients [26], G protein, a putative GTP-binding protein involved in Cl⁻ channel regulation. * potential sites of regulatory abnormalities in CF.
Table 1. 125I-Uptake in KCl-loaded apical membrane vesicles isolated from the HT-29 CL.19A colonocytes

Values are means ± S.E.M. of three to five membrane preparations. Apical membrane vesicles isolated from homogenates of confluent monolayers of HT-29 cl.19A colonocytes by differential Mg2+ precipitation [15] were suspended in buffer A (20 mM-Hepes/Tris, pH 7.0, 0.3 mM-mannitol, 6 mM-magnesium gluconate, 3 mM-Ca/EGTA (pCa 6) containing 0.15 mM-KCl (3-4 mg of protein/ml) and the indicated concentrations of guanine nucleotides. A single cycle of freezing-thawing in liquid nitrogen was employed to trap GTPyS and GDPS in the vesicle interior. Cyclic nucleotide specific, although the molecular basis of the activation at a site distal to cyclic GMP-dependent protein kinase in all tissues; (ii) a defect in cyclic GMP activation at a site distal to cyclic GMP-dependent protein kinase in the enterocyte; (iii) a defect in Ca2+-activation at a site distal to Ca2+-mobilization in enterocytes and sweat gland duct, but not in trachea and sweat gland coil; (iv) normal voltage activation of Cl− channels in excised patches of CF trachea and sweat gland membranes, arguing against a channel-recruitment defect or a mutation in the conductive part of the channel. Apparently the regulatory defect is not cyclic nucleotide specific, although the molecular basis of the tissue heterogeneity in Ca2+ response unmasked by the CF condition remains unclear. As indicated in Fig. 2, the CF abnormality in intestinal Cl− secretion most plausibly arises from (i) a defect in a regulatory component of the Cl− channel shared by the cyclic AMP, cyclic GMP and Ca2+ pathways (p25, G-protein, ...); (ii) the hyperactivation of a protein phosphatase promoting Cl− channel inactivation by excessive dephosphorylation. Phosphorylation studies in normal and CF enterocytes and a more detailed electrophysiological characterization of the intestinal Cl− channels are clearly needed to discriminate further between these possibilities. Furthermore, a definite identification of the CF gene and the gene-encoded protein might provide important new insights into the molecular basis of Cl− channel regulation and dysregulation in epithelial cells and may suggest new approaches in the rational design of anti-diarrhoea drugs aimed to mimic the anti-secretory action of the CF mutation at the intestinal level.


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