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Evidence for apical chloride channels in rabbit mandibular salivary glands

A chloride-selective microelectrode study

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Abstract. Double-barrelled, chloride-selective microelectrodes were used to study mandibular gland acinar cells at rest and during cholinergic stimulation. At rest, intracellular chloride activity was five times the expected equilibrium activity. During sustained stimulation with acetylcholine, chloride activity fell to three times the expected equilibrium activity. Thus, the gradient for chloride exit was reduced in the stimulated cell. These results lead to the conclusion that stimulation increases the permeability of the acinar cell to chloride. Experiments in which extracellular chloride was removed provided evidence that the permeability increase was due to opening of chloride channels located principally in the apical membrane of the acinar cell.

Key words: Salivary glands – Chloride channels – Chloride activity – Ion-selective microelectrodes

Introduction

When stimulated by cholinergic agonists, the rabbit mandibular salivary gland secretes a watery saliva comprising a hypotonic solution of sodium chloride containing some potassium and bicarbonate. Since the work of Thaysen et al. [24], the elaboration of this secretion has been understood in terms of a dual process related to different anatomical portions of the gland. The blind endpieces (or acini) of the ductal tree secrete a primary fluid that is essentially isotonic and plasma-like in composition. The ductal tree is the site of an absorptive process in which sodium and chloride are reabsorbed without concomitant water movement thus rendering the saliva hypotonic. Also in the ducts, small amounts of potassium and bicarbonate are secreted into the saliva. There is thus a primary secretion formed by the acini and a secondary modification of this primary secretion accomplished by the ducts. Evidence supporting this "two-stage" hypothesis comes from micropuncture sampling of regions of the duct lumina near the acini [15, 27] and from studies of the isolated main excretory duct [3, 11].

Knowledge of the cellular mechanisms underlying these processes remains limited. In the isolated, perfused, whole gland, substitution of perfusion chloride with isethionate results in the inhibition of secretion by about 67%. The remaining secretion is apparently sustained by bicarbonate transport [6]. Chloride thus appears to have an important role in the primary secretion process. In order to improve our understanding of this role we have employed double-barrelled, chloride-selective microelectrodes to monitor acinar intracellular chloride activity.

In the isolated rabbit mandibular gland, sustained stimulation with acetylcholine causes an initially very high rate of secretion which subsequently declines over a period of half an hour to a lower flow rate that is sustained over several hours [5]. We have therefore studied glands at rest, during the initial stages of stimulation, and after prolonged stimulation when the glands were in the steady state stage of secretion.

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Methods

Microelectrodes. Microelectrodes were prepared by a method similar to that of Zeuthen et al. [28]. Cleaned, borosilicate glass capillaries were glued together and twisted and pulled in a modified Campden puller. The longer barrel of each micropipette blank was then exposed to dimethyldichlorosilane vapour at room temperature for 90 s. The blanks were baked in an oven at 110° C for at least 1 h, and then sufficient chloride-selective liquid ion-exchanger (LIX) (Corning 477913) was placed in the silanised barrel to fill it to the shoulder. A batch of such micropipettes was left overnight under vacuum to allow the LIX to completely fill the tip. Just before use, the silanised barrels were back-filled with 0.5 M KCl above the LIX and the other, voltage-sensing barrel was filled with 0.1 M potassium citrate. This latter filling solution was used in order to reduce the possibility of leakage of potassium ions [10] and eliminate problems of chloride ion leakage from the voltage-sensing barrel. Such microelectrodes had the following characteristics: linear slopes in mixed solutions of KCl and $KHCO_3$ (varying KCl at constant $KHCO_3$ of 25 mM) ranged from −49 mV to −58 mV per decade change in activity; limits of detection ranged from 8 mM to 2 mM; and selectivity coefficients (Cl^- over HCO_3^-) ranged from 0.3 to 0.08. Individual ion activities were evaluated from tables published by Bates et al. [2]. Each microelectrode was calibrated after a successful impalement, usually immediately after withdrawal in order to reflect as closely as possible the properties of the microelectrode during the impalement.

Animals and solutions. Half-lop rabbits of either sex weighing 2–3 kg, supplied by the Medical School Animal Unit at Manchester University, were killed by anaesthetic overdose (sodium pentobarbitone, May&Baker, Dagenham, England) administered through an ear vein followed im-

mediately by a bolus of air through the same vein. Both mandibular glands were then removed from the animal.

Glands and gland tissue were maintained in a solution of the following composition (in mM): NaCl 120, choline HCO_3 21, K_2SO_4 2.25, KH_2PO_4 1, $CaSO_4$ 1.5, glucose 5.6. This was gassed with a mixture of 95% O_2 and 5% CO_2; pH was thus 7.4. When chloride-free solutions were required, sodium chloride was replaced mole for mole with sodium gluconate or sodium isethionate. Initially, isethionate was used; however, it was found to interfere with the chloride selective barrel of the microelectrode and subsequently gluconate was used instead. Measurements of basolateral membrane potential (V_b) from isethionate experiments are included in the results but not intracellular chloride activity (a_iCl).

The presence of gluconate or isethionate depresses the calcium activity in solutions [8]. Control experiments were carried out using solutions in which the calcium activity of gluconate solutions was matched with that in chloride-containing control solutions. This was achieved by measuring the calcium activities of the two types of solutions using a calcium electrode and adding enough $CaSO_4$ to the gluconate solution to bring the calcium activity to that of the control solution. The osmolalities of the two types of solution did not differ by more than 5 mOsm.

Secretion was evoked by the presence of 10^{-5} M acetylcholine in the solutions. In order to avoid any possibility that an adrenergic component of stimulation was present in the experiments, propanolol and phentolamine were present in all solutions at concentrations of 2.5×10^{-6} M.

Protocol. After removal of the mandibular glands, a small portion of one gland was dissected free from the rest of the gland and pinned in the experimental chamber. Solutions flowed through the chamber by gravity at a rate of approximately 16 ml/min. Different solutions could be admitted to the chamber without disturbing the microelectrode by using an eight way tap connecting the solution reservoirs to the chamber. A heat exchanger connected to a water circulator maintained the experimental chamber at a temperature of 38° C ± 1° C.

The initial stages of secretion were studied in continuous impalements of single cells. The effects of prolonged stimulation (greater than 30 min) were studied by comparing the values obtained from several cells before stimulation with values from other cells during prolonged stimulation.

Criteria for acceptance of an impalement were: a rapid deflection in the potential recorded by the voltage-sensing barrel; a sustained membrane potential at or above the initial potential recorded at the peak of the deflection; variation of the sustained potential by not more than ± 1 mV for a period of at least 1 min prior to the experimental manipulation; a rapid deflection on leaving the cell; and a return to within 4 mV of the original base-line potential.

Data are given as means ± SEM.

Results

Initial effects of stimulation

Stable impalements of rabbit mandibular acinar cells proved possible and could be maintained for up to 15 min. Figure 1 shows one impalement which was made while the gland was at rest and sustained during 5 min exposure to, and recovery from, acetylcholine (10^{-5} M). Table 1 summarises the data from four such impalements. The basolateral membrane potential (V_b) rapidly depolarizes and then repolarizes as has been described by others [19, 25]. However, contrary to these previous reports, in none of our impalements did cells hyperpolarize beyond the control value. This could be due to the different protocols employed. In these previous experiments, cells were exposed only briefly to acetylcholine (i.e. for seconds), either directly or by nerve stimulation. In such experiments it is difficult to distinguish events caused by application of the stimulus from those caused by its removal. Hyperpolarisation may be caused by the latter. Thus, in some of our impalements (e.g. Fig. 1), when the acetylcholine was removed, an hyperpolarization of V_b was observed. On those occasions when we have employed very brief stimulation we have always observed a depolarization followed by a repolarization and then a subsequent hyperpolarization.

The value of V_b in resting cells was -62.8 ± 4.2 mV (Table 1). This is very similar to the value found by Nishiyama and Petersen using single-barrelled microelectrodes filled with 3 M KCl [19]. Thus, our double-barrelled microelectrodes, filled with 0.1 M potassium citrate in the voltage barrel, behave similarly to conventional electrodes. In resting cells, a_iCl was 43.6 ± 3.7 mM. The ratio of the observed value of a_iCl to the predicted equilibrium value [$a_iCl(obs)/a_iCl(eq)$] is therefore 5.19 ± 0.55, indicating that chloride is actively accumulated by the resting gland. When acetylcholine was admitted to the bath, both a_iCl and $a_iCl(obs)/a_iCl(eq)$ fell. In other words, when the gland is stimulated the driving force for chloride exit from the cells declines (Table 1). These changes are reversed when stimulation is halted.

Long-term stimulation

In addition to short-term, continuous stimulation we have also studied the effects of long-term, continuous stimulation. In these experiments gland pieces were exposed to acetylcholine for more than 30 min. Values obtained under control conditions for V_b, a_iCl and $a_iCl(obs)/a_iCl(eq)$ were compared with values obtained from separate impalements obtained during stimulation. The results are summarised in Table 2. When compared with unstimulated cells, long-term stimulated cells have significantly lower values for V_b and a_iCl. The ratio $a_iCl(obs)/a_iCl(eq)$ consequently shows a similar significant difference between unstimulated and stimulated cells (4.95 ± 0.33 and 2.69 ± 0.21 respectively). In the long-term then, cells are depolarized and the gradient driving chloride exit is markedly reduced although still considerable. These changes are similar to those observed during 5 min exposure to acetylcholine.

Effects of Cl^- substitution

The use of gluconate or isethionate as replacement anions for chloride results in a depression of solution calcium activity [8]. When the calcium activities of the chloride-substituted solutions were measured using a calcium electrode, isethionate solution was found to reduce calcium activity to 67% of the control solution, and gluconate solution to 13% of control. Extracellular calcium activity is known to be important in the regulation of secretion in the rabbit man-

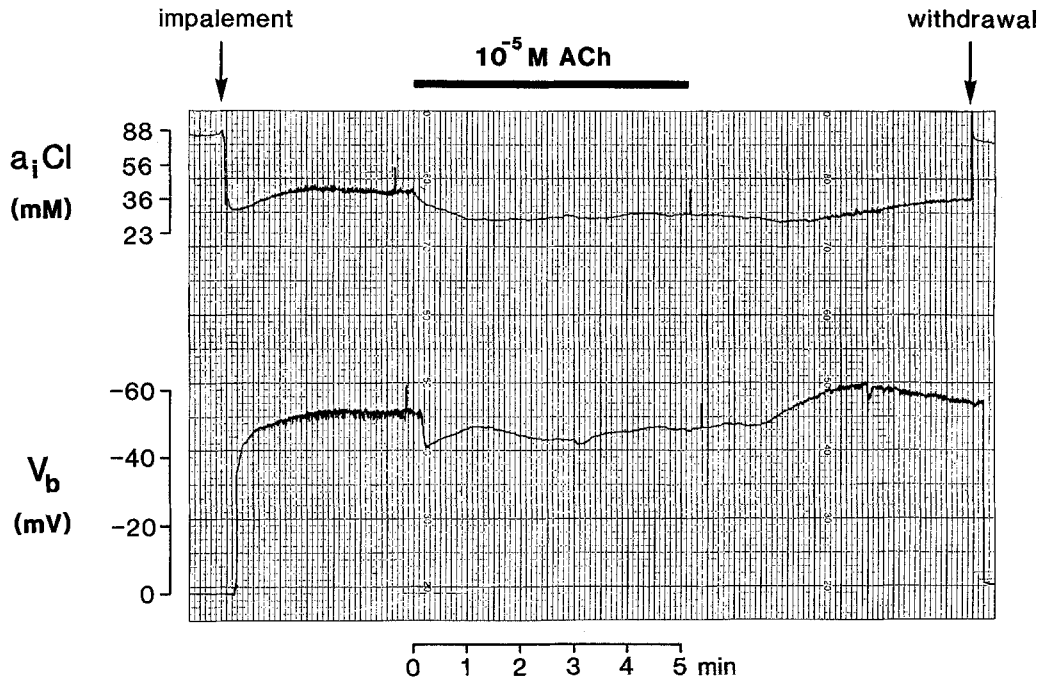

Fig. 1. An impalement of an initially unstimulated mandibular gland acinar cell. After stabilization of the basolateral membrane potential (V_b), the cell was stimulated with $10^{-5}M$ acetylcholine (*ACh*). a_iCl is the intracellular chloride activity

Table 1. Summary of four impalements similar to Fig. 1

		Control	Ach stimulation			Control		
	Time (min)	0	a*	1	4	5	6	8
a_iCl (mM)	mean	43.6	42.5	32.4	32.2	30.5	28.9	37.6
	SEM	3.67	1.85	2.63	3.34	3.37	1.01	4.63
V_b (mV)	mean	−62.8	−46.3	−54.0	−44.3	−53.3	−65.3	−66.8
	SEM	4.19	2.93	5.55	7.86	6.43	7.49	4.46
$\frac{a_i\text{Cl(obs)}}{a_i\text{Cl(eq)}}$	mean	5.19	2.91	2.81	1.95	2.57	4.14	5.21
	SEM	0.55	0.22	0.43	0.31	0.38	1.09	0.79

Abbreviations: a_iCl, intracellular chloride activity; V_b, basolateral membrane potential; a_iCl(obs)/a_iCl(eq), the ratio of the observed intracellular chloride activity to the equilibrium intracellular chloride activity predicted from V_b. n for all values is 4

* This time point is the point of maximum depolarization of V_b. It occurred at slightly different times in different cells, but always within the first few seconds after stimulation with acetylcholine (10^{-5} M)

dibular gland and exposure to an apparent calcium activity of 0.2 mM (gluconate solution) would be expected to inhibit markedly secretion in the first few minutes of exposure [14]. It was thus necessary to examine whether the reduced calcium activity had any effect upon V_b or a_iCl. When data gathered from experiments in which the calcium activity of the gluconate solution was controlled were compared to those from experiments in which calcium activity was not controlled no difference in behaviour of V_b or a_iCl was observed. We thus conclude that depression of calcium activity per se did not have any effect on V_b or a_iCl and data from both types of experiments have therefore been combined.

In unstimulated cells, replacement of chloride resulted in little or no change in V_b during the first 30 s. When the change in bath potential caused by the chloride-free solution was taken into account (+ 8 mV) this meant that a transient depolarisation of V_b had taken place (Fig. 2a). This depolarisation reached a peak at 30 s and amounted to 5.7 ± 2.24 mV ($n = 7$, $p < 0.05$). This was followed by an hyperpolarisation which, after 2 min, was 4.2 ± 1.99 mV ($n = 5$, $p > 0.05$) above the control value. Throughout this period a_iCl steadily declined.

In cells that had been continuously stimulated for more than 30 min, replacement of extracellular chloride had different effects on V_b, although a_iCl again showed a steady

Table 2. The effects of prolonged (> 30 min) stimulation with acetylcholine (10^{-5} M) in mandibular gland acinar cells

	a_iCl (mM)	V_b (mV)	$\frac{a_iCl(obs)}{a_iCl(eq)}$
	mean ± SEM (n)		
Unstimulated	41.7±2.1 (14)	−59.2±1.8 (20)	4.95±0.33 (14)
Stimulated	33.6±2.1 (10)	−53.0±1.8 (11)	2.69±0.21 (10)

Abbreviations as in Table 1. The stimulated and unstimulated values of all three parameters were significantly different when assessed by the unpaired t-test ($p < 0.05$)

decline (Fig. 2b). When adjusted for the bath potential change, depolarisation was not observed; instead a steady hyperpolarisation occurred which, after 2 min amounted to 15.1 ± 2.47 mV ($n = 8$, $p < 0.001$).

Discussion

In models of secretion in exocrine glands, chloride transport across the epithelial cells of the glands is the major driving force for fluid secretion [16, 20, 21]. These models can include paired Na^+/H^+ and Cl^-/HCO_3^- exchangers as well as Na^+, K^+, 2 Cl^--cotransport [16, 20], or only the cotransporter [21]. In both types of model, the initial events of secretion are viewed as an elevation of intracellular messenger (e.g. calcium or cyclic AMP) causing an increase in the basolateral potassium conductance (see e.g. [21]).

Both models require that intracellular chloride activity be maintained above its predicted equilibrium activity (calculated across the apical membrane) during stimulation since chloride exit is thought to occur passively through chloride-selective channels in the apical membrane. It is thought that the acinar epithelium is electrically leaky [21] so that V_b is approximately equal to the apical membrane potential. Assuming this to be the case, this prediction is fulfilled by our data, which show that a_iCl is elevated two to three times above the expected equilibrium activity in the stimulated acinar cell (Table 2). There is thus a gradient favouring chloride exit. Our data also show, however, that a_iCl is accumulated to an even greater extent in the resting cell (about five times above the predicted equilibrium level) so that the driving force for chloride exit is greater in the resting acinar cell than in the stimulated cell. A major role for chloride in secretion presupposes parallel increases in chloride transport with increases in secretory rate. This can only be reconciled with our observations if, at the onset of secretion, the resistance to flow of chloride across the cell is decreased so that a reduced gradient can drive an increased rate of chloride exit. Thus our data are consistent with the hypothesis that stimulation of acinar cells results in an increase in the chloride conductance of the apical membrane. This is also consistent with the observation of calcium-sensitive chloride channels in mouse and rat lacrimal gland acinar cells which are similar to salivary acinar cells [9, 17]. Using the calcium sensitive fluorophore quin-2, Ansah et al. [1] have shown an increase in calcium activity of rabbit mandibular gland cells upon exposure to acetylcholine. Thus, the elements required by the models for the onset of secretion all seem to be present.

The changes in V_b are also consistent with the models. The initial depolarization can be readily explained by the

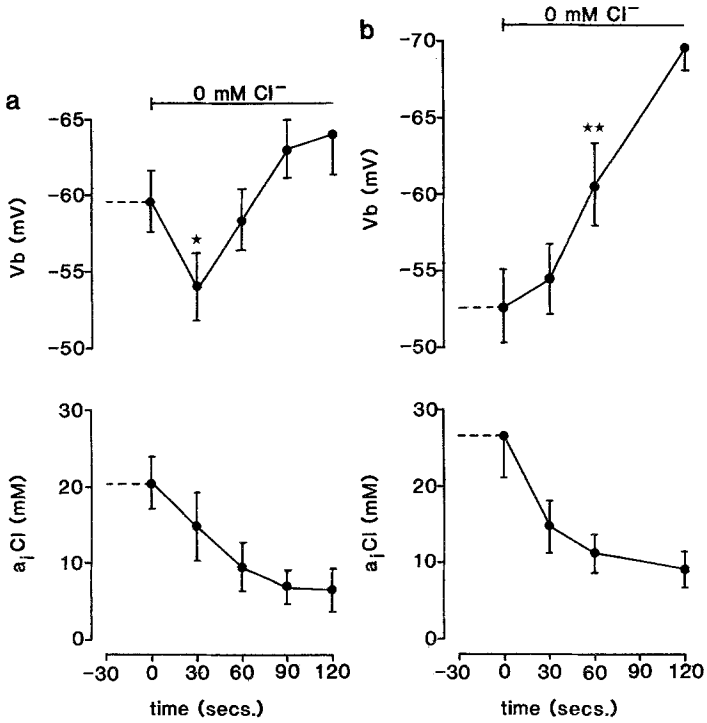

Fig. 2. a The effects of chloride free solution on basolateral membrane potential (V_b), and on intracellular chloride activity (a_iCl) in unstimulated mandibular gland acinar cells. *Data points* have been adjusted for an 8 mV liquid junction potential change due to the chloride free solution. Data from calcium controlled and calcium uncontrolled experiments have been combined (see Results). n is 7 for V_b except the last point where n is 5; n is 4 for a_iCl except the last point where n is 3. * = $p < 0.05$ (paired t-test). **b** The same as *a* except that the cells are continuously stimulated with acetylcholine. n is 14 for V_b except the last point where n is 8; n is 9 for a_iCl except the last point where n is 6. ** = $p < 0.001$ (paired t-test)

opening of chloride channels in the cell and the repolarisation by a subsequent increase in potassium channel conductance [21].

Chloride channels have been demonstrated in the apical membranes of trachea [26], rectal glands [12], and cornea [22] (although these channels all appear to be sensitive to cyclic AMP rather than calcium). No certain evidence for the location of these channels in mandibular glands exists because of the inaccessibility of the apical membrane. However, close examination of our chloride substitution data does suggest, albeit indirectly, that the major location of the chloride conductance change in mandibular glands is the apical membrane. When the gradient for chloride exit across the basolateral membrane is increased, as in chloride substitution experiments, the response of the basolateral membrane potential will depend on the relative conductance for chloride. If a significant chloride conductance is present then a transient depolarisation would be observed followed by an hyperpolarisation as intracellular chloride left the cell. By adopting the Goldman-Hodgkin-Katz formalism (Eq. (1) [13]) we can estimate the expected changes in V_b for various values of α, the permeability ratio of chloride and potassium (P_{Cl}/P_K).

$$V_b = RT/F \ln[(C_K^o + \alpha C_{Cl}^i)/(C_K^i + \alpha C_{Cl}^o)]. \quad (1)$$

Where R, T and F have their usual meanings; o and i extracellular and intracellular concentrations respectively; C_K

potassium concentration; C_{Cl} chloride concentration. For calculation, C_K^o was taken as 3 mM, C_K^i 100 mM, C_{Cl}^o 88 mM. Other values were taken from Tables 1 and 2.

Assuming that the basolateral membrane is the site of the conductance changes affecting V_b, inserting the non-stimulated values for V_b and a_iCl from Table 1 into Eq. (1) gives a value for basolateral membrane α of approximately 0.2. Stimulation with acetylcholine depolarises V_b by an average of 17 mV which could be brought about by increasing α to 0.5. The subsequent repolarisation of V_b would thus be explained adequately by the fall in a_iCl to 34 mM with α remaining at about 0.5. This would be the steady state for as long as stimulation is maintained (Table 2). For a basolateral membrane α of 0.5 we would expect to observe a transient depolarisation of about 10 mV when extracellular chloride is removed. This is clearly not the case and so we conclude that a change in the chloride conductance of the basolateral membrane does not account for the observed changes in V_b.

A small depolarisation in V_b could be occurring which we are failing to detect. The standard error of the observed change in V_b at 30 s is 1.3 mV which suggests that a depolarisation of the order of 3 mV would not be detected. If this were the case, then a 3 mV depolarisation would imply an α of approximately 0.15. However, this would mean that the actual value of the electromotive force (EMF) across the basolateral membrane was -69 mV, very different from the observed V_b. This would imply that the apical membrane EMF was depolarized compared with the basolateral membrane which would shunt the basolateral EMF via the leaky paracellular pathway [21]. A relatively depolarised apical membrane is of course readily explained by the presence of a significant chloride conductance, i.e. a large α. Thus, even if a small depolarisation of V_b was escaping detection, this would still lead to the conclusion that the major location of cellular chloride conductance was the apical membrane.

Interestingly, after adjusting for the expected liquid junction potential change, cells in the resting state do show a transient depolarisation of V_b when chloride is replaced. This is consistent with an α of $0.2-0.25$. Since this depolarisation is severely attenuated, if not actually lost, in the stimulated state, basolateral α must have decreased with stimulation. An obvious mechanism for this process is an increase in a basolateral potassium conductance relative to a chloride conductance. An increase in a potassium conductance upon stimulation has been inferred from the loss of potassium which accompanies secretion [21] and established by both tracer [4] and patch-clamp studies [21]. Again, this is consistent with the notion that the initial changes in V_b and a_iCl with stimulation are to be explained by apical membrane processes and not basolateral membrane processes. This result also encourages us to believe that we would have detected depolarisations smaller than those predicted by our GHK analysis and thus increases our confidence in our conclusion.

Finally, if the permeability to chloride of the paracellular pathway is significant, then when chloride is replaced in the basolateral bathing solution (the situation in our experiments) large changes in transepithelial potential would result and would be reflected in V_b. This is not likely to be the case since, when secretion from the perfused mandibular gland is inhibited by furosemide or bumetanide in normal chloride conditions, the concentration of chloride in the secreted fluid is very low [6, 7], indicating a low permeability of the paracellular pathway to chloride. Consequently, the observed changes in V_b can be taken to be due largely to cellular processes.

In conclusion, the work that we have presented here provides evidence that the initial steps in secretion include the opening of chloride channels in the acinar cells. Our chloride substitution experiments suggest, albeit indirectly, that these channels are not located in the basolateral membrane. In addition, in the resting state, our data suggest that a significant chloride conductance (relative to potassium) exists in the basolateral membrane. A recent report containing similar studies in mouse lacrimal gland acinar cells reached similar conclusions [23].

Our observations of the gland during the first few minutes of stimulation and after prolonged stimulation show that V_b and a_iCl reach a steady state well before the tachyphylaxis of the secretory flow is completed. Nonetheless, dissipation of the accumulated 'head' of chloride within the first few minutes of stimulation would result in a surge of chloride entering the duct lumen and this surge may contribute to the initially very high secretion rates observed in this gland. However, Murakami et al. [18] have recently reported that in the rat mandibular gland, choline can evoke secretion without tachyphylaxis. This points to a step in the regulatory mechanism of secretion as the major cause of tachyphylaxis.

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