Biochem. J. (1981) **196**, 771–780
Printed in Great Britain

Effects of trifluoperazine and pimozide on stimulus–secretion coupling in pancreatic B-cells

Suggestion for a role of calmodulin?

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(*Received 18 December 1980/Accepted 30 January 1981*)

The possible involvement of calmodulin in insulin release was evaluated by studying the effects on intact islets of trifluoperazine and pimozide, two antipsychotic agents known to bind strongly to calmodulin in cell-free systems. Trifluoperazine (10–100 μM) produced a dose- and time-dependent inhibition of the two phases of glucose-stimulated insulin release. The effect was not reversible by simple washing of the drug, but could be prevented by cytochalasin B or theophylline. Trifluoperazine also inhibited the release induced by glyceraldehyde, oxoisocaproate, tolbutamide or barium, but not that stimulated by 10 mM-theophylline or 1 mM-3-isobutyl-1-methylxanthine. Pimozide (0.5–10 μM) also produced a dose-dependent inhibition of insulin release triggered by glucose, leucine or barium, but did not affect the release induced by methylxanthines. Glucose utilization by islet cells was not modified by trifluoperazine (25 μM), which slightly increased cyclic AMP concentration in islets incubated without glucose. The drug did not prevent the increase in cyclic AMP concentration observed after 10 min of glucose stimulation, but suppressed it after 60 min. Basal or glucose-stimulated Ca^{2+} influx (5 min) was unaffected by 25 μM-trifluoperazine, whereas Ca^{2+} net uptake (60 min) was inhibited by 20%. Glucose-stimulated Ca^{2+} uptake was almost unaffected by pimozide. In a Ca^{2+}-free medium, trifluoperazine decreased Ca^{2+} efflux from the islets and did not prevent the further decrease by glucose; in the presence of Ca^{2+}, the drug again decreased Ca^{2+} efflux and inhibited the stimulation normally produced by glucose. In the absence of glucose, trifluoperazine lowered the rate of Rb^{+} efflux from the islets, decreased Rb^{+} influx (10 min), but did not affect Rb^{+} net uptake (60 min). It did not interfere with the ability of glucose to decrease Rb^{+} efflux rate further and to increase Rb^{+} net uptake. The results show thus that trifluoperazine does not alter the initial key events of the stimulus–secretion coupling. Its inhibition of insulin release suggests a role of calmodulin at late stages of the secretory process.

The concentration of cytoplasmic free Ca^{2+} is believed to control a variety of cellular events (Berridge, 1975; Rasmussen & Goodman, 1977). Recent evidence suggests that certain effects originally ascribed to Ca^{2+} itself are mediated by calmodulin, a ubiquitous Ca^{2+}-dependent regulatory protein (Cheung, 1980; Means & Dedman, 1980).

The importance of Ca^{2+} for stimulation of insulin release has long been recognized, but the mechanisms by which the ion triggers exocytosis of insulin granules remain elusive. Calmodulin is present in pancreatic islet cells (Sugden *et al.*, 1979; Valverde *et al.*, 1979; Hales *et al.*, 1980), where it may be involved in the metabolism of cyclic AMP (Sugden *et al.*, 1979; Valverde *et al.*, 1979; Sharp *et al.*, 1980) and in the regulation of the activity of a Ca^{2+}-dependent protein kinase (Shubart *et al.*, 1980; Gagliardino *et al.*, 1980).

The role of calmodulin in the functioning of intact cells cannot be studied directly. Certain antipsychotic agents that bind to calmodulin and inhibit its effects have been proposed as valuable tools to evaluate, indirectly, the involvement of calmodulin in various cellular processes (Weiss & Levin, 1978). One of these agents, trifluoperazine, has recently been shown to inhibit insulin release *in vitro* (Sugden

0306-3283/81/060771-10$01.50/1

et al., 1979; Shubart *et al.*, 1980; Krausz *et al.*, 1980). The present study is an attempt to unravel the possible roles of calmodulin in the stimulus–secretion coupling in pancreatic B-cells. It evaluates the effects of trifluoperazine (a phenothiazine) on various aspects of the B-cell function and compares some of these with the effects of pimozide (a diphenylbutylpiperidine), which binds to calmodulin with an even greater affinity than trifluoperazine (Weiss & Levin, 1978).

Materials and methods

All experiments were performed with islets isolated by collagenase digestion of the pancreas of fed male Wistar rats and incubated or perifused in a bicarbonate-buffered medium (Henquin, 1980).

The incubation and perifusion techniques utilized to study insulin release and the method for measurement of immunoreactive insulin have been reported previously (Henquin & Lambert, 1976). At the concentrations used, none of the substances tested interfered with the immunoassay. Glucose utilization by islet cells was measured by the production of ^3H_2O from D-[5-^{3}H]glucose (Ashcroft *et al.*, 1972). The technical aspects of the method have been described elsewhere (Henquin & Lambert, 1976). The perifusion system utilized to monitor the efflux of $^{86}Rb^+$ or $^{45}Ca^{2+}$ from preloaded islets and the method used to measure the uptake of $^{86}Rb^+$ or $^{45}Ca^{2+}$ by islet cells have been described previously (Henquin, 1978, 1980; Henquin & Lambert, 1976).

The islet content in cyclic AMP was measured as follows. After pre-incubation for 30 min in the presence of 3 mM-glucose, batches of five islets were incubated for 10 or 60 min in 1 ml of medium. The islets were then transferred, with a needle, into 0.4 ml of ice-cold acetate buffer (50 mM; pH 6.2). After sonication (5 s), the tubes containing the islets were heated for 3 min at 90°C. Islet cyclic AMP concentrations were determined by radioimmunoassay, with a commercially available kit (New England Nuclear, Boston, MA, U.S.A.) after acetylation of standards and samples. Reliability of the procedure was assessed by measuring cyclic AMP concentrations after treatment of the tissue with cyclic AMP phosphodiesterase. After incubation, two series of batches of 10 islets from the same rats were transferred into 0.4 ml of ice-cold Tris buffer (10 mM; pH 7.0) supplemented with 10 mM-$MgCl_2$; after sonication and heat treatment, the samples were incubated for 90 min at 37°C without or with 20 m-units of cyclic AMP phosphodiesterase (EC 3.1.4.17). The samples were then heated again at 90°C for 3 min and diluted with 0.4 ml of acetate buffer. After such treatment the cyclic AMP content was decreased from 15.1 ± 0.7 to 0.5 ± 0.3 fmol/islet.

Trifluoperazine was a gift of Smith, Kline and French S.A. (Brussels, Belgium) and was directly dissolved, each day, in the appropriate solutions; pimozide was provided by Janssen Pharmaceutica (Beerse, Belgium) and dissolved in dimethyl sulphoxide. Other sources were as follows: chlorpromazine from Specia (Paris, France); rotenone and ^2H_2O from Sigma Chemical Co. (St. Louis, MO, U.S.A.); ionophore A23187 from Calbiochem (San Diego, CA, U.S.A.); cyclic AMP phosphodiesterase from Boehringer (Mannheim, Germany); D600 from Knoll A.G. (Ludwigshaven, Germany); cytochalasin B from ICI Pharma (Brussels, Belgium); tolbutamide {*N*-[(butylamino)carbonyl]-4-methylbenzenesulphonamide} from Hoechst A.G. (Frankfurt, Germany). Radiochemicals were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) Other reagents were obtained from the same sources as those indicated in previous publications (Henquin & Lambert, 1976; Henquin, 1980).

Results are presented as means ± S.E.M. or S.D. for efflux experiments. The statistical significance of differences between experimental groups was assessed by Student's *t* test for unpaired data.

Results

Effects of trifluoperazine on insulin release

In the absence of glucose, basal insulin release was unaffected by 25 or 50 μM-trifluoperazine, but was increased from 0.33 ± 0.02 to 0.84 ± 0.14 ng/islet per 60 min ($P < 0.005$) by 100 μM-trifluoperazine. In the presence of 10 mM-glucose, trifluoperazine produced a dose- and time-dependent inhibition of insulin release (Fig. 1*a*). The lowest effective dose tested was 10 μM (20%, $P < 0.001$). Pre-incubation of the islets in the presence of trifluoperazine augmented the inhibitory effect of the drug. At the concentration of 200 μM, trifluoperazine markedly augmented basal insulin release. This effect was not suppressed by omission of extracellular Ca^{2+} (results not shown) and may be due to damage of B-cells. It likely explains why this high concentration of trifluoperazine was apparently less effective in inhibiting glucose-stimulated insulin release than lower concentrations (Fig. 1*a*).

The kinetics of the inhibition of glucose-stimulated insulin release by trifluoperazine is shown in Fig. 2. Introduction of the drug during the second phase of release produced a progressive, dose-dependent fall in the rate of insulin secretion. If trifluoperazine (25 μM) was present before glucose stimulation, the early and the late phases of secretion were inhibited. This inhibitory effect appeared irreversible as no rise in secretion was observed 30 min after removal of trifluoperazine (Fig. 2).

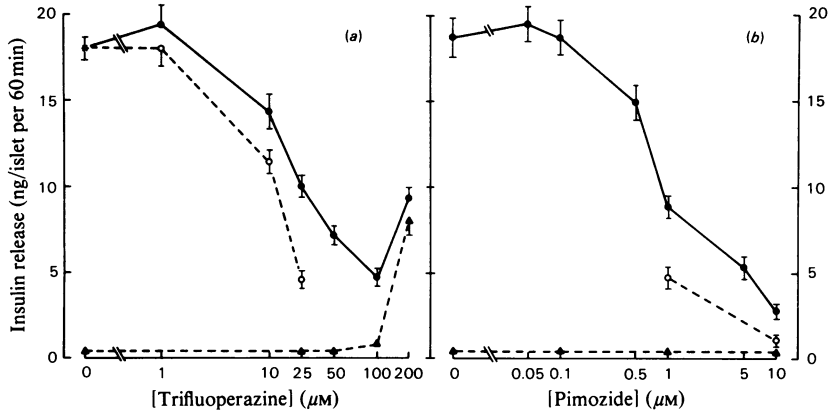

Fig. 1. *Effects of trifluoperazine and pimozide on insulin release by incubated rat islets*
After 30min of pre-incubation in the presence of 3mM-glucose, batches of three islets were incubated for 60min in a medium containing no glucose (▲) or 10mM-glucose (● and ○) and supplemented with the indicated concentrations of trifluoperazine (*a*) or pimozide (*b*). In one series of experiments (○), the tested drug was also present during the pre-incubation period. At the end of the incubation, a portion of the medium was taken for measurement of immunoreactive insulin. Values are means ± S.E.M. of 24 batches of islets in (*a*), except for controls in the presence of glucose where $n = 48$, and of 14 batches of islets in (*b*).

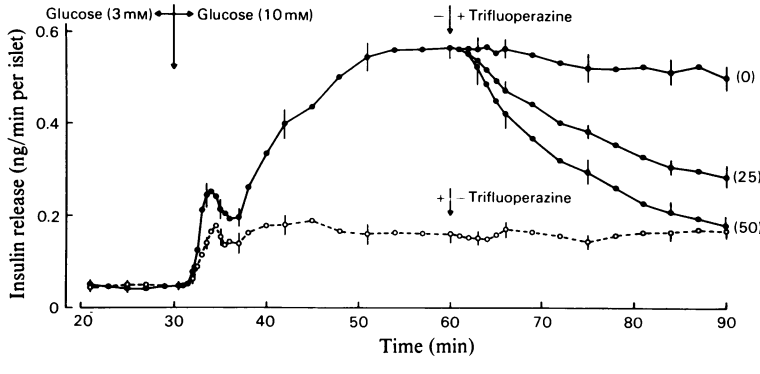

Fig. 2. *Effects of trifluoperazine on the dynamics of insulin release by perifused rat islets*
Immediately after isolation, groups of 15 islets were placed in perifusion chambers. The concentration of glucose was increased from 3mM to 10mM at 30min as shown. In one series of experiments (●), trifluoperazine was added at 60min as indicated, at the concentration (μM) indicated in parentheses. In the other series of experiments (○), trifluoperazine (25μM) was present from the start and withdrawn at 60min as indicated. Values are means ± S.E.M. for four experiments in each group.

The inhibitory potency of trifluoperazine was modulated by extracellular Ca^{2+} (Table 1). When the concentration of $CaCl_2$ was lowered from 2.5 to 1mM, control insulin release triggered by 10mM-glucose was unaffected, but its inhibition by trifluoperazine was slightly augmented. When the concentration of $CaCl_2$ was increased to 5mM, both the control effect of glucose and its inhibition by trifluoperazine were somewhat decreased.

The potentiators of glucose-induced insulin release, cytochalasin B and theophylline, decreased the inhibition by trifluoperazine. The absolute increase in insulin release brought about by 2mM-theophylline was only slightly lower in the presence than in the absence of trifluoperazine (13 compared with 16ng/islet) (Table 1).

Specificity of the effects of trifluoperazine

As detailed in Table 1, the inhibitory effect of trifluoperazine was not restricted to the stimulation of insulin release by glucose. Thus the insulinotropic effects of glyceraldehyde, oxoisocaproate (4-methyl-

Table 1. *Effects of trifluoperazine on insulin release stimulated by various agents*

Islets were first pre-incubated for 30 min in the presence of 3 mM-glucose. Batches of three islets were then incubated for 60 min in the indicated conditions. The various test substances were present only during the incubation period, whereas trifluoperazine was also present during the pre-incubation period. The normal concentration of $CaCl_2$ was 2.5 mM; when barium was used, the incubation medium was devoid of Ca^{2+}. Values are means ± S.E.M. for numbers of batches of islets given in parentheses. Significance levels: the effect of trifluoperazine was always significant ($P < 0.001$), except when indicated by * (not significant); the effect of the test substances was always significant ($P < 0.001$), except when indicated by † (not significant) or § ($P < 0.05$).

	Insulin release (ng/islet per 60 min)	
Test substance (mM)	No trifluoperazine	Trifluoperazine (25 μM)
(*a*) Glucose (0 mM)		
—	0.33 ± 0.03 (20)	0.30 ± 0.02 (20)*
Glyceraldehyde (10)	12.3 ± 0.7 (20)	5.9 ± 0.4 (20)
Oxoisocaproate (10)	10.1 ± 0.5 (24)	6.4 ± 0.4 (24)
Barium (2.5)	2.11 ± 0.12 (15)	0.54 ± 0.06 (15)
Theophylline (10)	2.73 ± 0.20 (16)	5.9 ± 0.3 (16)
3-Isobutyl-1-methylxanthine (1)	2.61 ± 0.19 (16)	4.6 ± 0.2 (16)
(*b*) Glucose (4 mM)		
—	0.37 ± 0.06 (18)	0.44 ± 0.05 (18)*
Tolbutamide (0.075)	2.60 ± 0.13 (22)	1.85 ± 0.12 (22)
(*c*) Glucose (10 mM)		
—	15.5 ± 0.6 (30)	4.2 ± 0.3 (30)
Calcium (1)	16.0 ± 0.9 (16)†	3.3 ± 0.3 (16)§
Calcium (5)	12.9 ± 0.8 (16)§	6.2 ± 0.3 (16)
Cytochalasin B (0.01)	25.5 ± 1.0 (14)	11.7 ± 0.9 (14)
Theophylline (2)	31.4 ± 1.3 (15)	17.2 ± 1.2 (15)

Table 2. *Effects of trifluoperazine on insulin release stimulated by 3-isobutyl-1-methylxanthine*

Islets were first pre-incubated for 30 min in the presence of 3 mM-glucose, with or without trifluoperazine. Batches of three islets were then incubated for 60 min in the absence of glucose, but in the presence of 1 mM-3-isobutyl-1-methylxanthine, in the conditions detailed in the Table. Values are means ± S.E.M. for 48 batches of islets with 3-isobutyl-1-methylxanthine alone and of 14–18 batches in the different test conditions. Significance levels: *, $P < 0.05$; **, $P < 0.001$, versus controls (same column); †, $P < 0.001$, versus same condition without trifluoperazine (same line).

	Insulin release (ng/islet per 60 min)	
Test conditions	No trifluoperazine	Trifluoperazine (25 μM)
Controls	2.51 ± 0.30	4.85 ± 0.19†
20°C	0.28 ± 0.03**	0.36 ± 0.03**
Rotenone (5 μM)	0.53 ± 0.05**	0.64 ± 0.06**
^2H_2O (75%)	1.40 ± 0.11**	1.32 ± 0.07**
Compound D600 (25 μM)	2.52 ± 0.23	5.26 ± 0.24†
Ca^{2+} (0) + EGTA (0.2 mM)	1.80 ± 0.14*	3.04 ± 0.21**,†

2-oxopentanoate), barium substitution for Ca^{2+} and of tolbutamide were also decreased by trifluoperazine. By contrast, the release triggered, in the absence of glucose, by high concentrations of theophylline or 3-isobutyl-1-methylxanthine was increased by trifluoperazine.

The unexpected potentiation, by trifluoperazine, of the release of insulin triggered by methylxanthines was studied in greater detail to ascertain that it was not due to a mere damage of B-cells (Table 2). The effect of 3-isobutyl-1-methylxanthine alone was abolished when the incubations were carried out at 20°C, almost suppressed by rotenone and markedly inhibited, when ^2H_2O was partially substituted for ^1H_2O. In all these conditions, trifluoperazine was without effect. The Ca^{2+}-antagonist, compound D600, did not affect the releasing property of 3-isobutyl-1-methylxanthine nor its increase by

Table 3. *Effects of pimozide on insulin release stimulated by various agents*

After 30min of pre-incubation in the presence of 3mM-glucose, but in the absence of pimozide or test substance, batches of three islets were incubated for 60min in 1ml of medium containing the indicated additions. Ca^{2+} was omitted from the medium supplemented with barium. Values are means ± S.E.M. of 14 batches of islets, except in the presence of 10mM-glucose alone or with 1 μM-pimozide, where $n = 26$. Significance levels: $^*P < 0.001$, versus controls without pimozide.

Test substance (mM)	Insulin release (ng/islet per 60min)		
	No pimozide	Pimozide (1 μM)	Pimozide (10 μM)
(*a*) Glucose (0mM)			
—	0.26 ± 0.03	0.27 ± 0.03	0.25 ± 0.04
Leucine (20)	1.96 ± 0.13	1.05 ± 0.08*	0.58 ± 0.07*
Barium (2.5)	3.28 ± 0.29	1.51 ± 0.09*	0.79 ± 0.05*
Theophylline (10)	2.46 ± 0.17	2.23 ± 0.21	2.21 ± 0.16
(*b*) Glucose (10mM)			
—	17.9 ± 0.7	9.8 ± 0.4*	2.5 ± 0.2*
Theophylline (2)	41.2 ± 1.8	30.0 ± 0.9*	14.4 ± 0.7*

trifluoperazine. In a Ca^{2+}-free medium enriched with EGTA, the effect of the methylxanthine was only partially decreased, and still potentiated by trifluoperazine, although less markedly (70%) than in the presence of Ca^{2+} (93%) (Table 2).

Effects of pimozide on insulin release

Pimozide produced a dose-dependent inhibition of glucose-stimulated insulin release, without affecting basal release (Fig. 1*b*). In the presence of 10mM-glucose, the lowest effective ($P < 0.05$) dose tested was 0.5 μM-pimozide and half inhibition was observed at a concentration of about 1 μM-pimozide. Pre-incubation of the islets with pimozide before stimulation by glucose accentuated the inhibitory effect of the drug (Fig. 1*b*). Phentolamine (20 μM) did not prevent the inhibition of release by 5 μM-pimozide (5.6 ± 0.3 and 5.4 ± 0.4ng of insulin/islet per 60min, in the presence and in the absence of phentolamine respectively; $n = 12$).

The inhibitory effect of pimozide was not specific for glucose-induced insulin release, but was also observed when secretion was stimulated by leucine or barium (Table 3). In the presence of these two stimuli, 1 μM-pimozide again inhibited insulin release by approx. 50%. By contrast, the releasing effect of 10mM-theophylline was not decreased by pimozide even at the concentration of 10 μM. Furthermore, a lower concentration of theophylline (2mM) potentiated insulin release triggered by 10mM-glucose not only in control islets, but also when the releasing effect of the sugar was inhibited by 1 or 10 μM-pimozide (Table 3).

As trifluoperazine, but not pimozide, potentiated the release of insulin induced by methylxanthines, the possibility was considered that this effect of trifluoperazine was due to its phenothiazine structure. Therefore the effects of chlorpromazine, another phenothiazine that also binds to calmodulin (Weiss & Levin, 1978), were also compared with those of pimozide. Islets were first pre-incubated with pimozide (1 μM) or chlorpromazine (25 μM) and then stimulated with 3-isobutyl-1-methylxanthine (1mM) in the presence of the drugs. Control insulin release (3.8 ± 0.25ng/islet per 60min) was not modified by pimozide (3.4 ± 0.14), but was increased by chlorpromazine (8.1 ± 0.54; $P < 0.001$, $n = 12$). In the same conditions, chloropromazine inhibited insulin release stimulated by 10mM-glucose from 15.2 ± 0.9 to 5.3 ± 0.5ng/islet per 60min ($P < 0.001$, $n = 12$). It thus seems that the potentiation of methylxanthine-induced release is a property of phenothiazine molecules rather than the consequence of inactivation of calmodulin.

Effects of trifluoperazine on glucose metabolism

Glucose metabolism by islet cells was not altered by 25 μM-trifluoperazine. After 60min of incubation with 10mM-glucose, glucose utilization amounted to 108.3 ± 3.9pmol/islet in controls and 113.5 ± 4.4pmol/islet in the presence of trifluoperazine ($n = 15$).

Effects of trifluoperazine on islet cyclic AMP concentrations

In control islets, glucose increased cyclic AMP content more markedly after 10 than after 60min of incubation (Table 4). In a glucose-free medium, cyclic AMP concentrations were slightly higher in the presence than in the absence of trifluoperazine. The drug did not prevent the stimulation by glucose after 10min, but suppressed it after 60min. The marked increase in cyclic AMP content brought about by 3-isobutyl-1-methylxanthine was not altered by trifluoperazine (Table 4).

Table 4. *Effects of trifluoperazine on cyclic AMP concentrations in islet cells*

After preliminary incubation for 30 min in the presence of 3 mM-glucose without or with trifluoperazine, the islets were incubated for 10 or 60 min in the indicated conditions. Values are means ± S.E.M. for the numbers of batches of islets given in parentheses. Significance levels: *, $P<0.05$; **$P<0.001$, versus appropriate controls without glucose; †, $P<0.05$; ††, $P<0.01$; †††, $P<0.005$, versus appropriate controls in the absence of trifluoperazine.

Test conditions (mM)	Cyclic AMP (fmol/islet)	
	No trifluoperazine	Trifluoperazine (25 μM)
(*a*) Incubation for 10 min		
Glucose (0)	13.1 ± 0.7 (16)	16.8 ± 0.9 (16)†††
Glucose (10)	24.9 ± 1.8 (16)**	24.5 ± 2.8 (16)*
Glucose (0) + 3-isobutyl-1-methylxanthine (1)	105 ± 6 (11)**	102 ± 10 (11)**
(*b*) Incubation for 60 min		
Glucose (0)	12.5 ± 0.4 (11)	14.8 ± 0.7 (11)††
Glucose (10)	17.3 ± 1.1 (11)**	14.3 ± 0.6 (11)†

Table 5. *Effects of trifluoperazine on Ca^{2+} uptake by islet cells*

After preliminary incubation for 30 min, batches of eight islets were incubated for 5, 30 or 60 min at 37°C in 100 μl of medium layered on silicone oil. The concentration of glucose was the same during the pre-incubation and the incubation period and, when tested, trifluoperazine was present also during the pre-incubation period. The medium also contained 2.5 mM-$^{45}CaCl_2$ (sp. radioactivity 10 Ci/mol) and 0.25 mM-[6,6′-^{3}H]sucrose (sp. radioactivity 0.1 Ci/mmol). Values are means ± S.E.M. of 14 batches of islets in (*a*) and (*c*) and of 17 batches in (*b*). Significance levels: *, $P<0.005$, **, $P<0.001$, versus controls without trifluoperazine; †, $P<0.001$, versus glucose (0 mM).

Glucose concn. (mM)	Ca^{2+} uptake (pmol/islet)		
	No trifluoperazine	Trifluoperazine (25 μM)	Trifluoperazine (50 μM)
(*a*) Incubation for 5 min			
0	8.9 ± 0.5	8.7 ± 0.5	8.1 ± 0.5
10	15.9 ± 0.6†	14.3 ± 0.6†	12.5 ± 0.5**,†
(*b*) Incubation for 30 min			
0	11.4 ± 0.5	10.4 ± 0.6	—
6	14.4 ± 0.5†	15.5 ± 0.7†	—
10	24.0 ± 1.1†	23.0 ± 0.9†	—
(*c*) Incubation for 60 min			
0	14.9 ± 1.0	11.0 ± 0.4*	10.0 ± 0.3**
10	27.7 ± 1.2†	22.5 ± 0.8*,†	16.0 ± 0.7**,†

Effects of trifluoperazine and pimozide on Ca^{2+} fluxes

Table 5 shows that glucose (10 mM) stimulated Ca^{2+} influx (estimated by the 5 min uptake) in islet cells and that this effect was slightly decreased by 50 μM-, but not by 25 μM-trifluoperazine. After 30 min of incubation, Ca^{2+} uptake was increased by 6 or 10 mM-glucose. Trifluoperazine, at the concentration of 25 μM, did not interfere with this stimulation. After 60 min, however, both basal and glucose-stimulated Ca^{2+} uptake were decreased by trifluoperazine, already at the concentration of 25 μM.

Pimozide had very little effect on Ca^{2+} uptake by islet cells. After 60 min of incubation with 10 mM-glucose, control uptake (25.6 ± 1.0 pmol/islet, $n = 12$) was unaffected by 1 μM-pimozide (26.3 ± 1.2) and decreased by only 12% in the presence of 10 μM-pimozide (22.5 ± 1.0, $P<0.05$).

The effects of glucose and trifluoperazine on Ca^{2+} efflux from perifused islets are illustrated in Fig. 3. In the presence of extracellular Ca^{2+}, trifluoperazine produced a progressive decrease in the rate of $^{45}Ca^{2+}$ efflux; it also markedly and irreversibly inhibited the rise in efflux rate, which normally follows the addition of glucose to the medium (Fig. 3*a*). In the absence of extracellular Ca^{2+}, trifluoperazine also decreased the rate of $^{45}Ca^{2+}$ efflux, but did not prevent nor did it alter the further inhibition caused by the subsequent addition of glucose to the medium (Fig. 3*b*).

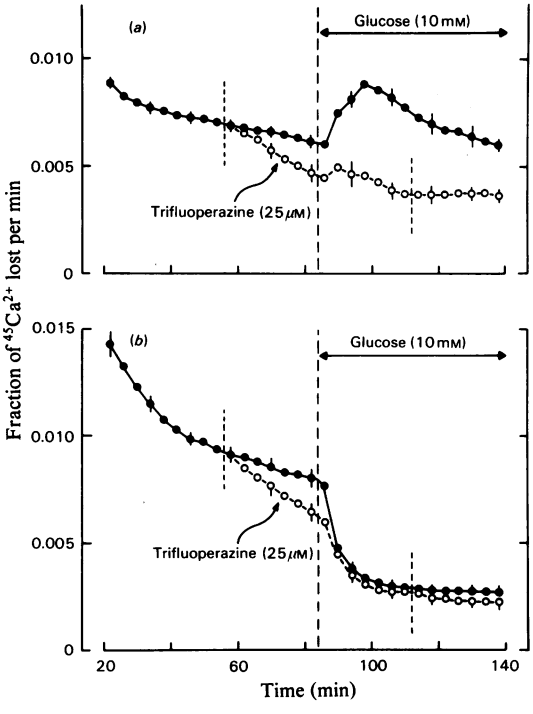

Fig. 3. *Effects of trifluoperazine on $^{45}Ca^{2+}$ efflux from perifused rat islets*

The experiments were started in the absence of glucose, which was added, at the concentration of 10mM, at 84min and maintained until the end. Trifluoperazine (25μM) was added at 56min and withdrawn at 112min (○). (*a*) The medium contained 2.5mM-Ca^{2+}. (*b*) Ca^{2+}-free medium. Values are means ± S.D. for four experiments in each group.

Effects of trifluoperazine on Rb^+ fluxes

In the absence of glucose, the rate of $^{86}Rb^+$ efflux from perifused islets declined slowly and regularly (Fig. 4*a*, ····). Addition of trifluoperazine (50μM) to the medium resulted in a progressive decrease in the efflux rate. This effect was similar in the presence or in the absence of Ca^{2+} (Fig. 4*a*), but was not reversible on withdrawal of trifluoperazine (not illustrated).

In control islets, glucose (10mM) rapidly, markedly and reversibly decreased the rate of $^{86}Rb^+$ efflux (Fig. 4*b*). An initial lowering of the rate of efflux by trifluoperazine (25μM) did not prevent the subsequent addition of glucose from decreasing it further. Although the mean fractional efflux rate between 60 and 70min was lower ($P<0.05$, $n=4$) in the simultaneous presence of trifluoperazine and glucose (0.0128 ± 0.0003) than in the sole presence of glucose (0.0138 ± 0.0002), both effects were clearly not additive.

In the presence of extracellular Ca^{2+}, ionophore A23187 markedly increases the efflux rate of $^{86}Rb^+$ from islet cells (Henquin, 1979). Trifluoperazine (25μM) progressively inhibited this effect without decreasing the uptake of Ca^{2+} brought about by the ionophore (results not shown).

To evaluate the influx and the net uptake of K^+ by islet cells, the uptake of the tracer $^{86}Rb^+$ was measured after 10 and 60min respectively. After 10min of incubation in the absence of glucose, K^+ influx was decreased from 225 ± 7pmol/islet to 172 ± 5pmol/islet ($P<0.001$, $n=24$) by 25μM-trifluoperazine. The net uptake of K^+ was not different, however, in control islets (403 ± 13pmol/islet) and in trifluoperazine-treated islets (398 ± 13pmol/islet, $n=24$), likely because the decrease in influx was compensated for by the fall in efflux rate also brought about by trifluoperazine (Fig. 4). Glucose (10mM) increased K^+ net uptake in control islets (632 ± 17pmol/islet, $P<0.001$, $n=24$) as well as in the presence of trifluoperazine (623 ± 25pmol/islet).

Discussion

Experiments in cell-free systems have shown that trifluoperazine, pimozide and other antipsychotic agents bind to calmodulin in a Ca^{2+}-dependent fashion and that this binding prevents the activation of several enzymes by the regulatory protein (Weiss & Levin, 1978). Calmodulin is present in islet cells (Sugden *et al.*, 1979; Valverde *et al.*, 1979). In insulinoma and islet extracts, trifluoperazine inhibits Ca^{2+}-dependent protein phosphorylation (Shubart *et al.*, 1980; Gagliardino *et al.*, 1980). It also prevents activation of Ca^{2+}-dependent cyclic AMP phosphodiesterase by islet extracts (Sugden *et al.*, 1979).

The effects of trifluoperazine and pimozide on the functioning of intact islets have thus been studied, with the hope that they would point to stages in the stimulus–secretion coupling of insulin release that depend on calmodulin. However, alternative or unspecific effects of these agents were also considered and possibly ruled out.

Phenothiazines and pimozide are powerful antagonists of dopamine and phenothiazines also inhibit α-adrenergic receptors (Baldessarini, 1980). These properties cannot explain their effects on insulin release as dopamine and α-adrenergic agonists themselves are inhibitors in this (J.-C. Henquin, unpublished work) and other systems (Smith & Porte, 1976). In certain tissues, pimozide enhances noradrenaline release from nerve terminals (Hope *et al.*, 1977); such an effect is unlikely to mediate the inhibition of insulin release by pimozide, as it is not antagonized by phentolamine. These neuroleptic drugs are fat-soluble and surface-active agents that

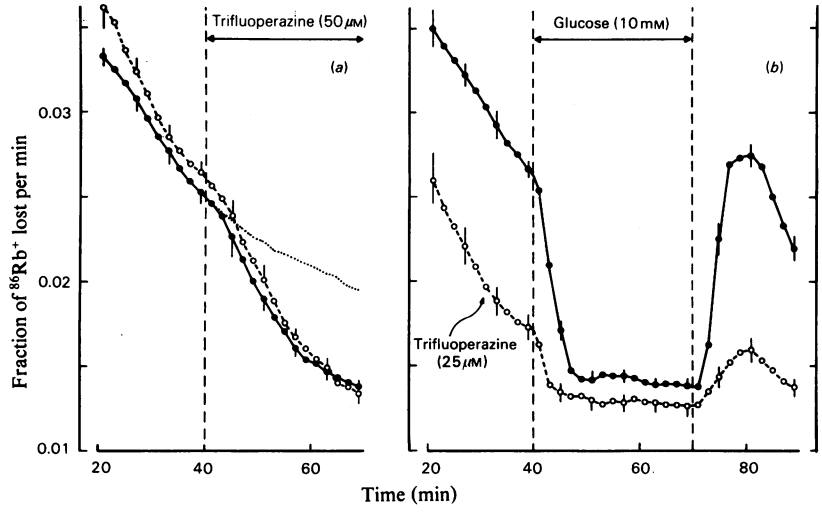

Fig. 4. *Effects of trifluoperazine on $^{86}Rb^+$ efflux from perifused rat islets*

(*a*) The experiments were carried out in the absence of glucose and in the absence (○) or in the presence (●) of 2.5 mM-$CaCl_2$. Trifluoperazine (50 μM) was added at 40 min as indicated. ····, control experiments in the presence of Ca^{2+} and absence of trifluoperazine. Values are means ± S.D. of three experiments. (*b*) The experiments were started in the absence of glucose, which was added, at the concentration of 10 mM, at 40 min and withdrawn at 70 min. In one series of experiments (○), trifluoperazine (25 μM) was present throughout. Values are means ± S.D. for four experiments.

readily adsorb on to plasma membranes, where they displace bound Ca^{2+} and induce electrical stabilization (Seeman, 1972). They can, however, be taken up by various types of cells (Ilundain & Naftalin, 1979; Ohmiya & Mehendale, 1979; Salhab & Dujovne, 1979; Laduron *et al.*, 1978) and may thus also exert intracellular effects.

Trifluoperazine produced changes in ionic fluxes, which may reflect a membrane action of the drug. First, it decreased both influx and efflux of $^{86}Rb^+$, most likely by modifying the passive permeability to K^+. Thus, blockade of the Na^+/K^+ pump by ouabain does not decrease K^+ efflux (J.-C. Henquin, unpublished work) and, in contrast with trifluoperazine, lowers the net uptake of K^+ by islet cells (Henquin, 1980). Such a decrease in K^+ permeability is expected to increase insulin release (Henquin *et al.*, 1979) and thus cannot be the cause of the inhibition observed here. Secondly, trifluoperazine could, like chlorpromazine in other tissues (Seeman, 1972), also inhibit Na^+ influx. Such an effect would not seem sufficient to account, alone, for the marked inhibition of insulin release. Thus, tetrodotoxin, a more potent and specific blocker of Na^+ channels, does not affect insulin secretion in our system (J.-C. Henquin, unpublished work). Thirdly, at the concentration of 25 μM, trifluoperazine did not produce any displacement of membrane-bound Ca^{2+} that could be detected by a rise in $^{45}Ca^{2+}$ efflux. On the contrary, it decreased the efflux rate. Preincubation for 30 min with the drug also failed to impair Ca^{2+} influx (5 min uptake) and Ca^{2+} net uptake was decreased only slightly after 60 min of incubation. That trifluoperazine does not act as a 'Ca^{2+}-antagonist' is supported further by the observation that it can inhibit K^+- or ouabain-induced insulin release without decreasing Ca^{2+} uptake (Shubart *et al.*, 1980). A similar dissociation between insulin release and Ca^{2+} uptake was observed with pimozide.

At the concentration of 25 μM, trifluoperazine did not cause any obvious damage in islet cells, although its effects appeared to be irreversible. Several observations suggest that it did not interfere with the recognition of glucose by B-cells; it did not impair glucose utilization (the present study; Gagliardino *et al.*, 1980) or oxidation (Sugden *et al.*, 1979) by the islets and also inhibited the release of insulin triggered by glyceraldehyde; the presence of the drug did not prevent the sugar from exerting its normal effects on Rb^+ and Ca^{2+} fluxes. It appears, therefore, that the initial sequence of events (glucose metabolism, decrease in K^+ permeability in B-cells, influx of Ca^{2+}) is not altered by trifluoperazine.

As in many tissues (Wolff & Brostrom, 1979), calmodulin activates a Ca^{2+}-dependent adenylate cyclase in rat islets (Valverde *et al.*, 1979; Sharp *et al.*, 1980). This may explain the Ca^{2+}-dependent

rise in cyclic AMP concentrations triggered by glucose in islet cells. Unexpectedly, trifluoperazine slightly increased cyclic AMP concentrations in islets incubated without glucose and did not impair the stimulation by the sugar after short incubation; it suppressed it only after 60min, when the change in cyclic AMP in control islets was also much smaller. Obviously the net effect of trifluoperazine depends on a balance between inactivation of synthesis and of degradation of the nucleotide. Depending on the brain area, phenothiazines have been found to decrease or to increase the intracellular concentration of cyclic AMP (for references, see Levin & Weiss, 1977). Anyhow, given the secondary role currently ascribed to cyclic AMP in the stimulation of insulin release (Sharp, 1979), the present results permit the conclusion that the effect of trifluoperazine is not primarily due to alteration of cyclic AMP formation.

The data discussed so far and the evidence that trifluoperazine and pimozide also inhibit the release induced by metabolized substrates unrelated to glucose or by non-metabolized agents like sulphonylureas (the present study; Gagliardino *et al.*, 1980) or K^+ and ouabain (Shubart *et al.*, 1980) suggest that its effects are probably due to an interference with late steps of the stimulus–secretion coupling. This would also explain their inhibition of barium-induced insulin release; thus the cation is thought to substitute for Ca^{2+} in activating the releasing machinery (Somers *et al.*, 1976). As calmodulin seems to be involved in the activation of actomyosin ATPase in non-muscle cells and in the assembly–disassembly of microtubules (Means & Dedman, 1980), the microtubular–microfilamentous system of the B-cells is a potential target for trifluoperazine. In this respect, it is noteworthy that cytochalasin B partially restored the release inhibited by trifluoperazine. The effect of the drug could also be due to the inhibition of Ca^{2+}–calmodulin-dependent phosphorylation of islet proteins (Shubart *et al.*, 1980; Gagliardino *et al.*, 1980), but the role of these phosphorylations in the releasing process remains to be clarified. Finally, the exocytotic process itself could be activated by calmodulin. The two latter possibilities have been considered in studies with other secretory tissues (De Lorenzo *et al.*, 1979; Baker & Whittaker, 1980; Baker & Knight, 1980). Anyhow, all of them meet with the problem of explaining the effects of methylxanthines. The present study and one previous report (Krausz *et al.*, 1980) show that glucose-stimulated release is not inhibited by trifluoperazine or pimozide in the presence of theophylline or 3-isobutyl-1-methylxanthine; they also show that high concentrations of these methylxanthines remain able to stimulate insulin secretion in the presence of the drugs. At first sight, these observations rule out an effect of trifluoperazine at the exocytotic site. It is possible, however, that the considerable increase in cyclic AMP produced by these agents and/or their direct mobilization of bound Ca^{2+} (Sehlin, 1976; Sugden & Ashcroft, 1978) antagonise or compensate for the inactivation of calmodulin by the inhibitors.

Two other effects of trifluoperazine deserve a short comment. First, the drug decreased the basal rate of Ca^{2+} efflux from islet cells in the presence and in the absence of extracellular Ca^{2+}, without interfering with the influx of the cation. This effect could be due to inhibition of a Na^+/Ca^{2+} exchange, but also raises the possibility that, as in erythrocytes (Vincenzi & Larsen, 1980), a calmodulin-activated system (Ca^{2+}-dependent ATPase) participates in the extrusion of Ca^{2+} from islet cells. Evidence for the existence of such a system has been published recently (Pershadsingh *et al.*, 1980). Secondly, trifluoperazine decreased the activation of the Ca^{2+}-sensitive K^+ permeability present in islet cells (Henquin, 1979). This effect could obviously be due to a direct action of the drug on the K^+ channels. However, a recent report (Lassen *et al.*, 1980) suggests that, in erythrocytes, Ca^{2+} control of the K^+ permeability may be mediated by calmodulin. Such an interpretation would also be consistent with the present findings in islet cells.

In conclusion, trifluoperazine and pimozide inhibit insulin release without interfering with the known key events of the stimulus–secretion coupling. None of their studied effects can satisfactorily explain the inhibition of secretion. Their exact mechanism of action can only be speculated on. If, in intact tissues as in tissue extracts, they inactivate the Ca^{2+}–calmodulin complex, the present study suggests possible roles for the regulatory protein in the B-cell functioning. It does not seem, however, that the step(s) of the secretory sequence sensitive to calmodulin can be defined further with the use of these antipsychotic agents on intact islets.

I thank M. Nenquin and F. Mathot for skilled assistance and M. Nenquin for editorial help. This investigation was supported by grant 3.4509.75 from the Fonds de la Recherche Scientifique Médicale, Brussels, and a grant-in-aid from Hoechst-Belgium S.A.

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