Biochemical Pharmacology, Vol. 32, No. 2, pp. 367–371, 1983.
Printed in Great Britain.

0006-2952/83/020367-05 $03.00/0

SHORT COMMUNICATIONS

In vitro inhibition of the biosynthesis of slow reacting substance of anaphylaxis (SRS-A) and lipoxygenase activity by quercetin

(*Received* 28 *January* 1982; *accepted* 24 *June* 1982)

There is much experimental evidence suggesting that slow reacting substance of anaphylaxis (SRS-A) is an important mediator in inducing the bronchospasm of human allergic asthma. Conceivably, the study of compounds that inhibit the biosynthesis of SRS-A in animal model systems could provide leads to drugs which may be therapeutically useful for the treatment of asthma.

Quercetin (2-3,4-dihydroxyphenyl]-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one), a flavonoid, has been reported to inhibit antigen-induced histamine release from actively sensitized rat peritoneal mast cells [1–3]. This compound bears some structural resemblance to both disodium cromoglycate (a known inhibitor of anaphylactic mediator release) and nordihydroguaiaretic acid (an inhibitor of lipoxygenase activity and SRS-A biosynthesis) [4–7]. This observation prompted us to determine if quercetin is both a mediator release inhibitor and an inhibitor of the biosynthesis of SRS-A. In addition, since a key enzyme in the biosynthesis of SRS-A is a Δ^5-lipoxygenase, quercetin was also examined for its effect on lipoxygenase catalyzed reactions, utilizing Δ^{12}-lipoxygenase from human platelets and Δ^5-lipoxygenase from rat basophilic leukemia (RBL-1) cells. The results of these studies are the subject of this communication.

Methods and results

The reagents used were: egg albumin (five times crystallized), atropine sulfate, pyrilamine maleate, quercetin, indomethacin, heparin and glutathione (Sigma Chemical Co., St. Louis, MO); disodium cromoglycate (DSCG) (Fisons Ltd., Loughborough, England); and ionophore A23187, nordihydroguaiaretic acid (NDGA), [1-^{14}C]arachidonic acid (60 mCi/mmole) and 5,8,11,14-eicosatetraynoic acid (ETYA, synthesized at Hoffmann-La Roche Inc., Nutley, NJ). The [1-^{14}C]-5-HETE standard was a gift of Dr. Walter Hubbard (Vanderbilt University, Nashville, TN), and the rat basophilic leukemia cells were provided by Dr. Anthony Kulczycki (Washington University, St. Louis, MO).

Effects of drugs on in vitro *IgE-dependent antigen-induced histamine release from passively sensitized rat peritoneal cells.* This model system was utilized to confirm the mediator release inhibitory activity of quercetin and to compare its activity with that of DSCG and NDGA. The methodology employed was the same as that reported earlier [8]. Briefly, a mixed population of rat peritoneal cells (containing 5–10% mast cells) was passively sensitized by incubating with reaginic rat antiserum prepared against egg albumin. Triplicate samples of sensitized cell suspensions (2 ml, final volume) were challenged with antigen, with or without the test compound added at the same time as antigen. The amount of antigen-induced histamine release from the cells was expressed as a percentage of their total histamine content. As shown in Fig. 1, quercetin and disodium cromoglycate were potent inhibitors of histamine release in this IgE-dependent test system. The IC_{50} values (drug concentrations that produced 50% of the maximum obtainable inhibition) for both drugs were approximately 2 μM. In contrast, NDGA, at concentrations up to 100 μM, had no significant effect on histamine release.

Effects of drugs on in vitro *ionophore A23187-induced SRS biosynthesis in rat peritoneal cells.* The ability of quercetin to inhibit SRS-A biosynthesis was studied in the rat peritoneal cell system described below. In these studies, the inhibitory activity of quercetin was compared to that of NDGA and ETYA, another known SRS-A biosynthesis inhibitor [6, 7]. Peritoneal cells were isolated by lavage from male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 180 and 220 g, which were fasted overnight. The procedure used was a modification of the method of Herzig and Kusner [9]. The buffer used was Hanks' balanced salt solution containing 50 μg/ml sodium heparin buffered to pH 6.9 with 5% (v/v) of 0.1 M sodium phosphate. The cells were isolated by centrifugation at 350 *g* for 5 min at 0–4° and resuspended in buffer to a concentration of approximately 2×10^6 cells/ml. Triplicate samples of cell suspensions (2 ml, final volume) were preincubated for 10 min at 37° with shaking in the presence or absence of various concentrations of test drug. The test drugs were dissolved in dimethyl sulfoxide (DMSO) prior to addition to cell suspensions. The final concentration of DMSO added to the cells was 0.25%. This level had no effect on ionophore-induced SRS biosynthesis. The cells were subsequently challenged with 0.5 μM ionophore A23187 which produces a submaximal stimulation of SRS biosynthesis. After a 10-min incubation with ionophore, SRS biosynthesis was terminated by placing samples in a boiling water bath for 10 min and centrifuging for 10 min at 2000 *g* at 4° to remove cellular debris. The concentrations of SRS (units/ml) present in the resulting supernatant fractions were quantitated by bioassay using an isolated segment of guinea pig ileum [10]. One unit of SRS was defined as the amount eliciting a contraction response of the ileum equivalent to that obtained with 5 ng of histamine. The SRS contractions are inhibited completely by the antagonist FPL 55712, sodium 7-[3-(4-acetyl-3-hydroxy-2-propyl-phenoxy)-2-hydroxy-propoxy]-4-oxo-8-propyl-4*H*-1-enzopyran-2-carboxylic acid (0.5 μM).

The data presented in Fig. 2 demonstrate that quercetin inhibited ionophore A23187-induced SRS biosynthesis in rat peritoneal cell *in vitro* in a dose-dependent manner. The IC_{50} for quercetin was approximately 4–5 μM. The reference compound, ETYA (IC_{50} 3–4 μM), and NDGA (IC_{50} 5–7 μM) demonstrated approximately similar inhibitory potencies.

Effect of quercetin on in vitro *antigen-induced histamine release, SRS-A release and SRS-A biosynthesis in actively sensitized guinea pig lung fragments.* This additional model system was used to confirm the ability of quercetin to inhibit both antigen-induced histamine release and SRS-A biosynthesis. A modification of the methods described by Hitchcock [11] was used in the IgG_1-dependent test system. Male Hartley guinea pigs (Charles River Laboratories) were actively sensitized to egg albumin by an intraperitoneal injection of antigen (10 ml in 1 ml of 0.9% sodium chloride) 28–48 days prior to use. The lungs were removed from animals killed by exsanguination and were chopped into approximately 1 mm^3 fragments with a McIlwain tissue slicer. Lung fragments were combined and washed with

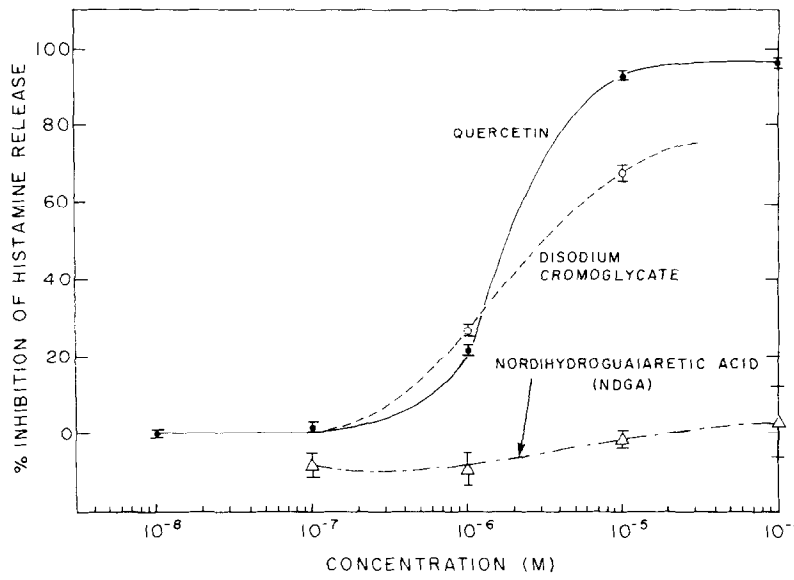

Fig. 1. Effects of quercetin, disodium cromoglycate and nordihydroguaiaretic acid (NDGA) on *in vitro* anaphylactic histamine release from passively sensitized rat peritoneal cells. Passively sensitized cells (1×10^6 cells/ml) were challenged with 10 μg egg albumin/ml in the presence or absence of various concentrations of quercetin (●—●), disodium cromoglycate (○---○) or NDGA (△·····△), added at the same time as antigen. In the absence of drug, antigen-induced histamine release was 30.8 ± 0.2%, after correcting for a spontaneous release of 7.0 ± 0.3%. The results are presented as the percent inhibition of this histamine release. Each point on the graph represents the mean percent inhibition ± the standard error of triplicate determinations in a typical experiment. Each drug was tested in two to five experiments.

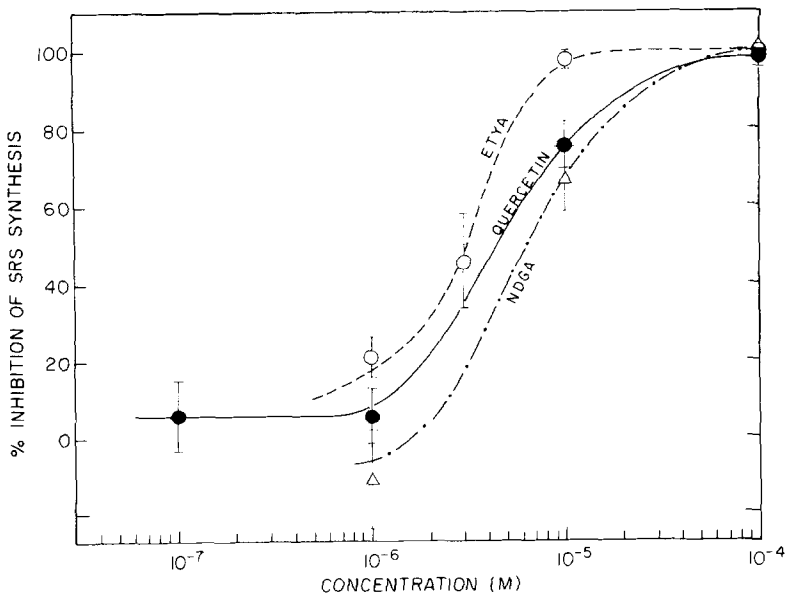

Fig. 2. Effects of quercetin, nordihydroguaiaretic acid (NDGA), and 5,8,11,14-eicosatetraynoic acid (ETYA) on *in vitro* ionophore A23187-induced SRS synthesis in rat peritoneal cells. The cells (2×10^6 cells/ml) were preincubated for 10 min in the presence or absence of test drug prior to incubation with 0.5 μM ionophore. In the absence of drug, ionophore-induced SRS biosynthesis was 40–50 units SRS/10^6 cells. The results are presented as the percent inhibition of this SRS biosynthesis. Each point on the graph represents the mean percent inhibition ± the standard error of triplicate determinations.

ice-cold Tyrode's solution which was aerated with a 95% O_2/5% CO_2 mixture. Assays were performed in triplicate by preincubating 150-mg aliquots of the pooled lung fragments in 5 ml of Tyrode's solution for 10 min at 37° with shaking in the presence or absence of various concentrations of quercetin. The tissue suspensions were subsequently challenged for 15 min with egg albumin (40 μg/ml) and filtered to remove tissue.

The resulting filtrates of incubated tissue suspensions were assayed for both histamine and SRS-A release. Histamine release was determined by mixing a 1-ml aliquot of filtrate with an equal volume of 0.8 N perchloric acid and storing overnight at 0–4°. Samples were centrifuged for 10 min at 2000 *g* (0–4°), and the supernatant fractions were assayed for histamine using an automated spectrofluorometric method [12]. The amount of histamine released was expressed as a percentage of the total histamine content of the lung fragments. The total histamine content was obtained by homogenizing 150-mg aliquots of tissue fragments in 5 ml Tyrode's solution using an all-glass tissue grinder and heating the homogenate for 10 min in a boiling water bath, prior to centrifuging for 10 min at 2000 *g* (0–4°), mixing resulting supernatant fractions with an equal volume of 0.8 N perchloric acid, recentrifuging, and assaying final supernatant fractions for histamine.

The filtrates of incubated tissue suspensions were assayed for SRS-A activity utilizing the isolated guinea pig ileum bioassay. The amount of SRS-A in this filtrate was termed "SRS-A release". In addition, the total amount of SRS-A biosynthesized *de novo* by the lung fragments was determined by homogenizing antigen-challenged samples (150 mg tissue fragments/5 ml assay mixture), heating the homogenate for 10 min in a boiling water bath, centrifuging for 10 min at 2000 *g* (0–4°), and quantitating the SRS-A in the resultant supernatant fraction by bioassay. The SRS-A present in this supernatant fraction represents the "total SRS-A biosynthesis" induced by antigen.

The data presented in Table 1 demonstrate that 10^{-3} M quercetin inhibited the IgG_1-mediated, antigen-induced histamine release from actively sensitized guinea pig lung fragments. The IC_{50} for inhibition of histamine release was between 10^{-4} and 10^{-3} M. It can also be seen that this compound inhibited SRS-A release and total SRS-A biosynthesis in this test system. Quercetin was a more potent inhibitor of SRS-A biosynthesis and release (IC_{50} values were $< 10^{-5}$ M) than of histamine release. In contrast, 10^{-3} M disodium cromoglycate (the reference inhibitor of mediator release) had no effect on either histamine or SRS-A release in this system [13]. As described in the legend of Table 1, all of the SRS-A which was biosynthesized was also released from the antigen-challenged tissue. It was also observed that quercetin was a less potent inhibitor of antigen-induced histamine release in the guinea pig lung fragment system than in the rat peritoneal cell system described above. These findings may be explained by the poorer penetration of drugs into tissue than into free cells or by differences in the species or sensitizing antibodies.

Effects of drugs on Δ^{12}-lipoxygenase activity. The antioxidant NDGA is believed to inhibit SRS-A biosynthesis by inhibiting a lipoxygenase catalyzed reaction [14]. The reported antioxidant activity of quercetin [15] and its structural similarity to NDGA suggested a similar mechanism of action for quercetin. In addition, it has been reported recently that flavonoids structurally related to quercetin inhibit the lipoxygenase activity from soybeans, rat lung, and rat spleen [16]. To study the activity of quercetin toward lipoxygenases, an assay for Δ^{12}-lipoxygenase was employed. The activity of this enzyme was determined by measuring the enzymatic conversion of [1-^{14}C]arachidonic acid to [1-^{14}C]-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid ([1-^{14}C]-12-HPETE) which leads to the formation of the 12-hydroxy derivative ([1-^{14}C]-12-HETE). Human platelets were used as a source of Δ^{12}-lipoxygenase. The platelets were washed in Dulbecco's phosphate-buffered saline (PBS) containing 1.3 mM ethylenediaminetetraacetic acid and then lysed by freezing and thawing three times. The platelet lysate was homogenized using a glass–Teflon homogenizer, and the homogenate was centrifuged for 1 hr at 100,000 *g*. The enzyme in the supernatant fraction was partially purified by precipitation with 50% saturated ammonium sulfate at 0°. The partially purified enzyme was assayed at 37° using 3.1 μM (0.18 μCi/ml) [1-^{14}C]-arachidonic acid as the substrate in PBS containing 1 mM glutathione. After a 2-min incubation, the reaction was stopped by addition of citric acid and diethyl ether. The ethereal extract was analyzed by silica gel TLC using isooctane–methylethylketone–acetic acid (100:9:1) as the developing solvent. The major radioactive product ([1-^{14}C]-12-HETE) and unconverted substrate ([1-^{14}C]arachidonic acid) were located using a Berthold TLC scanner. The R_f values for these two materials were 0.67 and 0.95 respectively. In addition, there was a small amount of unidentified radioactive polar products which remained near the origin (R_f 0.04). The radioactivity in appropriate regions of the chromatogram was quantitated by liquid scintillation counting. The effects of drugs on Δ^{12}-lipoxygenase activity were determined by preincubating the enzyme for 10 min at 25° in the presence or absence of

Table 1. Concentration-dependent influence of quercetin on *in vitro* anaphylactic histamine release, SRS-A release and SRS-A biosynthesis in actively sensitized guinea pig lung fragments*

Conc (M)	% Inhibition of:		
	Histamine release	SRS-A release	SRS-A biosynthesis
10^{-5}	9.9 ± 1.8 (NS)†	75 ± 1 ($P < 0.01$)	66 ± 4 ($P < 0.01$)
10^{-4}	16.4 ± 2.4 (NS)	91 ± 0.7 ($P < 0.001$)	92 ± 1 ($P < 0.001$)
10^{-3}	72.4 ± 9.6 ($P < 0.001$)	91 ± 0.7 ($P < 0.001$)	100 ± 0 ($P < 0.001$)

* Lung fragments from five actively sensitized guinea pigs were challenged with antigen. Antigen alone induced a 12.8 ± 0.8% release of histamine, after correcting for a spontaneous release of 3.5 ± 1.0%. In the absence of drug, antigen induced a release of SRS-A activity (1.65 ± 0.17 units/mg lung) and *de novo* biosynthesis of SRS-A (1.51 ± 0.16 units/mg lung). Quercetin exhibited no SRS-A antagonism activity at the levels used in this study. The P values indicate the level of statistical significance of the release or biosynthesis attained in the presence of the drug in comparison to that attained with antigen alone.

† Value that was not significantly different from control.

various concentrations of drug prior to addition of [1-^{14}C]arachidonic acid.

Quercetin and the reference compounds ETYA and NDGA caused a concentration-dependent inhibition of human platelet Δ^{12}-lipoxygenase activity. Quercetin inhibited Δ^{12}-lipoxygenase activity with the same potency (IC_{50} 4–5 μM) that it inhibited SRS-A biosynthesis in ionophore A23187-stimulated rat peritoneal cells. Similarly, NDGA inhibited Δ^{12}-lipoxygenase with approximately the same potency (IC_{50} 5 μM) that it inhibited SRS-A biosynthesis in the rat cells. In contrast, ETYA was a more potent inhibitor of Δ^{12}-lipoxygenase activity (IC_{50} 0.05 μM) than of SRS-A biosynthesis in rat cells (IC_{50} 3–4 μM).

Effect of quercetin on Δ^5-lipoxygenase activity. Since the specific lipoxygenase which initiates SRS-A biosynthesis is thought to be a Δ^5-lipoxygenase, quercetin was also tested for its effect on Δ^5-lipoxygenase from rat basophilic leukemia (RBL-1) cells. The activity of this enzyme was determined by measuring the catalytic conversion of [1-^{14}C]arachidonic acid to [1-^{14}C]-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid ([1-^{14}C]-5-HPETE) which leads to the formation of the 5-hydroxy derivative ([1-^{14}C]-5-HETE). The Δ^5-lipoxygenase was derived from the supernatant fraction of lysed RBL-1 cells using a modification of the method previously described by Jakschik and Lee [17]. Briefly, RBL-1 cells were lysed by homogenization in ice-cold buffer (50 mM Tris–HCl buffer, pH 7.2, containing 1 mM EDTA and 14 μM indomethacin). The homogenate was centrifuged at 4° at 49,000 *g* for 20 min and the resulting supernatant fraction was used as the source of Δ^5-lipoxygenase. The enzyme was assayed at 37° using 6.7 μM (0.39 μCi/ml) [1-^{14}C]arachidonic acid as the substrate in 50 mM Tris–HCl buffer, pH 7.2, containing 1 mM glutathione, 2 mM $CaCl_2$, 14 μM indomethacin, and 0.25 to 0.50 mM EDTA. The mixture was incubated for 10 min, and the reaction was stopped by the addition of citric acid and diethyl ether. The ethereal extract containing [1-^{14}C]-5-HETE and unreacted substrate was analyzed by silica gel TLC using the same methods as described above for the Δ^{12}-lipoxygenase assay. The [1-^{14}C]-5-HETE was identified by co-chromatography with an authentic, chemically synthesized [1-^{14}C]-5-HETE standard. The R_f values for [1-^{14}C]-5-HETE, unconverted [1-^{14}C]arachidonic acid, and unidentified radioactive polar products were 0.49, 0.95, and 0.04 respectively. The effect of quercetin on Δ^5-lipoxygenase activity was determined by preincubating the enzyme for 10 min at 30° in the presence or absence of various concentrations of the drug prior to addition of substrate. Quercetin caused a concentration-dependent inhibition of the RBL-1 cell-derived Δ^5-lipoxygenase activity. Quercetin was a more potent inhibitor of RBL-1 cell Δ^5-lipoxygenase activity (IC_{50} 0.2 μM) than of human platelet Δ^{12}-lipoxygenase activity (IC_{50} 4–5 μM).

Discussion

The studies presented in this communication confirm previous observations that quercetin and DSCG inhibit antigen-induced (IgE-mediated) histamine release from passively sensitized rat peritoneal cells. NDGA did not have this property. It has been suggested that quercetin and DSCG have a similar mechanism of action as mediator release inhibitors. Both drugs are believed to inhibit antigen-stimulated histamine release from rat peritoneal mast cells by blocking antigen-induced Ca^{2+} entry into the cell, but neither drug affects the release stimulated by the Ca^{2+} ionophore A23187, which bypasses this normal pathway of Ca^{2+} influx [1–3]. The ionophore A23187-stimulated rat peritoneal cell system thus provides a convenient model in which to directly examine the effect of drugs on SRS biosynthesis, since mediator release inhibitors that block ligand-mediated Ca^{2+} entry would not be expected to inhibit this ionophore system. The present studies demonstrate that quercetin, like NDGA and ETYA, inhibits SRS biosynthesis in this model system. Ionophore A23187 is thought to induce SRS biosynthesis in these cells by activating the Ca^{2+}-dependent hydrolysis of membrane phospholipids, liberating arachidonic acid, which is further metabolized by Δ^5-lipoxygenase, initiating the pathway to SRS formation [18]. The finding that quercetin inhibits Δ^5-lipoxygenase and Δ^{12}-lipoxygenase suggests that a lipoxygenase may be one of the sites at which quercetin inhibits SRS biosynthesis in the rat peritoneal cells.

In the present studies, quercetin was also found to inhibit antigen-induced (IgG_1-mediated) histamine release and SRS-A biosynthesis in actively sensitized guinea pig lung fragments. In this system, quercetin could conceivably inhibit *de novo* SRS-A biosynthesis by two mechanisms: (1) inhibition at the level of antigen-induced Ca^{2+} influx through the cell membrane; and (2) inhibition of a Δ^5-lipoxygenase step after Ca^{2+} influx. The observation that quercetin is a more potent inhibitor of SRS-A biosynthesis than of histamine release in the guinea pig lung fragment model (Table 1) is consistent with this hypothesis. Another possible mechanism that is presently being explored is that quercetin may inhibit a phospholipase A_2 to prevent the release of arachidonic acid from membrane phospholipids and thereby inhibit SRS-A biosynthesis.

In summary, quercetin has been shown to be a potent inhibitor of histamine release and SRS-A biosynthesis *in vitro*. Since quercetin was also shown to be a potent inhibitor of Δ^5-lipoxygenase activity from RBL-1 cells and Δ^{12}-lipoxygenase activity from human platelets, one of the mechanisms by which this drug inhibits SRS-A biosynthesis may involve the inhibition of a Δ^5-lipoxygenase. These studies suggest that quercetin may be an interesting prototype for a drug which is both an inhibitor of general mediator release (like DSCG) and an inhibitor of SRS-A biosynthesis (like NDGA).

Acknowledgement—We are grateful to Mrs. Jeanne Harris for her excellent secretarial contributions involved in the preparation of this manuscript.

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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.