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Calcium ionophore plus excision induce a large conductance chloride channel in membrane patches of human colon carcinoma cells HT-29cl.19A

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Abstract. In excised inside-out membrane patches of the human colon carcinoma HT-29cl.19A cells a large conductance (373 ± 10 pS) chloride channel was found. Channel activity could only be observed after excision of patches from cells incubated with calcium ionophore. The channel was never observed in cell-attached patches. The channel was strongly voltage dependent, being open only between +30 and −30 mV clamp potentials. The selectivity sequence among anions, deduced from reversal potentials, was I > Br > Cl > F > gluconate. The P_{Na}/P_{Cl} was 0.09. Although a similar type of channel has been described earlier, this is the first report stating its appearance in patches of intestinal epithelial cells requiring the combined action of Ca^{2+} ionophore and excision, suggesting its control by an intracellular compound.

Key words. Maxi-chloride channel; HT-29; intestinal epithelium; electrolyte transport; Ca^{2+}-ionophore.

Chloride secretion through the apical membrane of epithelial cells is induced by activation of Cl^- channels by intracellular second messengers (for review see Frizzell[1]). Recent findings suggest that non-rectifying, small conductance, cAMP-dependent Cl^- channels of about 6 pS may carry the Cl^- current[2,3]. In the cell clone HT-29cl.19A, forskolin (i.e. cAMP) induced chloride secretion by an increase in the Cl^- conductance of the apical membrane[4]. Cell-attached patch clamp experiments revealed the activation of these non-rectifying small Cl^- channels by forskolin and their further activation by the phorbol ester PDB (Bajnath et al.[5], and results submitted).

Carbachol evoked Cl^- secretion via increase of the Ca^{2+} concentration and activation of protein kinase C (PKC)[6,7]. We investigated whether we could find single channel activity in cell-attached patches after increasing intracellular Ca^{2+}. In a few cell-attached patches, after stimulation with carbachol or a Ca^{2+} ionophore, linear 13 pS channel activity was found (20 channels in 95 patches, to be reported elsewhere). Surprisingly, however, after excision of patches from ionophore-treated cells, large conductance Cl^- channels became active. This report is restricted to the description of this maxi Cl^- channel. Although maxi-anion channels have been described in many different cell types, their presence in this cell clone, which is used as a model for intestinal epithelial cells[4,6–8] has to our knowledge not been reported before. In contrast to previously reported data, maxi channel-activity could only be found after incubation with Ca^{2+} ionophore followed by excision of the patch.

Materials and methods

HT-29cl.19A cells[8] were grown in Dulbecco's Modified Eagle's medium in cell culture flasks in a 5% CO_2/95% O_2 incubator at 37 °C. The medium was supplemented with 10% fetal calf serum and the following antibiotics in mg/l: penicillin 40, ampicillin 8, and streptomycin 90. Cells were subcultured after trypsinization on 35-mm petri dishes (10^5 cells/dish) and used 3–8 days after plating. Culture media and antibiotics were purchased from Gibco. Patch clamp experiments were carried out in a solution containing (mM): NaCl 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 10, pH 7.4. The pipette solution had the following composition (mM): NaCl 95, KCl 50, $CaCl_2$ 1.5, $MgCl_2$ 1.0, HEPES 10, pH 7.4. This solution was used in order to distinguish between the presence of anion or cation channels under excised patch conditions. To determine the anion selectivity, NaCl in the bath was replaced by an equal amount of the sodium salt of one of the following anions: iodide, bromide, fluoride, and gluconate. Data are presented as mean and SEM.

Experiments were performed at a temperature of 24–26 °C. Single channel experiments were performed according to Hamill et al.[9]. Patch pipettes were fabricated from borosilicate glass (Clark GC150-15TF) and pulled in two stages (List, Darmstadt, FRG). Filled with standard pipette solution, the tip resistances varied between 4 and 8 MΩ. Single channel currents were amplified (LM-EPC 7, List, Darmstadt, FRG), digitized by means of a pulse code modulator (Sony PCM-F1) and stored on a video-cassette recorder (Sony SL-HF 950 E/ES). For analysis, the records were reconverted into

analog form, and usually low pass filtered at 0.5 kHz using an 8-pole bessel filter (902LPF, Frequency devices, Haverhill, MA.) and transferred to a Tulip 386SX Computer. The data were analysed using a software package programmed by Julius de Vries (Erasmus University, Rotterdam, the Netherlands).

In all recordings the pipette potential (V_p) refers to the command voltage applied to the pipette interior with respect to the bath solution, which was grounded through an agar bridge containing the 140 mM NaCl solution and an Ag/AgCl electrode. V_p was corrected for the diffusion potential across the agar bridge when Cl^- was replaced[10]. Upward deflections represent outward currents, i.e. cations flowing from bath to pipette or anions from pipette solution to bath. To increase intracellular Ca^{2+} concentration, cells were exposed to 10^{-6} M of the Ca^{2+} ionophore A23187 (Sigma Chemical, St. Louis, MO). The final concentration of the carrier solution ethanol (0.1%) was without measurable effect. Patches were made in the middle of cell islands from 50–90% confluent monolayers grown in petridishes. Seals were formed before ionophore addition or just after its addition up to 120 min incubation in the presence of the drug. After giga seal formation the pipette potential was varied between +70 and −70 mV for 5 min. If no channel activity was observed during this period in the cell-attached configuration, the membrane patch was excised. After patch excision the pipette potential was clamped at +20 mV for a maximum of 5 min. If no channel activity occurred within this time the experiment was terminated.

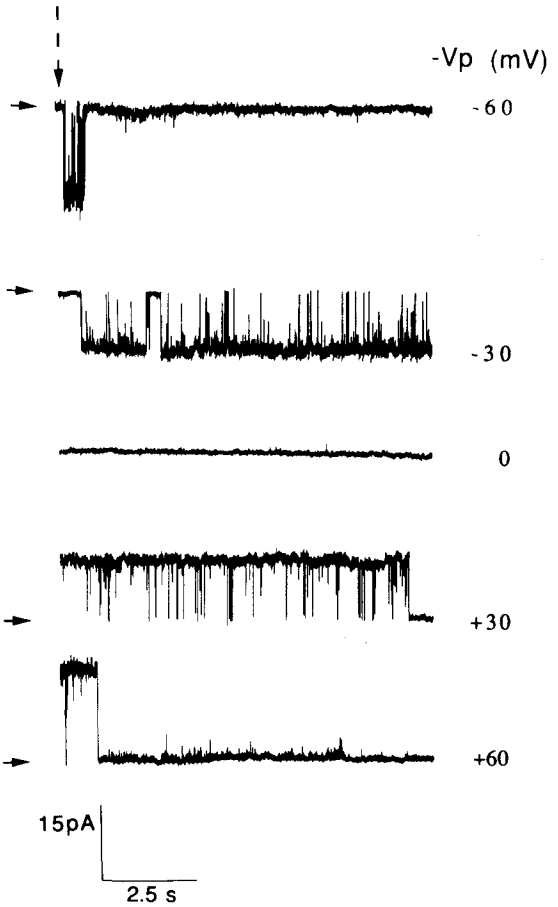

Figure 1. Single channel currents of an excised inside-out membrane patch from HT-29cl.19A cells. The closed state current levels are indicated by the horizontal arrows. The vertical arrow at the top of the tracing indicates time of the potential step to the value indicated at the right.

Results and discussion

After treatment with A23187, 57% of a total of 23 excised patches showed a large conductance channel while clamped at a pipette potential of +20 mV. Channel activity usually appeared within 4 min after excision and was present as long as the patch remained stable (5–30 min). With the same protocol but without drug-addition no large conductance channel could be found in 50 excised patches, nor were maxi channels found in 22 excised patches after incubation with forskolin nor in 27 patches after incubation with carbachol.

Figure 1 shows a typical record of channel activity at + and −30 mV and + and −60 mV clamp potentials in symmetrical chloride solutions. At positive clamp potentials, the currents are outward, the current reversed direction at 0 mV. This indicates that the channel is Cl^- selective. At clamp potentials between +30 and −30 mV the channel was most of the time in the open state (open probability 0.82–0.94). The open periods lasted for seconds, interrupted by brief channel closures of a few milliseconds. When the clamp potential exceeded +30 or −30 mV channels showed a very fast, reversible inactivation. Switching the clamp potential back to between +30 and −30 mV re-activated the channel.

Frequently, more than one conductance level could be observed. Figure 2 shows an example of a record at −30 mV clamp potential, which shows two different intermediate levels between the maximal open and the fully closed level. The amplitude-histogram constructed from this record (fig. 2, lower panel) shows, besides the zero current level, three other peaks at −3.8, −7.4, and −11.4 pA corresponding to 126, 246 and 372 pS, respectively. The full conductance state of the channel appears to be integer multiples of the lowest subconductance state. Our results show that the large-conductance Cl^- channel of the HT-29cl.19A cells may have at least three conductance states.

The transitions from the zero current level to the full conductance state (94%) far exceeded transitions from zero to a subconductance level. Intermediate levels were absent during prolonged periods of recording (fig. 1). Multiple transitions between the fully open and the fully closed level appear to be a common feature of large

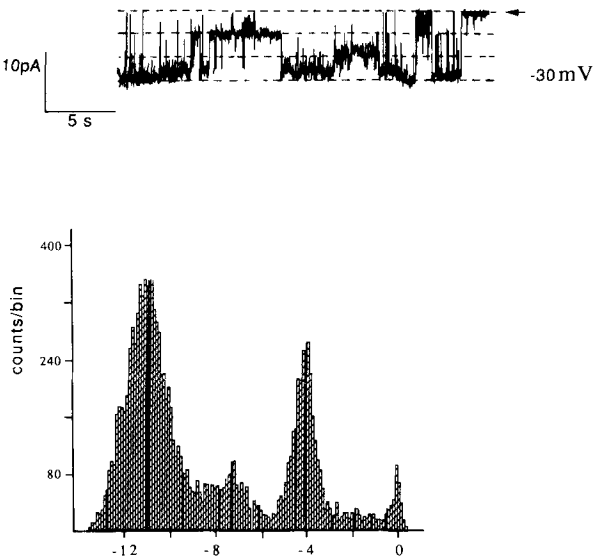

Figure 2. Multiple conductance states at −30 mV clamp potential (upper panel). Arrow indicates the fully closed state and the dashed lines the subconductance levels. Lower panel shows the amplitude histogram constructed from this record over 25 s.

conductance chloride channels[11-16]. These transitions are caused by the complex gating behaviour of one channel rather than the opening of different independent or artificially aggregated channels[13,15].

The single channel conductance was determined from the relation between the single channel current amplitude and the clamp potential. In symmetrical chloride solutions (i.e. pipette contained 50 KCl/95 NaCl solution and the bath solution contained 5 KCl/140 NaCl solution) the channel exhibits a linear I/V relation with a single channel conductance of 373 ± 10 pS and a reversal potential of 0.1 ± 0.4 mV, n = 13 (fig. 3). The conductance of the large-conductance channel of the HT-29cl.19A in 150 mM Cl^- solutions is very similar to those reported for other epithelial cells[11,14,17,18].

To determine the permeability ratio (P_{Na}/P_{Cl}) the bath solution was replaced by a solution containing 5 KCl/420 NaCl solution. From the shift in the reversal potential (fig. 3) a P_{Na}/P_{Cl} of 0.09 was calculated using the Goldman-Hodgkin-Katz equation. When the bath solution was changed to 5 KCl/47 NaCl + glucose, to compensate for osmolarity, the reversal potential shifted to −20 mV (fig. 3). Calculation of P_{Na}/P_{Cl} yielded again a value of 0.09.

The permeability ratio (P_{anion}/P_{Cl}) was calculated according to the Goldman-Hodgkin-Katz equation from the change in the reversal potential upon substitution of four different anions for chloride in the bath solution. The permeability sequence for the ions tested was: I(1.20) > Br(1.05) > Cl(1) > F(0.46) > Glu(0.24). A similar sequence for the anion selectivity has been observed in other large conductance anion channels[15,17,19,20]. The single channel currents showed rectification with gluconate and fluoride substitutions and the conductance for inward current with these anions was decreased to 128 ± 8 pS (n = 3) with gluconate and to 282 ± 10 pS (n = 3) with fluoride. Current rectification was less pronounced with iodide and bromide substitutions; apparently the difference in permeability between I, Br and Cl was too small to be detectable as an increase of the inward current.

The large conductance channel was not observed without preincubation with A23187 and was never observed in the cell attached patch configuration. In addition, this channel could not be activated by carbachol in cell attached patches and no channels could be found after patch excision of the carbachol activated cells. This differs from recent preliminary observations in the parental HT-29 cells[20], where a large-conductance chloride channel could be activated in cell-attached patches by ionomycin or by the muscarinic Ca-linked agonist carbachol. We propose that activation of the channel in HT-29cl.19A cells required elevation of intracellular

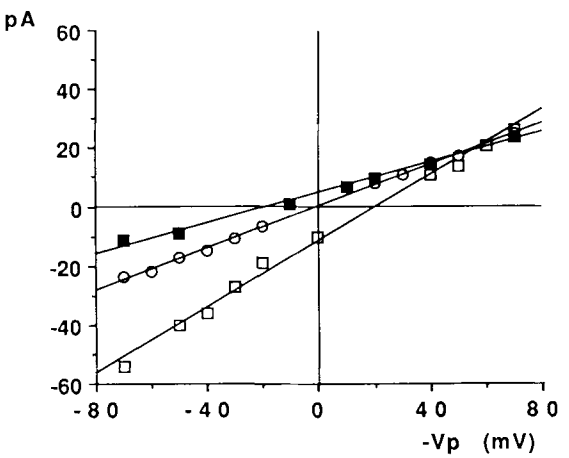

Figure 3. Plot of currents in the fully open state against pipette potential. Open circles (n = 13): symmetrical chloride solutions in pipette (50 KCl/95 NaCl) and bath (5 KCl/140 NaCl). The reversal potential shifted to 22 ± 2 mV (open squares, n = 3), when the bath solution was replaced by 5 KCl/420 NaCl solution. This is close to the theoretical value for the chloride equilibrium potential of 26.5 mV predicted by the Nernst equation. Replacing the bath solution by 5 KCl/47 NaCl shifted the reversal potential to −20 mV (filled squares, n = 1).

Reversal potentials and permeabilities relative to chloride

Anion	Reversal potential (mV)	P_{anion}/P_{Cl}	n
Iodide	5 ± 0.5	1.20	3
Bromide	1.8 ± 0.3	1.05	3
Chloride	0.1 ± 0.4	1.00	13
Fluoride	−17 ± 1.0	0.46	3
Gluconate	−30 ± 3.0	0.24	3

Values shown are mean ±SEM. n indicates the number of experiments. Chloride was replaced in the bath solution.

calcium to a high level and the removal of an intracellular suppressive factor by excision or the inactivation of a membrane bound enzyme. This idea is supported by the observation that after patch excision channel activity mostly occurred after a lag time (2 channels opened in the first min, 2 channels in the second min, 4 channels in the third min and 5 channels in the fourth min). During this period the blocking action of a putative factor may be washed away from the membrane patch and allow channel activation. Support for the hypothesis comes also from the observation that channel incidence increases after patch excision in other cells as well[11, 12, 21]. It may be that this putative suppressive factor is expressed in the clone 19A in a larger amount than in the parental cell line.

Our observation of the dependency of the channel activity on high Ca^{2+} plus excision seems to be unique. Activation by excision has been reported earlier e.g. for *MDCK* cells[12], but most of the large conductance anion channels are reported to be Ca^{2+} insensitive[11, 18, 19, 21–23]. The physiological function of large-conductance anion channels remains rather speculative[11, 12, 14–17]. The channel has been proposed to play a role in transepithelial chloride transport in several epithelia. However, a direct regulation of the channel by intracellular second-messengers, which are involved in the regulation of chloride transport (i.e. cAMP and Ca^{2+}) could not be shown in kidney epithelial cells[18]. Neither do our results support the idea that this channel is involved in the regulation of chloride transport in HT-29cl.19A cells. The apparently ubiquitous occurrence of this channel in cell membranes from many different tissues, however, suggests that it may fulfill some general, i.e. house-keeping, role in the control of cellular anion permeability. We postulate that if the channel has a function in the HT-29cl.19A cells at all, its activation requires an increase in intracellular Ca^{2+} and the down regulation of an inhibitory component.

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1 Frizzell, R. A., Trends neurosci. *10* (1987) 190.
2 Kartner, N., Hanrahan, J. W., Jensen, T. J., Naismith, A. L., Sun, S., Ackerly, C. A., Reyes, E. F., Tsui, L. C., Rommens, J. M., Bear, C. E., and Riordan, J. R., Cell *84* (1991) 681.
3 Tabcharani, J. A., Low, W., Elie, D., and Hanrahan, J. W., FEBS Lett. *270* (1990) 157.
4 Bajnath, R. B., Augeron, C., Laboisse, C. L., Bijman, J., de Jonge, H. R., and Groot, J. A., J. Membrane Biol. *122* (1991) 239.
5 Bajnath, R. B., Groot, J. A., de Jonge, H. R., and Bijman, J. Pflügers Arch. *420* (1992) R247.
6 Bajnath, R. B., Vaandrager, A. B., Dekker, K., de Jonge, H. R., and Groot, J. A., J. Membrane Biol. *127* (1992) 81.
7 Bajnath, R. B., van Hoeve, M. H., de Jonge, H. R., and Groot, J. A., Am. J. Physiol. *263* (1992) C759.
8 Augeron, C., Maoret, J. J., Laboisse, C. L., and Grasset, E., in: Ion-coupled Transport, p. 363. Eds F. Alvarado and C. H. Van Os. Elsevier, Amsterdam 1986.
9 Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J., Pflügers Arch. *395* (1981) 85.
10 Zuidema, T., Dekker, K., and Siegenbeek van Heukelom, J., Bioelectrochem. Bioenerg. *14* (1985) 479.
11 Becq, F., Fanjul, M., Mahieu, I., Berger, Z., Gola, M., and Hollande, E., Pflügers Arch. *420* (1992) 46.
12 Kolb, H. A., Brown, C. D. A., and Murer, H., Pflügers Arch. *403* (1985) 262.
13 Krouse, M. E., Schneider, G. T., and Gage, P. W., Nature *319* (1986) 58.
14 Nelson, D. J., Tang, J. M., and Palmer, L. G., J. Membrane Biol. *80* (1984) 81.
15 Schlichter, L. C., Grygorczyk, R., Pahapill, P. A., and Grygorczyk, C., Pflügers Arch. *416* (1990) 413.
16 Woll, K. H., and Neumcke, B., Pflügers Arch. *410* (1987) 641.
17 Schneider, G. T., Cook, D. I., Gage, P. W., and Young, J. A., Pflügers Arch. *404* (1985) 354.
18 Velasco, G., Prieto, M., Alvarez-Riera, J., Gascon, S., and Barros, F., Pflügers Arch. *414* (1989) 304.
19 Bevan, S., Gray, P. T. A., and Ritchie, J. M., J. Physiol., Lond. *348* (1984) 18P.
20 Hazama, A., Rabe, A., and Frömter, E., Pflügers Arch. *420* (1992) R242.
21 Schwarze, W., and Kolb, H. A., Pflügers Arch. *402* (1984) 281.
22 Blatz, A. L., and Magleby, K. L., Biophys. J. *43* (1983) 237.
23 Hanrahan, J. W., Alles, W. P., and Lewis, S. A., Biophys. J. *45* (1984) 300a.