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Biochemical Pharmacology, Vol. 32, No. 2, pp. 371–373, 1983.
Printed in Great Britain.

0006-2952/83/020371-03 $03.00/0

Effect of trifluoperazine on catecholamine secretion by isolated bovine adrenal medullary chromaffin cells

(*Received* 1 *February* 1982; *accepted* 14 *July* 1982)

Adrenal medullary chromaffin cells secrete the catecholamines epinephrine and norepinephrine by a calcium-dependent exocytotic process. There is increasing evidence that calmodulin may regulate a wide variety of cellular processes including enzymes whose activities are calcium-dependent, calcium transport in erythrocytes, actomyosin ATPase in smooth muscle, and protein phosphorylation in a variety of cells (for review see Ref. 1). Of particular interest is recent evidence indicating an essential role for calmodulin in neurotransmitter release by synaptosomes [2, 3] and insulin release by pancreatic islet cells [4]. In general, the actions of calmodulin on secretory processes appear to involve phosphorylation of cellular proteins [3]. We have isolated a calcium-binding protein from adrenal medulla that is present in high concentrations and has been shown to be calmodulin [5]. It seemed possible, therefore, that calmodulin may be involved in some aspect of the secretory process by chromaffin cells. Since the phenothiazine drug trifluoperazine (TFP) has been shown to inhibit calmodulin function *in vitro* [6, 7] and to affect the behavior of other secretory cells, we have examined its effect on secretion by isolated chromaffin cells.

Materials and methods

Physiological salt solutions. The media in these experiments were variations of Locke's solution with the composition: 154 mM NaCl, 2.6 mM KCl, 2.15 mM K_2HPO_4, 0.85 mM KH_2PO_4, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, pH 7.2, 298 mOsM. For experiments involving stimulation of the cells with medium containing elevated potassium, the medium was prepared with the isosmotic substitution of KCl for NaCl to a final KCl concentration of 112 mM. Dilution of this medium with an equal volume of cell suspension in Locke's medium produced a final potassium concentration of 56 mM. The solution used for perfusion of the adrenal glands during isolation of the cells was Locke's medium (low calcium medium) containing 0.1 mM calcium, 1.0 mM magnesium and 15 mg of phenol red per liter. After preparation, 95% O_2:5% CO_2 was bubbled through the solution for about 15 min, and the pH was adjusted to 7.4 with 1 N NaOH. Media containing elevated amounts of calcium were prepared as described by Seglen [8].

Preparation of cells. Bovine adrenal glands were obtained at a local slaughterhouse within 20 min of death and taken to the laboratory in ice. Cells were prepared from glands perfused in a manner similar to that described by Fenwick *et al.* [9] and collected and purified as described by Brooks [10]. The final cell pellet was resuspended at 500,000 cells/ml of 1% bovine serum albumin (BSA)-Locke's medium, and the suspension was placed in a 125-ml Erlenmeyer flask and incubated at 37° for 2 hr. The medium was changed once after the first hour of the incubation period. The isolated cells used in these experiments contained 60–70 μg of total catecholamine/10^6 cells.

Assay of catecholamine secretion. After the 2-hr incubation, the cell suspension was washed twice with fresh Locke's solution and used for experiments. Unless otherwise stated, cells were used at a concentration of 10^6/ml. For experiments involving preincubation of the cells with TFP, the cell suspension was added to tubes containing freshly prepared TFP, quickly mixed by inversion, and allowed to incubate for 5 min at 37°. Aliquots of each suspension were transferred to 1.5-ml polyethylene microfuge tubes containing a volume of 1 mM acetylcholine solution (Locke's solution for controls) to give a final acetylcholine concentration of 0.1 mM, and incubated for 10 min in a 37° water bath. For most experiments a final volume of 0.44 ml or 1.1 ml was used.

The control suspensions were divided into two groups. One group, designated as a zero-time control, was centrifuged for 30 sec in a Beckman Microfuge B at the start of the 10-min assay period, while the other was carried through the 10-min assay period. Cell suspensions, stimulated with acetylcholine in the presence or absence of TFP, were similarly carried through the 10-min assay period. Stimulation by exposure to elevated potassium medium was initiated by adding cell suspensions, pretreated with TFP in Locke's medium, to an equal volume of isosmotic 112 mM potassium Locke's solution. When appropriate, the potassium-enriched medium also contained TFP. Aliquots of all supernatant fractions were immediately assayed for catecholamine content. Solutions of acetylcholine and TFP were prepared in Locke's medium immediately before use.

Analytical measurements. The total catecholamine secretion for samples containing 10^6 cells was determined spectrophotometrically using epinephrine bitartrate as a standard [11]. For most experiments the norepinephrine content of individual samples was determined by high performance liquid chromatography (HPLC) using the method of Hegstrand and Eichelman [12]. For these measurements data were expressed as ng catecholamine secreted/10^5 cells. The significance of differences between means was determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. In instances where a nonparametric test was required, the Kruskal–Wallis one-way ANOVA test was used followed by the Mann–Whitney U test for comparison of means for different ranks. Significance was determined at the level of $P = 0.01$ or less.

All chemicals were of reagent grade and were purchased from the Fisher Chemical Co., Itaska, IL, except for the methanol (Omni Solv) used for HPLC, which was purchased from MCB Manufacturing Chemists, Inc., Cincinnati, OH. Collagenase (Type 1), bovine serum albumin (fraction V), acetylcholine and hexamethonium were purchased from the Sigma Chemical Co., St. Louis, MO. TFP was a gift from Smith Kline & French Laboratories, Philadelphia, PA.

Results and discussion

A dose–response curve for the effect of TFP on total catecholamine secretion by acetylcholine-stimulated chromaffin cells is shown in Fig. 1. The curve is biphasic with reduction of secretion to the level of unstimulated controls at 1 μM TFP. Increasing the TFP concentration to 10 μM resulted in an apparent increase in catecholamine secretion. A further increase to 100 μM TFP resulted in cell norepinephrine secretion that was 5-fold greater than the secretion

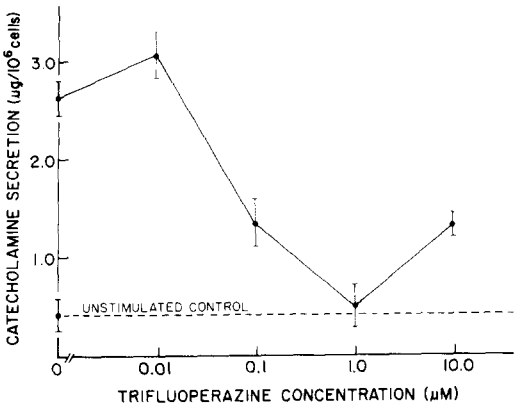

Fig. 1. Dose–response curve for the effect of TFP on total catecholamine secretion by acetylcholine-stimulated chromaffin cells. Cells were preincubated for 5 min at 37° with the indicated concentration of TFP, and aliquots were taken to determine the effect of stimulation on catecholamine secretion. The quantity of catecholamine present in the medium was determined spectrophotometrically. Each point represents the mean (five replicates) for a treatment group minus the mean of the zero time control ± $\sqrt{a^2 + b^2}$, where a and b represent the S.E. of treatment and zero-time means respectively.

by cells stimulated in the absence of the drug. However, much of this secretion was independent of acetylcholine stimulation since it occurred upon incubation of the cells with high concentrations of TFP in the absence of acetylcholine (Table 1).

Since low TFP concentrations produced inhibition of acetylcholine-induced secretion, we felt that it was necessary to eliminate any potential anticholinergic effect. Untreated and TFP-treated cells were depolarized by exposure to 56 mM potassium medium in order to bypass receptor-mediated stimulation by acetylcholine. As shown in Table 2, norepinephrine secretion by cells stimulated with 56 mM potassium medium was 46% greater than that by acetylcholine-stimulated cells. However, in the presence of TFP, acetylcholine-induced secretion was reduced to the level of the unstimulated control (TFP), while secretion induced by 56 mM potassium medium was reduced by only 21%. This indicates that the major effect of TFP was probably at the membrane level, perhaps through an anticholinergic action, although there was also a significant effect of the drug directly upon secretion itself. It may also be noted from this table that the effect of TFP at low concentrations was evident only for stimulated cells. There was no significant difference between zero-time and incubated control groups for TFP-treated and untreated cells.

If TFP depressed the entry of calcium into the cell or influenced the action of calcium in a manner independent of calmodulin, exposure of cells to elevated extracellular calcium should relieve the TFP inhibition of secretion. Cells were incubated in the presence or absence of 2 μM TFP in Locke's medium (2.2 mM calcium) for 5 min at 37°. Aliquots were then transferred to equal volumes of medium with 2.2 mM calcium or 20 mM calcium in the presence or absence of 2 μM TFP and acetylcholine. Norepinephrine secretion (ng/10^5 cells, six replicates) for the group was: normal calcium (2.2 mM), 48.5 ± 5.7; normal calcium + 2 μM TFP, 1.8 ± 1.4; elevated calcium (11.1 mM), 78.2 ± 4.5; and, elevated calcium + 2 μM TFP, 8.7 ± 1.4. As shown by the data, raising the extracellular calcium to 11.1 mM caused a significant increase in secretion, compared to the medium with 2.2 mM Ca^{2+}. However, 2 μM TFP still completely inhibited secretion ($P < 0.006$). Therefore, it appears that increasing extracellular calcium cannot override the functional disability caused by TFP, possibly because of limitations on calcium movement across the membrane or because of the inability of free calcium to replace the calcium–calmodulin complex in required reactions.

The effect of TFP on secretion, at least in chromaffin cells, is too complex to allow the simple explanation that

Table 1. Effect of high TFP concentrations on secretion of norepinephrine by acetylcholine-stimulated and unstimulated cells*

Treatment	Norepinephrine secretion (ng/10^5 cells)
Zero-time control	58.9 ± 1.6
Incubated control	73.1 ± 3.2
Acetylcholine	235.2 ± 6.8†
Acetylcholine + 10 μM TFP	99.6 ± 1.0†
10 μM TFP	88.9 ± 0.5†
Acetylcholine + 50 μM TFP	189.0 ± 1.4†
50 μM TFP	340.9 ± 1.8†
Acetylcholine + 100 μM TFP	1055.6 ± 17.8†
100 μM TFP	526.2 ± 8.0†

* Cells were suspended in Locke's medium in the presence or absence of TFP at the indicated concentrations and preincubated at 37° for 5 min. At the end of the incubation period, aliquots were taken to determine the effect of stimulation on catecholamine secretion. Each value represents the mean ± S.E. for six replicates.

† Significantly different from the incubated control group ($P < 0.01$).

Table 2. Inhibition of norepinephrine secretion by TFP-treated cells stimulated with acetylcholine or 56 mM potassium medium*

	Norepinephrine released (ng/10^5 cells)
Zero-time control	33.8 ± 2.0
Zero-time + 2 μM TFP	33.9 ± 1.52
Incubated control	42.6 ± 2.2
Incubated control + 2 μM TFP	49.3 ± 1.4
Stimulated, 0.1 mM acetylcholine	89.0 ± 5.0†
Stimulated, 0.1 mM acetylcholine + 2 μM TFP	53.0 ± 1.2
Stimulated, 56 mM potassium	129.8 ± 4.2†
Stimulated, 56 mM potassium + 2 μM TFP	103.0 ± 3.4†

* Cells were suspended at 2 × 10^6/ml in medium in the presence or absence of 2 μM TFP for a 5-min incubation period at 37°. Aliquots were then pipetted into microfuge tubes containing an equal volume of medium (controls), medium containing 0.2 mM acetylcholine, or 112 mM potassium medium. For TFP-treated groups, the media also contained 2 μM TFP. Each value is the mean ± S.E. for six replicates.

† Significantly different from the incubated control at $P < 0.01$.

inactivation of calmodulin results in inactivation of the secretory system. Inhibition of chromaffin cell secretion by low micromolar concentrations of TFP parallels the results of Schubart *et al.* [13] for protein phosphorylation and insulin secretion by hamster insulinoma cells. They found half-maximal inhibition of these two calcium-dependent functions at less than 3 μM TFP and hypothesized that the drug influenced secretion at an early calcium-dependent step in the secretory pathway. Krausz *et al.* [4] have demonstrated inhibition of an early step of insulin secretion by isolated pancreatic islets but at much higher TFP concentrations (30–100 μM). Our results with chromaffin cell secretion also indicate a predominant role of TFP at an early stage of the secretory process, probably with events surrounding activation of the cholinergic receptor, while events subsequent to membrane depolarization appear to be less affected. It also appears that sufficient calcium is available for the exocytotic phase of secretion since potassium-induced secretion is inhibited by only 21% in the presence of TFP (2.2 mM extracellular calcium).

Our results, and those of others [14, 15] with high TFP concentrations, indicate detrimental effects of the drug on cell function that are probably unrelated to an effect on calmodulin. Thus, considerable care must be exercised in the definition of calmodulin-dependent functions based upon treatment of cells with TFP concentrations in the high micromolar range.

Acknowledgements—We wish to thank Peck Meat Packing, Inc., for the gift of tissue and Smith Kline & French Laboratories for the gift of trifluoperazine. We are especially grateful to Dr. Frank L. Siegel for helpful suggestions.

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Biochemical Pharmacology, Vol. 32, No. 2, pp. 373–375, 1983.
Printed in Great Britain.

0006-2952/83/020373-03 $03.00/0

Identification of a new metabolite of tamoxifen in patient serum during breast cancer therapy

(*Received* 6 *May* 1982; *accepted* 23 *July* 1982)

Tamoxifen, ICI 46,474, *trans*-1-(*p*-β-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene, is a non-steroidal antiestrogen used as a palliative agent in the treatment of advanced breast cancer [1]. This antagonist competes with