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EFFECT OF CALCIUM ANTAGONISM OR CHELATION ON RABBIT LACRIMAL GLAND SECRETION AND MEMBRANE POTENTIALS

BY STELLA Y. BOTELHO AND DARLENE A. DARTT*

From the Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, U.S.A.

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SUMMARY

1. ACh-induced secretion from the main excretory duct, as well as ACh-induced hyperpolarizations and resting membrane potentials of superficial, probably acinar, cells were recorded from the rabbit lacrimal gland perfused *in vivo* with either control or test solutions.

2. During perfusion with test solution containing a Ca antagonist, verapamil (10^{-5} to 10^{-3} M), or a Ca chelator EGTA (10^{-4} or 10^{-3} M), ACh-induced secretion was only 30% of the control value, whereas the magnitude of the ACh-induced hyperpolarization was unchanged.

3. With 10^{-5} M-verapamil, but not with 10^{-3} M-verapamil or EGTA-containing solutions, the inhibition of ACh-induced flow was completely reversed either by replacing the test with control solution or by increasing the Ca concentration of the test solution twofold.

4. In addition, EGTA (10^{-4} M) solution containing the normal extracellular Ca^{2+} concentration inhibited secretion by 30%.

5. During perfusion with verapamil or EGTA test solutions the mean resting membrane potential of superficial, probably acinar, cells was depolarized by 15%.

6. It is concluded that although extracellular Ca is required for the major portion of ACh-induced secretion, the magnitude of the ACh-induced hyperpolarization of cells, which are most likely acinar, is independent of extracellular Ca.

INTRODUCTION

Evidence that extracellular calcium is involved in producing lacrimal gland secretion in response to cholinergic agonists exists in studies of whole lacrimal glands (Dreisbach, 1964; Pholpramool & Tangkrisanavinont, 1976) as well as of lacrimal gland segments (Keryer & Rossignol, 1976) and slices (Putney, Parod & Marier, 1977). In superfused segments of *in vitro* lacrimal glands, extracellular Ca may play a role in maintaining the resting potential (Pholpramool & Korppaibool, 1977) and in establishing cholinergic-induced hyperpolarization of acinar cells (Iwatsuki & Petersen, 1978*b*). The present study was undertaken to determine the effects, not

* Present address: Department of Physiology, Tufts University School of Medicine, Boston, Mass., U.S.A.

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only of Ca chelation, but also of Ca antagonists on cholinergic-induced secretion and membrane potentials in an *in vivo* preparation, which permits correlation of secretion and simultaneously recorded cell potentials (Botelho, Hisada & Fuenmayor, 1966; Hisada & Botelho, 1968; Kikkawa, 1970). This type of preparation was selected despite the fact that there are disadvantages related to determination of the electrical properties of cell membranes (Botelho, Fuenmayor & Hisada, 1978; Kanno, 1975; Petersen, 1974).

METHODS

All experiments were done on male white New Zealand rabbits weighing $3{\cdot}43 \pm 0{\cdot}03$ kg, $n = 40$ (Data are expressed as mean ± S.E. of the mean (n = number of determinations) except as stated otherwise). The animals were anaesthetized with intravenous sodium pentobarbitone (initial dose 35 mg/kg subsequently sustained with a total dose of 50-70 mg/kg in divided doses), immobilized with intravenous tubocurarine (2–6 mg/kg in divided doses) and respired with a Harvard pump (model 607). The methods for recording peak and average acetylcholine (ACh)-induced secretion from the main excretory duct as well as ACh-induced and resting potentials of superficial cells have been described previously (Botelho *et al.* 1978). In essence, to inject ACh chloride and to perfuse the gland with control solution or test solutions containing a Ca antagonist, verapamil, or a Ca chelator, ethylene-glycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA), the lingual artery was cannulated after isolation of the arterial supply of the gland. Data from those experiments in which the gland was stained and cleared within one min after a 0·3–0·5 ml. bolus of 1·0% Evans Blue (T1824) had been injected into the isolated arterial supply of the gland are included in the Results. The excretory duct of the lacrimal gland was cannulated with a tapered glass cannula and connected to a pressure transducer to measure flow (sensitivity 0·5 μl./min per mm deflexion) and volume (sensitivity 0·02 μl. per mm deflexion) of secretion. To record ACh-induced and resting potentials from superficial cells which were most likely acinar (Hisada & Botelho, 1968), the inferior lobe of the right lacrimal gland was exposed and decapsulated. Potentials (sensitivity 0·5 mV) were recorded between an intracellularly placed glass, K-acetate-filled micro-electrode (tip diameter < 1 μm and resistance from 10–80 MΩ) and a Ag–AgCl reference electrode, which was inserted into an agar pledget that had been impregnated with the appropriate perfusion solution and placed on the surface of the gland.

Protocol. The lacrimal gland was perfused with control solution at a constant rate of 0·513 ml./min for 1–3 hr in four rabbits, and with one of the test solutions immediately followed by control solution for 1 hr in six rabbits. At 10 min intervals throughout each experiment, a 15 μg/100 μl. bolus of ACh was injected into the dead space (200 μl.) of the lingual artery cannula and then, within 4 sec, flushed into the gland with a 300 μl. bolus of the perfusate. Prior to each injection of ACh, lacrimal gland secretion was reduced to minimal by placing 0·5% proparacaine on the cornea and conjunctiva (Botelho, Martinez, Pholpramool, van Prooyen, Janssen & de Palau, 1976). In addition, to ensure that cells were not leaky, we recorded ACh- induced responses only from cells with resting potentials which were -20 mV or more negative for at least 30 sec prior to the injection, except as otherwise indicated.

Solutions. All solutions were gassed with 95% O_2 and 5% CO_2 for at least 1 hr prior to the start of and throughout each experiment. The concentrations ($\times 10^{-3}$ M) of substances in the control solutions were NaCl 125, KCl 4·0, $NaHCO_3$ 25, $CaCl_2$ 2·2, $MgCl_2$ 1·0, glucose 5·5 and the pH and osmolarity were 7·31 and 323 m-osmole/l. respectively. Four verapamil with normal Ca ($2{\cdot}2 \times 10^{-3}$ M-$CaCl_2$) test solutions contained substances in the same concentrations as the control solution, and one of the following concentrations of verapamil HCl: 10^{-8} M, 10^{-5} M, 10^{-4} M or 10^{-3} M. The verapamil with high Ca test solution contained 10^{-5} or 10^{-3} M-verapamil HCl and substances in the same concentration as in the control solution, except $CaCl_2$, which was increased to $4{\cdot}4 \times 10^{-3}$ M. The two essentially Ca^{2+}-free test solutions contained the same concentrations of substances as the control solution, except for Ca^{2+} and either 10^{-4} M-EGTA ($= 10^{-8}$ M-Ca^{2+} calc.) or 10^{-3} M-EGTA ($= 10^{-9}$ M-Ca^{2+} calc.). The EGTA with normal Ca test solution contained 10^{-4} M-EGTA and the same concentrations of substances as in the control solution, including $2{\cdot}2 \times 10^{-3}$ M-Ca^{2+}. The osmolarity and pH of the verapamil and EGTA test solutions were similar to those of the control solution.

RESULTS

ACh-induced secretion

The ratio of ACh-induced peak/average flow during perfusion with all of the solutions was $4{\cdot}0 \pm 0{\cdot}05$ ($n = 327$). ACh-induced peak flow was $11{\cdot}92 \pm 1{\cdot}12$ μl./min ($n = 56$) during perfusion with control solution and was reduced to 84% and 30% of control during perfusion with 10^{-8} and 10^{-5} M-verapamil with normal Ca^{2+} solution respectively. Replacing the 10^{-5} M-verapamil with control solution or doubling the Ca^{2+} concentration in the 10^{-5} M-verapamil solution restored the ACh-induced secretion to control value. Despite the fact that the same degree of inhibition occurred during perfusion with 10^{-3} M- as with 10^{-5} M-verapamil, neither replacing the 10^{-3} M-verapamil with control solution nor doubling [Ca^{2+}] in the 10^{-3} M-verapamil solution completely reversed the inhibition. ACh-induced secretory rate was reduced to 31–34% of control when Ca^{2+} was decreased to 10^{-8} M or 10^{-9} M with EGTA, and unlike 10^{-8} M and 10^{-5} M-verapamil this inhibition of secretion was not reversed when these solutions were replaced with control solution. Even when the EGTA solution contained the normal concentration of Ca^{2+}, the ACh-induced peak flow was only 69% of control, but this inhibition was completely reversed when the EGTA solution was replaced with control solution.

Resting membrane potential

In the unstimulated gland, during perfusion with control solution, the membrane potential was $-30{\cdot}4 \pm 0{\cdot}8$ mV ($n = 66$), a value not significantly different from that previously reported in the artificially perfused or blood-perfused *in vivo* rabbit lacrimal gland (Botelho *et al.* 1978); it was 3·7 mV more negative than that reported in superfused *in vitro* rabbit lacrimal gland (Pholpramool & Korppaibool, 1977), and 12·1 mV less negative than that reported in superfused *in vitro* rat exorbital lacrimal gland (Iwatsuki & Petersen, 1978*a*). The discrepancies, however, could be due to species and technique differences. During perfusion with verapamil or with EGTA the resting potential was only 15% less negative than control (Table 1). Although this relatively slight depolarization could be accounted for by a slight increase in the number of depolarized cells (Table 1) the possibility that there was slight depolarization of all cells cannot be entirely eliminated. Finally, during perfusion with each of the solutions there was no correlation between the magnitude of the resting potential and the magnitude of ACh-induced hyperpolarization (Fig. 1).

ACh-induced potential changes

As has been reported (Botelho *et al.* 1978), when the gland was perfused with control solution ACh induced a change in potential, which was a hyperpolarization in 76·3%, of the impaled cells, a depolarization in 15·8% and a bi- or polyphasic potential in 5·3% of them; the potential in the remaining 2·6% of the cells did not change when ACh was administered. The distribution of these various responses was the same during perfusion with verapamil or with EGTA as with control solution. Since the predominant electrical response to ACh was hyperpolarization of the cell, ACh-induced hyperpolarization was analysed and found to be the same magnitude during perfusion with verapamil or with EGTA as with control solution (Table 2).

TABLE 1. Resting membrane potential

Solution (M)	Magnitude (mV)	Cells less negative than −16 mV (%)
Control	−30·4 ± 0·8 (66)	19 (244)
Verapamil		
10^{-8}	−29·3 ± 1·5 (18)	
10^{-5}	−27·2 ± 0·7 (60)*	32 (221)
10^{-4}	−27·4 ± 0·7 (62)*	38 (370)
10^{-3}	−25·4 ± 0·7 (30)*	36 (108)
EGTA		
10^{-4}	−25·5 ± 0·5 (61)*	38 (297)
10^{-3}	−27·5 ± 0·8 (42)*	34 (170)

Mean ± S.E. (number)

* Significantly different from control, $P < 0{\cdot}05$.

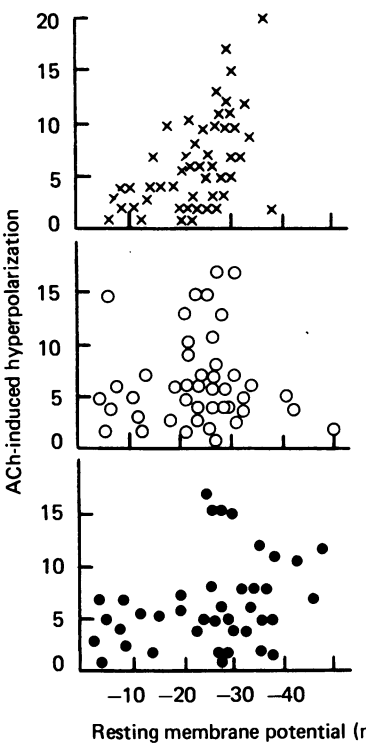

Fig. 1. ACh-induced hyperpolarization as a function of resting membrane potential when the gland was perfused with control (bottom), 10^{-4} M-verapamil (middle) or 10^{-4} M-EGTA (top) solutions. Each point represents a single impaled cell. Correlation coefficients (r) were 0·763, 0·577, and 0·786 during perfusion with control, 10^{-4} M-verapamil and 10^{-4} M-EGTA solutions respectively. Data in this Figure are from cells stimulated with ACh regardless of the resting potential prior to ACh injection. Therefore, the number of cells here is greater for any given solution than the number in Table 2.

TABLE 2. ACH-induced hyperpolarization*

Solution (M)	Magnitude (mV)
Control	8·3 ± 1·3 (29)
Verapamil	
10^{-8}	10·9 ± 1·7 (10)
10^{-5}	7·2 ± 1·4 (33)
10^{-4}	8·4 ± 1·2 (33)
10^{-3}	6·3 ± 0·7 (17)
EGTA	
10^{-4}	7·2 ± 0·8 (35)
10^{-3}	6·6 ± 0·8 (21)

Mean ± S.E. (number)

* To ensure that cells were not leaky, data in this table were obtained only from cells with resting potentials which were −20 mV and more negative for at least 30 sec prior to ACh injection (see Methods).

DISCUSSION

The inhibition of secretion, which occurred when the lacrimal gland was perfused with verapamil in concentrations of 10^{-5} M and less, probably resulted from decreased Ca influx since in such low concentrations verapamil or a verapamil derivative (D600) decrease Ca uptake in a number of secretory systems (Dreifuss, Grau & Nordmann, 1973; Eto, Wood, Hutchins & Fleischer, 1974; Kondo & Schulz, 1976; Malaisse, Herchuelz, Levy & Sener, 1977). Notable is the fact that the degree of inhibition of lacrimal secretion was no greater with 10^{-3} M than with 10^{-5} M verapamil, suggesting that 30% of ACh-induced secretion is independent of external Ca^{2+} ions, perhaps because an intracellular source is utilized or a portion of secretion does not require Ca^{2+} ions.

The finding that, in the lacrimal gland, verapamil inhibited ACh-induced secretion but did not change the ACh-induced hyperpolarization is consistent with a previous finding in another exocrine gland, the submandibular (Petersen, Poulsen & Thorn, 1967).

There are a number of possible explanations for the dissociation of ACh-induced secretion from ACh-induced hyperpolarization in the present study. First, if Ca influx is important in stimulus–secretion coupling, our data indicate that the major portion of ACh-induced secretion is dependent upon Ca influx but that the ACh-induced hyperpolarization which, in the rat exorbital lacrimal gland is primarily an increase in K conductance (Iwatsuki & Petersen, 1978*a*), is independent of Ca influx. If the initial transient K release (Putney & Parod, 1978) partially reflects K released during the increased K conductance of the ACh-induced hyperpolarization, then the fact that the ACh-induced hyperpolarization in the present study was independent of Ca influx agrees with the finding that the initial K release is independent of extracellular Ca^{2+} (Parod & Putney, 1978). Furthermore, both of these facts are not in disagreement with the conclusion of Iwatsuki & Petersen (1978*b*) that an increase in intracellular Ca^{2+} may mediate the ACh-induced hyperpolarization in the rat exorbital lacrimal gland, because ACh could release Ca^{2+} intracellularly in addition

to inducing an extracellular influx. Secondly, the hyperpolarization could have been recorded from a cell type, which was not responsible for the major portion of ACh-induced flow since lacrimal acinar and duct cells appear to have somewhat different secretory functions (Alexander, van Lennep & Young, 1972; Botelho & Martinez, 1973) and the acinar cell is the most likely cell impaled when a micro-electrode is inserted into the surface of the lacrimal gland (Hisada & Botelho, 1968). Two other possibilities are that there were more non-responsive cells, and structural damage occurred during perfusion with verapamil. We believe that these possibilities are unlikely because during perfusion with verapamil solutions at concentrations of 10^{-4} M or less there was no correlation between the magnitudes of the resting and ACh-induced potentials; the mean resting potential was only 9% less negative; there was only about a 13% increase in the number of cells with resting potentials less negative than -16 mV, and the verapamil-induced inhibition of flow was completely reversible. On the other hand, permanent changes did occur when the gland was perfused with 10^{-3} M-verapamil, since the inhibition of lacrimal gland flow was only about 30% reversed.

The same results were seen when the gland was perfused with the Ca chelator, EGTA, as when perfused with verapamil except that in the presence of a normal Ca concentration, EGTA produced 30% inhibition of lacrimal gland flow. Since this inhibition was completely reversed when the normal calcium EGTA solution was replaced with control solution, it is possible that the irreversibility seen with essentially Ca-free EGTA solution indicates that permanent damage occurs to cellular structures, from which Ca has been leached.

In summary, we have concluded that although influx of Ca^{2+} ions is required to produce the major portion of cholinergically-induced lacrimal gland flow, about 30% of the flow is independent of external Ca. The cholinergically-induced hyperpolarization of lacrimal gland cells is independent of external Ca and the ratio of ionic permeabilities which control the magnitude of the resting membrane potential is to a small extent dependent upon external Ca. Inhibiting Ca influx with verapamil is a better method than lowering the concentration of external Ca^{2+} ions with EGTA, to determine the effects of Ca influx on lacrimal gland secretion.

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