Effect of trifluoperazine on ^{3}H-labeled protein secretion induced by pentoxifylline, cholinergic or adrenergic agonists in rat lacrimal gland. A possible role of calmodulin?

Philippe Mauduit, Geneviève Herman and Bernard Rossignol

Laboratoire de Biochimie des Transports Cellulaires, Institut de Biochimie, Bât 432, Université de Paris-Sud, 91405 Orsay Cedex, France

Received 20 December 1982

In rat lacrimal gland, cholinergic, α- or β-adrenergic or methylxanthine stimulations of protein secretion are extracellular calcium dependent. 10 μM trifluoperazine (TFP) inhibited only cholinergic and α-adrenergic stimulations. Half maximal effect was observed at 30 μM, with all inducers except norepinephrine (3 μM). 10 or 30 μM TFP also suppressed the decrease of L-[^{3}H]leucine incorporation into protein due to carbamylcholine. 100 μM TFP inhibited protein secretion and L-[^{3}H]leucine incorporation. 500 μM TFP promoted cell lysis. It is suggested that: (a) at 100 μM TFP, inhibition is not specific for protein secretion; (b) at 30 μM TFP, inhibition could be related to a role of calmodulin in the secretory regulation process.

Calmodulin *Calcium* *Lacrimal gland* *Protein secretion* *Trifluoperazine*

1. INTRODUCTION

In rat lacrimal glands, protein secretion is chiefly controlled by muscarinic or α-adrenergic receptors. It has recently been shown that it could be also induced via β-receptors and by methylxanthines (specially pentoxifylline and isobutylmethylxanthine) [1–3]. β-Receptor activation and pentoxifylline entail an intracellular cAMP increase (submitted). Moreover, all these secretory stimulations are calcium dependent [2,3]. According to several studies, a great number of calcium dependent processes, including secretory process, seem to involve the ubiquitous calcium dependent regulatory protein (calmodulin) [4–6]. A careful study of the endocrine pancreas has been performed to investigate more extensively the actions of various phenothiazines and mainly trifluoperazine (TFP) [7], known for its high affinity for the calmodulin–calcium complex and as a potent inhibitor of calmodulin dependent processes [8]. The data on the effect of TFP on exocrine protein secretion stimulation, controlled by neurotransmitters is inconsistent. Recently, in the rat lacrimal glands, it has been shown that a protein kinase could be activated by calmodulin–calcium complex [9]. However, a relationship between this protein kinase and protein secretion has not been settled. Therefore, it seemed advisable to define a possible role of calmodulin in the secretory process from lacrimal gland fragments by exploring the action of TFP on protein release, induced either by secretagogues or by a methylxanthine. This last inducer bypasses the receptor level, but may be implicated in the regulation of cAMP and/or cellular calcium mobilization (submitted) [10].

2. MATERIALS AND METHODS

2.1. *Chemicals*

L-[^{3}H]Leucine was purchased from the C.E.A. Saclay, France. Carbamylcholine (Carbachol, Cch) was obtained from Mann Research Laboratories, NY. L-Isoproterenol bitartrate (IP) and papaverine (Pv) were from Sigma, St Louis, MO. Pentoxifylline (BL) and trifluoperazine dichlorhydrate

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

(TFP) were a kind gift respectively from Hoechst France and from Rhône-Poulenc, France. Arterenol bitartrate hydrochloride (norepinephrine, NE) was from Hoechst-Behring-Calbiochem, France.

2.2. *Biological material*

Fragments of lacrimal glands from male albino Sprague–Dawley rats (6–8 weeks old) were prepared as previously described [11].

2.3. *Incubation procedures*

Secretion experiments – Incubation procedure, pulse labeling (10 min instead of 3.5 min) and protein discharge were performed as previously described [11,12]. The protein pellet was dissolved in 0.5 N NaOH and the radioactivity was determined using aqualuma as scintillation cocktail on Packard Tri-Carb Scintillation Counter. Secretion was expressed as the amount of labeled proteins present in the incubation medium as a percentage of total labeled proteins in tissue and medium.

Labeled protein biosynthesis – After a 30 min equilibration in Krebs–Ringer bicarbonate (KRB) buffer, 70–90 mg of gland fragments were further incubated in 5 ml of the same buffer. TFP, when present, was added at the onset of the second incubation period (50 min); agonists and L-[^{3}H]leucine (2 μCi/ml) were added for the last 40 min. Then the fragments were washed once with 7 ml of KRB buffer supplemented with 1 mM L-[^{1}H]leucine, slightly dried, weighed and homogenized in 4 ml ice-cold 20% trichloroacetic acid, 0.1% phosphotungstic acid. The homogenates were centrifuged at 20000 × g for 15 min at 0°C. The pellets were resuspended in 4 ml of the same mixture and centrifuged once more in the same conditions. The supernatant was discarded and the pellet was dissolved in 1 ml N NaOH; an aliquot was used to determine the radioactivity incorporated into proteins on a Packard Tri-Carb Scintillation Counter. L-[^{3}H]Leucine incorporation was corrected from the specific radioactivity of the incubation medium and tissue weight.

2.4. *Lactate dehydrogenase discharge*

As an indicator of cell lysis, lactate dehydrogenase (LDH) discharge was determined according to Boehring's test. LDH released was assayed by the rate of decrease in absorbance at 340 nm after the addition of an aliquot of the incubation medium to assay-cocktail (final concentration 0.6 mM pyruvate, 0.18 mM NADH in 50 mM phosphate buffer, pH 7.5, at 37°C).

3. RESULTS

As shown in table 1, carbamylcholine, norepinephrine, isoproterenol via muscarinic, α-adrenergic and β-adrenergic receptors, respectively, promote an important labeled protein secretion. Moreover, pentoxifylline, a potent phosphodiesterase inhibitor, induces a large protein secretion without the intervention of a receptor step. In every case, extracellular calcium omission reduces the secretory response. In the absence of extracellular calcium, 50 μM EGTA nearly completely abolishes the protein secretion induced by carbamylcholine and norepinephrine, even though it has no effect on the unstimulated one. On the other hand, under the same conditions as above, EGTA has no added effect on the labeled protein discharge induced by pentoxifylline or isoproterenol. Cholinergic and noradrenergic (via α-adrenergic receptor) dependence on free extracellular calcium can be correlated to the increase in calcium uptake induced through the activation of their respective receptors [13,14]. Figure 1 shows the effect of increasing concentrations of trifluoperazine (TFP) on unstimulated and variously stimulated protein secretion. Unstimulated protein secretion was unaffected by TFP up to a 100 μM concentration, in contrast to the substantial increase observed for a 500 μM concentration. 100 μM TFP inhibited the stimulation of protein secretion by 95, 80, 80 and 70%, when it was induced by norepinephrine, carbamylcholine, isoproterenol and pentoxifylline, respectively. Noradrenergic stimulation was already inhibited by a TFP concentration of less than 1 μM. A 1 μM concentration of TFP slightly increased cholinergic stimulation and significant inhibition was first observed for a 10 μM concentration. The latter concentration had no significant effect on isoproterenol and pentoxifylline stimulation. However, the half maximal effect was observed at identical concentrations, about 30 μM of TFP. The inhibition of induced protein discharge was suppressed for TFP concentrations above 100 μM. A similar result was obtained for glucose induced insulin secretion in β-cells of rat pancreas [7].

Table 1

Effect of extracellular calcium omission on net protein secretion induced by various agonists

	KRB buffer	KRB buffer $-Ca^{2+}$	KRB buffer $-Ca^{2+}$ + EGTA
Cch 5 μM	22.6 ± 3.4%	8.0 ± 1.9%	3.5 ± 1.9%
NE 50 μM	18.3 ± 2.3%	4.1 ± 0.1%	2.0 ± 0.7%
IP 5 μM + Pv 10 μM	12.9 ± 1.6%	7.6 ± 0.8%	7.3 ± 2 %
BL 10 mM	16.2 ± 2.7%	7.3 ± 1.6%	5.5 ± 3.5%

^{3}H-Labeled protein secretion was performed in the appropriate KRB buffer (+/− Ca^{2+}, +/− 0.5 mM EGTA). Unstimulated secretions similar for each case and ranging from 3.2% to 4.3% were subtracted from the data obtained with each agonist. Each value is the mean ± SE of 3 to 5 separate experiments

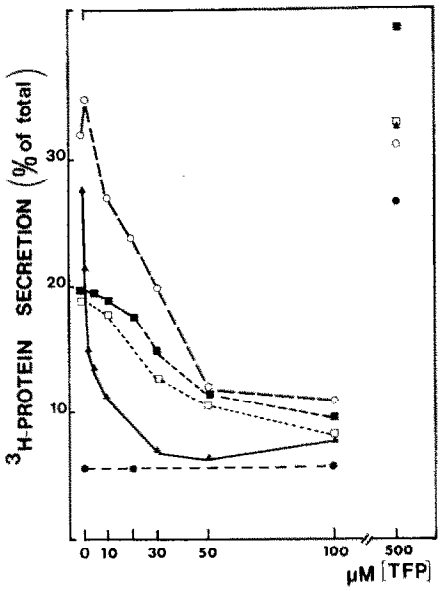

Fig.1. Effect of TFP on labeled protein secretion in rat lacrimal glands. ^{3}H-labeled protein discharge was performed in the presence of various concentrations of TFP for a time period of 50 min. 5 μM carbamylcholine (○), 50 μM norepinephrine (▲) and 10 mM pentoxifylline (■) were added 10 min after TFP. 5 μM isoproterenol (□) was added 10 min after TFP + 10 μM Pv. Unstimulated secretion (●). Each value is the mean SE of 3 to 8 separate experiments.

The very substantial protein output observed at 500 μM TFP could be related to cell damage. As shown in fig.2, LDH activity in the incubation medium increased both for the unstimulated and stimulated state of protein secretion. The results observed with TFP concentrations higher than 100 μM show a significant correlation between labeled protein and LDH output.

In order to define the specificity of TFP precisely, it was important to see the effect of this phenothiazine on another metabolic parameter. Figure 3 shows TFP effect on the incorporation of [^{3}H]leucine into proteins in both the absence or presence of carbamylcholine. A 100 μM concentration of TFP promoted an important inhibition of [^{3}H]leucine incorporation. On the other hand, TFP was without any significant effect, when used at 10 or 30 μM, whereas the 30 μM concentration significantly inhibited all the induced protein secretions tested. As was shown in the rat parotid gland [15], carbamylcholine inhibits the incorporation of [^{3}H]leucine into proteins in the rat lacrimal gland. This effect has been shown to be calcium dependent. It is interesting to point out that 10 and 30 μM TFP overcomes the effect of carbamylcholine, whereas a concentration of 100 μM still inhibits the incorporation.

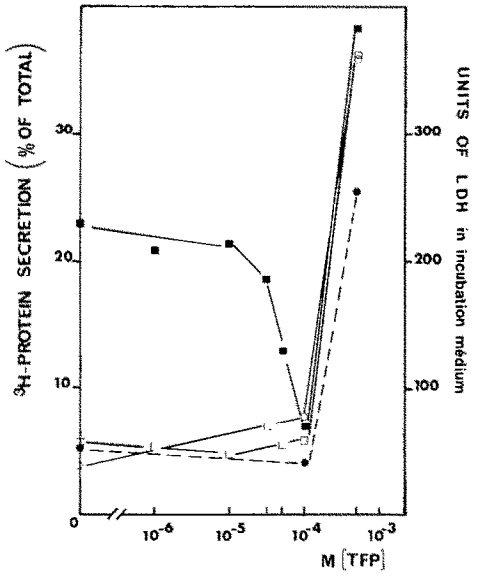

Fig.2. Effect of TFP on labeled protein and LDH discharge in rat lacrimal glands. ^{3}H-Labeled proteins (■,●) and LDH (□,○) released in the incubation medium were determined as described in section 2. Incubations were performed in the presence of varying concentrations of TFP for 50 min, in the absence (●,○) or presence of 10 mM pentoxifylline (■,□) for the last 40 min. One unit of LDH activity was defined as a $\Delta D.O_{340} = 10^{-3}$ D.O unit/g.min. This experiment is a representative one.

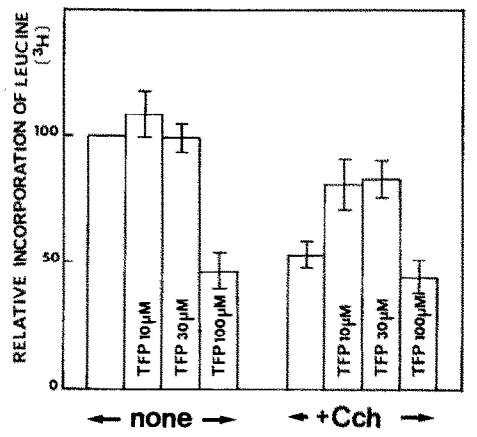

Fig.3. Effect of TFP on L-[^{3}H]leucine incorporation into proteins in the presence or absence of 5 µM carbamylcholine. L-[^{3}H]Leucine incorporation into proteins was determined as described in section 2. Incorporation in the presence of TFP and/or 5 µM carbamylcholine was expressed as a function of control incorporation (100). The result is the mean ± SE of 4 separate experiments.

4. DISCUSSION

As it was previously reported for insulin secretion in Langerhans islets [6,7], ^{3}H-labeled protein secretion from rat lacrimal glands is significantly inhibited by 30 µM TFP independently of the inducer. However, the protein secretion induced by pentoxifylline (a methylxanthine) seems to be less affected by the phenothiazine. This result can be compared to the effect of TFP on theophylline induced insulin secretion observed previously [7]. The nearly complete inhibition of norepinephrine induced protein secretion, which occurs for low concentrations of TFP, could be related to the competition exerted by the phenothiazine for the binding of the adrenergic agonist at the α-adrenergic receptor level, as reported for hepatocytes [16].

Up to 30 µM, TFP has not effect on [^{3}H]leucine incorporation into proteins, but 10 and 30 µM concentrations inhibit the decrease induced by carbamylcholine. This result can be compared to the one obtained on the cholinergic stimulation of protein secretion. It is noteworthy that a 10 µmol concentration of TFP did not affect the secretory response induced by a β-adrenergic agonist (isoproterenol) or by a methylxanthine (pentoxifylline), but inhibits the effect of carbamylcholine. Although a high concentration of TFP (100 µM) does not inhibit the unstimulated protein secretion nor induce LDH release, it inhibits [^{3}H]leucine incorporation into proteins. This result suggests that, for such a concentration, the effect of TFP on stimulated protein secretion could be nonspecific to the secretory process. Moreover, slightly higher concentrations of TFP promote an important LDH release, as an indicator of cell lysis.

In a recent paper [9], in rat lacrimal glands, the presence of calmodulin–calcium dependent protein kinase was reported, whose activity was shown to be inhibited by TFP concentrations ranging from 10 to 300 µM, with significant inhibition for 30 µM.

Taken together, the results obtained on β-cells and lacrimal glands suggest that calmodulin could play a key role in one or more step(s) of secretory process regulation. Thus calmodulin could be involved:

(1) Either in one or more calcium dependent step(s) of protein secretion stimulation, both

common to cholinergic and adrenergic agonists (such as protein kinase activation).

(2) Or in different ways: calmodulin dependent intracellular calcium movements, as reported for rat parotid gland [17] and human platelets [18]; calmodulin–calcium dependent adenylate cyclase activity [19–21].

The results obtained in the present study concerning the regulation of protein secretion, in the lacrimal glands, merit further investigation. However, as of now they provide additional information on the possible role of calmodulin in the calcium regulation of exocrine gland protein secretion.

ACKNOWLEDGEMENTS

Thanks are due to J. Dujancourt for her skilful technical assistance. This work was supported by the Centre National de la Recherche Scientifique (LA 272, ATP: Pharmacologie des recepteurs des neuromédiateurs) and Laboratories Hoechst.

REFERENCES

[1] Friedman, Z.Y., Lowe, M. and Zelinger, Z. (1981) Biochim. Biophys. Acta 675, 40–45.
[2] Herman, G., Mauduit, P. and Rossignol, B. (1981) Scand. J. Clin. Lab. Invest. 41, suppl.156, 233–236.
[3] Mauduit, P., Herman, G. and Rossignol, B. (1982) Biol. Cell 45, 281.
[4] Cheung, W.Y. (1980) Science 207, 19–27.
[5] Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Ann. Rev. Biochem. 49, 489–515.
[6] Gagliardino, J.J., Harrison, D.E., Christie, M.R., Gagliardino, E.E. and Ashcroft, S.J.H. (1980) Biochem. J. 192, 919–927.
[7] Henquin, J.C. (1981) Biochem. J. 196, 771–780.
[8] Weiss, B., Fertel, R., Figlin, R. and Uzunov, B. (1974) Mol. Pharmacol. 10, 615–625.
[9] Dartt, D.A., Guerin, V.J., Donowitz, M., Taylor, L. and Sharp, G.W.G. (1981) Biochem. J. 202, 799–802.
[10] Gylfe, E. and Hellman, B. (1981) Biochem. Med. 26, 365–376.
[11] Chambaut-Guerin, A.M., Muller, P. and Rossignol, B. (1978) J. Biol. Chem. 253, 3870–3876.
[12] Rossignol, B., Herman, G. and Clauser, H. (1969) Biochem. Biophys. Res. Commun. 34, 111–119.
[13] Keryer, G. and Rossignol, B. (1976) Am. J. Physiol. 230, 99–104.
[14] Parod, R.J., Leslie, B.A. and Putney, J.W.(1980) Am. J. Physiol. 239, 99–105.
[15] Kanagasuntheram, P. and Soh Chan Lim (1978) Biochem. J. 176, 23–29.
[16] Blackmore, P.F., El-Refai, M.F., Dehaye, J.P., Strickland, W.G., Hughes, B.P. and Exton, J.H. (1981) FEBS Lett. 123, 245–248.
[17] Kanagasuntheram, P. and Teo, T.S. (1982) FEBS Lett. 141, 233–236.
[18] White, G.C. and Raynor, I. and S. (1982) Biochem. Biophys. Res. Commun. 104, 1066–1072.
[19] Salter, R.S., Krinks, M.H., Klee, C.B. and Neer, E.J. (1981) J. Biol. Chem. 256, 9796–9798.
[20] Ausiello, D.A. and Hall, D. (1981) J. Biol. Chem. 256, 9796–9798.
[21] Thams, P., Capito, K. and Hedeskov, C. (1982) Biochem. J. 206, 97–102.