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DANTROLENE-INDUCED INHIBITION OF INSULIN RELEASE

A MECHANISM INDEPENDENT OF EFFECTS ON CALCIUM FLUXES

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Abstract—Dantrolene is felt to inhibit the release of Ca^{2+} from vesicular stores but only in response to certain stimuli; the mechanism responsible for its effects is unclear. Since our recent studies implicated arachidonic acid and other polyunsaturated fatty acids in Ca^{2+} mobilization and insulin release from pancreatic islets, we have now examined the effect of dantrolene on fatty acid-induced $^{45}Ca^{2+}$ efflux and insulin release. Dantrolene inhibited insulin secretion induced by exogenous unsaturated fatty acids as well as that caused by endogenous fatty acids (generated via the exogenous provision of pancreatic phospholipase A_2, or by *p*-hydroxymercuribenzoic acid, which prevents the reacylation of free fatty acids). In contrast, the effects of 50 mM K^+, 2 mM $BaCl_2$, 1 mM isobutylmethylxanthine or lysophosphatidylcholine were not impaired, suggesting that dantrolene does not inhibit nonspecifically the influx, mobilization or cellular effects of Ca^{2+}, or poison exocytosis in general. However, dantrolene did reduce insulin secretion triggered by 12-*O*-tetradecanoylphorbol-13-acetate, mezerein or exogenous phospholipase C, all of which can activate protein kinase C; this inhibition was not accompanied by alterations in $^{45}Ca^{2+}$ efflux. Furthermore, the $^{45}Ca^{2+}$ efflux induced by phospholipase A_2 or *p*-hydroxymercuribenzoic acid was not reduced by dantrolene. We conclude that the insulin secretion stimulated by unsaturated fatty acids involves two effects (one on Ca^{2+} fluxes, and one independent of Ca^{2+} mobilization). Dantrolene, in turn, may selectively probe such fatty acid-dependent insulin release; its inhibitory effect is predominantly, if not totally, independent of effects on Ca^{2+} fluxes, and may involve the inhibition of the effects of protein kinase C on exocytosis.

Ca^{2+} is widely accepted to be a critical factor in stimulus–secretion coupling in the pancreatic β cell; however, the control of Ca^{2+} fluxes in the islet is multifactorial and involves both intra- and extracellular sources. Recently, attention has been focused on two probable mediators of the release of intracellular Ca^{2+} stores: *myo*-inositol 1,4,5-trisphosphate (IP_3)† [1] and arachidonic acid (AA) [1, 2, ‡]. We observed that dantrolene (1-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin) could reduce AA-induced $^{45}Ca^{2+}$ efflux from intact rat islets and inhibit the concomitant insulin release [2, ‡, §]. The exact mechanism of action of dantrolene is unclear. It is widely felt to inhibit Ca^{2+} release from sarcoplasmic reticulum [3] or from endoplasmic reticulum [4] perhaps by blocking "the movement of a natural calcium ionophore" [3]. However, it does not impede the release of vesicular Ca^{2+} induced by all stimuli. For example, Ca^{2+}- or IP_3-induced Ca^{2+} release from islets [5, 6] and from other tissues [7–10] appears to be unresponsive to dantrolene. In view of the inhibitory effects of dantrolene on AA-induced effects in the pancreatic islet, we considered the possibility that this drug might be a specific probe of the release of Ca^{2+} stores and/or of insulin secretion induced by polyunsaturated fatty acids such as AA. To test that hypothesis further, we have examined the effect of dantrolene on $^{45}Ca^{2+}$ efflux and insulin release induced by free fatty acids (FFA) as well as by pharmacologic agents having relatively circumscribed mechanisms of action. These studies were carried out at the substimulatory glucose concentration of 1.7 mM in order to obviate possible effects of dantrolene on the potentiating effects of glucose [4].

MATERIALS AND METHODS

The methods used for the isolation of intact islets of Langerhans from fed male Sprague–Dawley rats have been described in detail, as have the procedures

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† Abbreviations: IP_3, *myo*-inositol 1,4,5-trisphosphate; PHMB, *p*-hydroxymercuribenzoic acid; PLA_2, phospholipase A_2; PLC, phospholipase C; lyso-PC, lysophosphatidylcholine; FFA, free fatty acid; AA, arachidonic acid; TFP, trifluoperazine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; IBMX, isobutylmethylxanthine; RIA, radioimmunoasay; and EGTA, ethyleneglycolbis(aminoethylether)tetra-acetate.

‡ S. A. Metz, *Sixty-ninth Meeting of the Endocrine Society*, Abstr. 255, p. 85 (1987).

§ S. Metz, manuscript submitted for publication.

used to study insulin release during batch-type, static 30-min incubations of freshly-isolated islets [11–14]. For studies of $^{45}Ca^{2+}$ efflux, islets were prelabeled to isotopic equilibrium [15, 16] overnight (×18–20 hr) in 100–125 μCi/ml $^{45}Ca^{2+}$, using previously described materials and techniques [14]. Except where indicated, the glucose concentration during incubations was 1.7 mM.

The phospholipase A_2 (PLA_2) was from porcine pancreas (Sigma). Arachidonic acid, docosahexaenoic and α-linolenic acids were from Nu-Chek Prep (Elysian, MN). Eicosapentaenoic acid was from Aldrich (Milwaukee, WI). Phospholipase C (PLC) was from *Clostridium perfringens* (chromatographically purified; type XII; Sigma). Trifluoperazine (TFP), *p*-hydroxymercuribenzoic acid (PHMB), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), mezerein and 4α-phorbol 12,13-didecanoate were from Sigma. The phospholipids used were: egg lysophosphatidylcholine (lyso-PC; Avanti-Polar Lipids, Birmingham, AL) and synthetic 1-oleoyl-lyso-PC, or lyso-PC from soybeans (Sigma). Lyso-PC was made up in stock solutions of chloroform, chloroform–methanol (1:1), or 0.1 N NaOH. Two to five microliters of each stock solution (or its diluent) was added to experimental tubes; organic solvents were then allowed to evaporate (by air or use of an argon stream) and test compounds were vortexed into solution in 0.75 to 1.0 ml Krebs–Ringer bicarbonate buffer with a composition as previously described [11–14]. Stock solutions of dantrolene (a gift of Norwich-Eaton Pharmaceuticals, Norwich, NY) were made up in dimethyl sulfoxide (DMSO); 2–5 μl samples of these stock solutions (or equal amounts of DMSO, alone, in all control tubes) were added to the incubation medium. Dantrolene (or trifluoperazine) was present during both preincubation period (24 min for $^{45}Ca^{2+}$ efflux; 30 min for insulin release) as well as the actual experimental (incubation) period (18 min for $^{45}Ca^{2+}$ efflux; 30 min for insulin secretion). A concentration of 100 μM dantrolene was used throughout these studies, as was used in the studies of Janjic *et al.* [4], which also involved pancreatic islets. This concentration is probably at or above the maximal solubility of the drug in aqueous medium. Our preliminary studies had indicated that this concentration of dantrolene inhibits $^{45}Ca^{2+}$ efflux stimulated by 33 μM arachidonic acid by 51% and reduces the associated insulin release by 65% [2]. Dantrolene does not have obvious toxic effects on the islets at this concentration, as assessed by the absence of consistent alterations in basal $^{45}Ca^{2+}$ efflux or basal insulin release, and only very selective effects on stimulated $^{45}Ca^{2+}$ efflux or exocytotic insulin release (see Results).

Insulin was measured by RIA as previously described [11, 12]; 100 μM dantrolene, when added to insulin standards, did not alter nonspecific binding, the slope or shape of the standard curve, or the recovery of insulin (recovery of 10 through 1225 μU added insulin = 95 ± 9%, N = 10 concentrations of insulin).

Results are expressed as μU insulin per 10 islets per 30 min for insulin release, and percent of the last two basal (reference) values for $^{45}Ca^{2+}$ efflux, as has been described [14]. Data are reported as mean ± SEM where (N) = number of observations (i.e. batches of islets derived from a sample population of islets obtained from four to eight rat pancreata). Statistical analyses were carried out by non-paired *t*-testing for single comparisons, or by analysis of variance (ANOVA) where multiple comparisons were required. If ANOVA revealed an overall treatment effect, contrast analysis was performed at individual time points using one-way ANOVA followed by Duncan's multiple range test. A value of $P < 0.05$ was considered significant.

RESULTS

Insulin release. We have reported previously that unsaturated fatty acids stimulate non-toxic, insulin release when provided in Ca^{2+}-free medium (plus 0.2–0.6 mM EGTA); dantrolene inhibited the insulin release induced by 33 μM AA by 65% in these studies [2]. We have reconfirmed this effect for the current studies: dantrolene blocked the effects of 82 μM AA by 69 ± 7% (df 8; $P < 0.01$). The stimulatory effect of exogenous arachidonic acid is greater than that of α-linolenic or docosahexaenoic acid; however, the insulin release induced by the latter fatty acids was also reduced (by 86 ± 4 and 71 ± 3%, respectively, df = 14 each; $P < 0.001$) by dantrolene (Table 1, Expt. 1). Eicosapentaenoic acid (49 μM) also significantly augmented insulin release (df 12; $P < 0.02$), an effect inhibitable by dantrolene (−73 ± 14%, df = 14, $P < 0.05$).

To stimulate the accumulation of free fatty acids endogenously, phospholipase A_2 was provided [11]. This led to dose-dependent insulin release (300–3000 mU/ml) which was largely, albeit not totally, inhibited by dantrolene ($P < 0.001$ by ANOVA and Duncan's multiple range test; Fig. 1). The organic mercurial *p*-hydroxymercuribenzoic acid (PHMB) also leads to the accumulation of endogenous, unesterified arachidonate (and probably other fatty acids) by inhibiting the re-esterification of fatty acids with lysophospholipids [17, 18]; as previously reported [13, 19], PHMB (100–200 μM) also induces insulin release. PHMB-induced secretion was partially (30–52%; df 18 and 8, respectively) reduced by dantrolene (Table 1; Expts. 2 and 3). Both exogenous phospholipase A_2 and PHMB can also induce the concomitant accumulation of lysophospholipids in islets [11, 17]. However, at insulinotropic concentrations (50–75 μg/ml), the effect of exogenous lysophosphatidylcholine was totally resistant to inhibition by dantrolene (Table 1, Expt. 3). At higher concentrations (150–200 μg/ml), lyso-PC-induced insulin release, whether induced by egg lyso-PC (containing mixed fatty acid residues) or synthetic oleoyl-lyso-PC, was only slightly and inconsistently blocked by dantrolene (Table 1, Expts. 4 and 5). Overall, this effect was not statistically significant ($\bar{x} = -28 \pm 10\%$; N = 5 separate experiments).

To confirm the findings using dantrolene, a second probe (trifluoperazine; TFP) was used. TFP also inhibits arachidonate-induced Ca^{2+} mobilization and insulin release [2, *]. It blunted the effects of 2000 mU/ml phospholipase A_2 by 69% (df 10,

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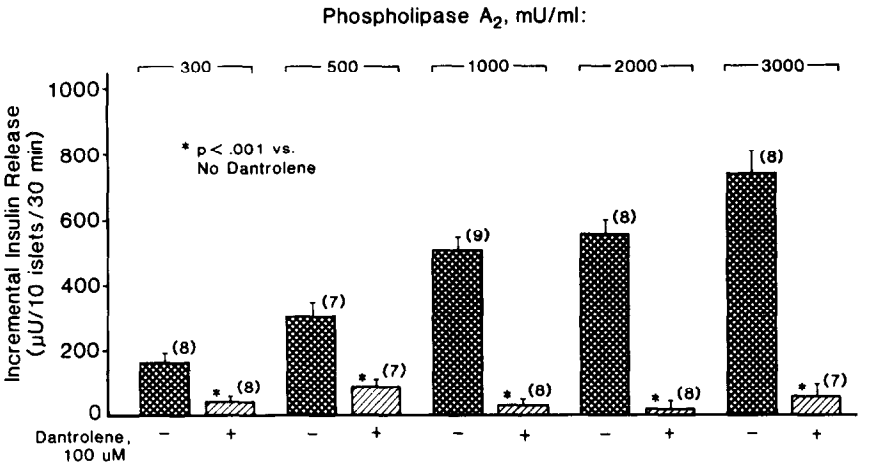

Fig. 1. Effect of dantrolene (100 μM) on increasing concentrations of porcine pancreatic phospholipase A_2. The individual studies were performed on three separate days. Data are expressed as mean ± SEM for incremental insulin release (i.e. above basal release rates).

$P < 0.001$; Table 1, Expt. 6) and of 3000 mU/ml (by 42 ± 7%; df 16; $P < 0.001$). TFP, like dantrolene, had no inhibitory effect on insulin release induced by 60 μg/ml lyso-PC (not shown) but had a slight effect on that due to 150 μg/ml lyso-PC (df 9; $P < 0.01$; Table 1, Expt. 6).

To examine the possibility that dantrolene is a non-specific Ca^{2+} antagonist, or a generalized poison of Ca^{2+}-dependent exocytosis, the effects of 50 mM K^+, 2 mM $BaCl_2$ (in Ca^{2+}-free medium) and 1 mM isobutylmethylxanthine (IBMX) were examined. The former triggers Ca^{2+} influx from the extracellular space, whereas Ba^{2+} selectivity mobilizes Ca^{2+} from extracellular stores; IBMX has both actions [14]. Dantrolene did not reduce the insulin release induced by any of the three (Table 1, Expts. 7–9). To examine the possibility that dantrolene might block some unrecognized effect of fatty acids or of phospholipases to disorder membranes, a known detergent (digitonin) was provided, likewise in Ca^{2+}-free medium; its effect also was resistant to dantrolene (Table 1, Expt. 10).

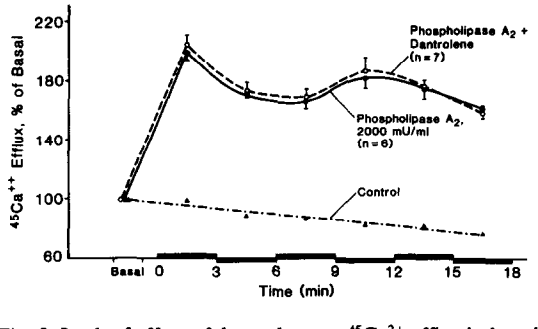

Fig. 2. Lack of effect of dantrolene on $^{45}Ca^{2+}$ efflux induced by 2000 mU/ml phospholipase A_2. Data are mean ± SEM for the number of observations in parentheses. Absolute basal values (cpm/100 islets/3 min) were: control (8850± 263); phospholipase A_2 alone (8300 ± 382); and phospholipase A_2 plus dantrolene (7610 ± 201).

To study further the specificity and mechanisms of action of dantrolene, we studied two agents that can activate protein kinase C: exogenous phospholipase C, which cleaves phospholipids to yield diacylglycerol [20, 21] the endogenous activator of protein kinase C, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a direct activator of protein kinase C. At 125 mU/ml (df 14; $P < 0.001$; Table 1, Expt. 11) or 75 mU/ml (data not shown) of phospholipase C, the resultant insulin release was nearly totally blocked by dantrolene. TPA-induced insulin release was also reduced 49% by dantrolene (df 14; $P < 0.001$; Table 1, Expt. 12). A non-phorbol activator of protein kinase C (mezerein) also augmented insulin release; this effect was reduced 41% by dantrolene (df 22; $P < 0.01$; Table 1, Expt. 13). A phorbol which does not bind to, or activate, protein kinase C (4α-phorbol 12,13-didecanoate, 2 μM) did not stimulate insulin release.

$^{45}Ca^{2+}$ Efflux. Since the activation of protein kinase C is felt to stimulate insulin release in a fashion largely independent of changes in Ca^{2+} fluxes or increases in cytosolic Ca^{2+} concentration [22, 23] these data with phsopholipase C and TPA suggested a second, Ca^{2+}-independent effect of dantrolene on secretion. Therefore, the efflux of $^{45}Ca^{2+}$ from prelabeled islets was studied. Both phospholipase A_2 and PHMB promoted rapid $^{45}Ca^{2+}$ efflux; this effect tended to be more rapid in onset and more sustained after phospholipase A_2 treatment (Figs. 2 and 3). However, the effects of both of these agonists on Ca^{2+} efflux were completely unaffected by dantrolene (Figs. 2 and 3). Furthermore, phospholipase C (125 mU/ml) and TPA (2 μM) induced only a very small and delayed efflux of $^{45}Ca^{2+}$ (Table 2); even this minor effect was unchanged by dantrolene (Table 2, Expts. 1 and 2). In fact, TPA-stimulated Ca^{2+} efflux tended to be greater in the presence of dantrolene than in its absence ($P < 0.05$ by ANOVA). At a 4-fold higher concentration (500 mU/ml), phospholipase C elicited greater Ca^{2+} efflux, although this was still modest (Table 2, Expt. 3). This increment

Table 1. Insulin release induced by various agonists and its response to dantrolene or trifluoperazine

Condition	Insulin (μU/10 islets/30 min)		Comments
Expt. 1 (Ca^{2+}-free medium plus 0.6 mM EGTA)			
a. Control	204 ± 45	(3)	
b. Dantrolene, 100 μM	113 ± 13	(2)	
c. α-Linolenic acid, 49 μM	617 ± 79	(8)	
d. α-Linolenic acid, 49 μM + dantrolene	169 ± 16	(8)	Incremental response (d − b) reduced 86 ± 4% vs control (c − a), df 14, $P < 0.001$
e. Docosahexaenoic acid, 49 μM	830 ± 88	(8)	
f. Docasehexaenoic acid, 49 μM + dantrolene	294 ± 19	(8)	Incremental response (f − b) reduced 71 ± 3% vs control (e − a); df 14, $P < 0.001$
Expt. 2			
a. Control	61 ± 6	(6)	
b. Dantrolene, 100 μM	70 ± 6	(5)	
c. PHMB, 200 μM	280 ± 12	(9)	
d. PHMB + dantrolene, 100 μM	222 ± 9	(11)	Incremental response (d − b) reduced 30 ± 4% vs control (c − a); df 18, $P = 0.001$
Expt. 3			
a. Control	124 ± 14	(3)	
b. Lyso-PC (egg), 50 μg/ml	391 ± 69	(6)	
c. Lyso-PC, 50 μg/ml + dantrolene, 100 μM	540 + 116	(6)	
d. Lyso-PC, 75 μg/ml	743 ± 141	(6)	
e. Lyso-PC, 75 μg/ml + dantrolene, 100 μM	662 ± 147	(6)	−13 ± 24% (df 10, P = NS) vs (d)
f. PHMB, 100 μM	326 ± 29	(5)	
g. PHMB, 100 μM + dantrolene, 100 μM	221 ± 6	(5)	−52 ± 3% vs (f), df 8, $P < 0.01$
Expt. 4			
a. Control	50 ± 12	(3)	
b. Lyso-PC (egg), 150 μg/ml	1286 ± 106	(6)	
c. Lyso-PC, 150 μg/ml + dantrolene, 100 μM	995 ± 103	(6)	−23 ± 8% vs (b); df 10, P = NS
Expt. 5			
a. Control	138 ± 21	(4)	
b. Oleoyl-lyso-PC, 150 μg/ml	1562 ± 202	(8)	
c. Oleoyl-lyso-PC, 150 μg/ml + dantrolene, 100 μM	944 ± 124	(8)	−43 ± 9% vs (b); df 14, $P < 0.05$
d. Oleoyl-lyso-PC, 200 μg/ml	1863 ± 170	(9)	
e. Oleoyl-lyso-PC, 200 μg/ml + dantrolene, 100 μM	1764 ± 139	(9)	−6 ± 8% vs (d); df 16, P = NS
Expt. 6			
a. Control	88 ± 5	(4)	
b. Trifluoperazine (TFP), 50 μM	169 ± 16	(4)	
c. Egg lyso-PC, 150 μg/ml	1805 ± 65	(6)	
d. Egg lyso-PC, 150 μg/ml + TFP	1436 ± 114	(5)	Incremental response (d − b) reduced 26 ± 7% vs control (c − a); df 9, $P < 0.01$
e. PLA_2, 2000 mU/ml	484 ± 36	(6)	
f. PLA_2, 2000 mU/ml + TFP	294 ± 24	(6)	Incremental response (f − b) reduced 69 ± 6% vs control (e − a); df 10, $P < 0.001$
Expt. 7			
a. Control	103 ± 16	(6)	
b. Dantrolene, 100 μM	99 ± 20	(6)	
c. 2 mM $BaCl_2$	381 ± 26	(5)	
d. $BaCl_2$ + dantrolene	474 ± 28	(5)	
Expt. 8			
a. Control	134 ± 19	(3)	
b. Dantrolene, 100 μM	104 ± 3	(2)	
c. IBMX, 1 mM	240 ± 11	(5)	
d. IBMX + dantrolene	217 ± 9	(5)	Incremental response (d − b) not significantly different from control response (c − a); df 8

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Table 1—*continued*

Condition	Insulin (μU/10 islets/30 min)		Comments
Expt. 9			
a. Control	154 ± 22	(5)	
b. 50 mM K^+	356 ± 26	(8)	
c. 50 mM K^+ + 100 μM dantrolene	428 ± 39	(8)	P = NS vs (b); df 14
Expt. 10 (Ca^{2+}-free medium; EGTA = 0.2 mM)			
a. Control	148 ± 10	(4)	
b. Digitonin, 20 μg/ml	365 ± 35	(5)	
c. Digitonin + dantrolene, 100 μM	440 ± 48	(4)	P = NS vs (b); df 7
Expt. 11			
a. Control	165 ± 28	(4)	
b. Phospholipase C, 125 mU/ml	624 ± 79	(8)	
c. Phospholipase C, 125 mU/ml + dantrolene	177 ± 30	(8)	−93 ± 6% vs (b); df 14, P < 0.001
Expt. 12			
a. Control	38 ± 4	(3)	
b. Dantrolene, 100 μM	42 ± 6	(4)	
c. TPA, 2 μM	646 ± 35	(8)	
d. TPA + dantrolene	348 ± 26	(8)	Incremental response (d − b) reduced 49 ± 4% vs (c − a); df 14, P < 0.001
Expt. 13			
a. Control	76 ± 17	(4)	
b. Dantrolene, 100 μM	50 ± 8	(4)	
c. Mezerein, 2 μM	431 ± 32	(12)	
d. Mezerein + dantrolene	261 ± 22	(12)	Incremental response (d − b) reduced 41 ± 6% (df 22; P < 0.01) compared to control (c − a)

All tubes contained 2.5 mM $CaCl_2$ (and no EGTA) unless otherwise stated. Data are expressed as mean ± SEM, where (N) = number of observations. Statistical analyses are by non-paired *t*-testing.

in Ca^{2+} efflux was reduced by dantrolene such that values returned to efflux rates similar to those seen at the lower concentration of phospholipase C. During this study, dantrolene reduced the areas under the curve (Table 2) for phospholipase C-induced Ca^{2+} efflux by 49 ± 5% (df 10; P < 0.01).

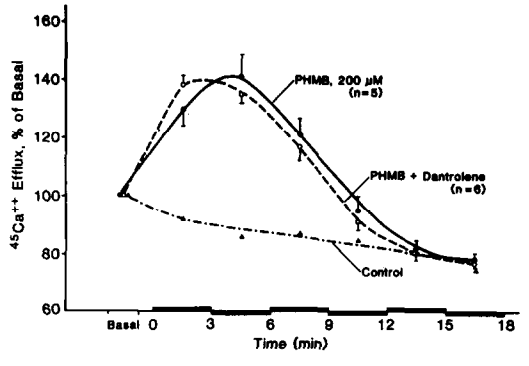

Fig. 3. Lack of effect of dantrolene on $^{45}Ca^{2+}$ efflux induced by 200 μM PHMB. Data are mean ± SEM for the number of observations in parentheses. Absolute basal values (cpm/100 islets/3 min) were: control (5470 ± 235); PHMB (6970 ± 692); and PHMB plus dantrolene (6170 ± 303).

DISCUSSION

Three probes were used to assess the effects of unesterified fatty acids in the islet: (1) exogenous provision; (2) exogenous phospholipase A_2, to release endogenous *sn*-2-position fatty acids from phospholipids; and (3) PHMB, to impede the reacylation of lysophospholipids with FFA [17, 18]. All three maneuvers yielded generally similar effects on $^{45}Ca^{2+}$ efflux and insulin release this paper, and Ref. 12 and 13). However, the temporal patterns of $^{45}Ca^{2+}$ efflux (and the degrees of inhibitability of the associated insulin secretion) seen with PHMB or phospholipase A_2 treatment were not identical. For example, the effects of PHMB on $^{45}Ca^{2+}$ efflux were less sustained, and PHMB-induced insulin release was less susceptible to inhibition by dantrolene. This is probably due to the fact that the action of PLA_2 is restricted to the hydrolysis of phospholipids of the outer plasmalemmal leaflet, whereas PHMB leads to the accumulation of endogenously generated lipid hydrolysis products derived largely from the cytoplasmic surface of the membrane. Therefore, the composition of these lipid products, and the rapidity of their release, would be expected to differ from those generated *de novo* by exogenous phospholipase A_2 (see discussion in Refs. 5, 11 and 17). Thus, phospholipase A_2 and PHMB

Table 2. Effects on $^{45}Ca^{2+}$ efflux of phospholipase C (PLC) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the presence or absence of dantrolene

		$^{45}Ca^{2+}$ Efflux*								
	Basal (=100%) (cpm/100 islets/3 min)	minutes 0–3	4–6	7–9	10–12	13–15	16–18	19–21	22–24	25–27
I. TPA										
a. Control (6)	(8040 ± 1070)	90 ± 2	84 ± 3	82 ± 2	82 ± 4	82 ± 4	76 ± 5	69 ± 6	61 ± 4	64 ± 3
†b. TPA,2 μM (8)	(8710 ± 539)	99 ± 4	98 ± 3‡	93 ± 2‡	94 ± 4	93 ± 3	93 ± 3‡	88 ± 2‡	87 ± 3‡	85 ± 2‡
c. TPA + dantrolene, 100 μM (7)	(7980 ± 328)	100 ± 4	96 ± 4	101 ± 4	98 ± 4	92 ± 3	94 ± 4	89 ± 4	91 ± 4	88 ± 4
II. Phospholipase C										
a. Control (6)	(6000 ± 493)	94 ± 1	90 ± 1	87 ± 1	83 ± 1	83 ± 2	80 ± 1	80 ± 1	74 ± 1	72 ± 2
†b. PLC, 125 mU/ml (14)	(6520 ± 300)	94 ± 1	92 ± 1	89 ± 1	89 ± 1‡	91 ± 1‡	84 ± 2	84 ± 1	83 ± 1‡	81 ± 2‡
c. PLC + dantrolene, 100 μM (14)	(5880 ± 213)	93 ± 1	91 ± 1	89 ± 1	89 ± 2	89 ± 2	84 ± 2	83 ± 2	84 ± 2	81 ± 2
III. Phospholipase C										
a. Control (5)	(5810 ± 248)	95 ± 2	87 ± 3	83 ± 4	87 ± 2	78 ± 2	77 ± 3	76 ± 4	68 ± 3	71 ± 2
†b. PLC, 500 mU/ml (6)	(6100 ± 439)	105 ± 3	110 ± 6‡	103 ± 4‡	108 ± 3‡	109 ± 4‡	113 ± 7‡	120 ± 6‡	111 ± 4‡	117 ± 8‡
†c. PLC + dantrolene, 100 μM (6)	(6080 ± 456)	101 ± 5	97 ± 2‡	98 ± 2	100 ± 2	96 ± 2‡	95 ± 3‡	95 ± 4‡	95 ± 4‡	91 ± 3‡

* Data are presented as percent of the mean of two basal values (=100%) immediately preceding the start of the incubation period. Values are mean ± SEM for the number of observations given in parentheses.

† $P < 0.001$ vs entire row immediately above (ANOVA).

‡ $P < 0.05$ or greater vs time point immediately above (Duncan's multiple range test).

are similar but not identical probes. In addition, it is possible that direct, non-specific effects of pharmacologic agents like PHMB (such as inhibition of Ca^{2+} extrusion mechanisms [24]) may have contributed to the observed results.

The data indicate that dantrolene, at the concentration used, considerably reduces the insulin release associated with the exogenous provision of unsaturated fatty acids, or with their endogenous accumulation. Similar effects on the insulin secretion induced by PLA_2 were seen using trifluoperazine which, while not specific in that effect, does reduce the effects in the islet of arachidonate on $^{45}Ca^{2+}$ efflux and insulin release [2]. Phospholipase A_2 [11] and PHMB [17, 18] can also generate lysophospholipids, which, like fatty acids, can initiate insulin release [11–13, 19, 25]. However, the effect of lysophospholipids was totally resistant to dantrolene and trifluoperazine at lower concentrations of the agonist, and remained largely resistant even at higher concentrations. Exogenously-provided lyso-PC traverses the membrane bilayer and is then catabolized in islets by a lysophospholipase to produce free fatty acids [17]; thus, at high concentrations of lyso-PC, it is likely that the small, variable inhibitory effects of dantrolene and trifluoperazine were due to blockade of the effects of some FFA released from the lyso-PC. We cannot, however, totally exclude an alternate possibility—namely, that at higher concentrations, some lyso-PC could cross the plasma membrane unmetabolized and directly release Ca^{2+} from intracellular organelles, and enhance insulin release in a dantrolene-sensitive fashion. It is likely that the dantrolene-*resistant* fraction of the insulin release induced by FFA, PHMB or PLA_2 was due to the concomitant production of lysophospholipids; however, we have not tested the alternative possibility that such secretion would be inhibited by higher concentrations of dantrolene and thus could be fatty acid-dependent.

In contrast, the effects on insulin release of agents which trigger the influx of extracellular Ca^{2+} or the mobilization of intracellular Ca^{2+} stores were not blocked by dantrolene; the effect of a detergent, digitonin, was also resistant to inhibition. Likewise, in the studies of Janjic *et al.* [4], dantrolene (at the same concentration) failed to reduce insulin release triggered by leucine, ouabain or arginine. Thus, dantrolene is not acting as a non-specific inhibitor of Ca^{2+}-activated process or as a universal poison of exocytosis, nor does it seem to act merely to protect the beta cell against the disordering of membranes by detergents such as fatty acids.

Interestingly, however, the effects of exogenous phospholipase C, and of TPA and mezerein, on insulin release also were blunted by dantrolene. One could ask whether these treatments also release unesterified fatty acids. Phospholipase C cleaves phospholipids to generate diacylglycerol; it is possible that the diacylglycerol, in turn, was hydrolyzed to yield fatty acids (via the combined actions of diglyceride and monoacylglycerol lipases). Exogenous phospholipase C has been shown to generate not only diacylglycerol [20] but also arachidonate or its metabolites [26] in some other cells. Alternatively, PLC (indirectly via the generation of diacylglycerol) or TPA and mezerein (directly) might activate protein kinase C, which in turn has been shown to activate phospholipase A_2 in many cells [27–30]. Indeed, the effects of TPA on insulin release are blunted by an inhibitor of phospholipase A_2 [14]. While we cannot exclude such a unitary hypothesis in the absence of direct measurements of FFA release, we feel that this is somewhat unlikely to be the major effect of TPA and PLC (at lower concentrations) since these agonists, unlike fatty acids, stimulated only minimal Ca^{2+} efflux. (Only at 500 mU/ml did PLC elicit Ca^{2+} efflux which could be blunted by dantrolene, suggesting some release of FFA at higher concentrations of PLC.) The minimal $^{45}Ca^{2+}$ efflux stimulated by 125 mU/ml phospholipase C also makes it unlikely that exogenous PLC hydrolyzed anionic phospholipids and, thereby, directly released sufficient stores of Ca^{2+} bound in the plasma membrane [31] to explain the concomitant insulin release. Furthermore, it is unlikely that PLC degraded anionic phospholipids or generated inositol trisphosphate (IP_3) since bacterial PLC, unlike mammalian PLC, preferentially acts on phosphatidylcholine, not upon inositol phosphoglycerides [32, 33]. For these reasons, we tentatively propose the alternative unitary hypothesis that TPA, PLC and fatty acids all stimulate insulin release (at least in part) via the activation of protein kinase C (or a closely related signalling system) and that dantrolene may inhibit this kinase or its effects on exocytosis. it is unlikely that the inhibitory effects of dantrolene on phospholipase A_2- or C-stimulated insulin release could be due simply to a direct inhibition of phospholipases since (1) the $^{45}Ca^{2+}$ efflux triggered by exogenous phospholipase A_2 or phospholipase C (low concentration) was *not* inhibited by dantrolene (in contrast to the insulin release); (2) a known islet phospholipase A_2 inhibitor (bromphenacyl bromide) does not inhibit the effects of *exogenous* PLA_2 in the islet unless the enzyme is directly preincubated with high concentrations of the inhibitor ([11, 12] and S. Metz, unpublished observations); and (3) dantrolene does not inhibit phospholipase C (as judged by IP_3 formation), at least in rat liver [34]. Furthermore, dantrolene inhibition of the effects of polyunsaturated fatty acids does not reflect possible actions on their metabolic fate (i.e. oxygenation or esterification into membranes) since the effects of FFA do not require such transformations [2].

Direct assessments of protein kinase C activity will be required to confirm or refute a role of fatty acids in the activation of protein kinase C. However, the current, as well as previous, studies of the effects of arachidonic acid and of dantrolene do lend some support to the formulation that they both have important actions aside from those on Ca^{2+} fluxes, and that these actions may involve protein kinase C. Thus, AA continues to elicit further progressive, non-toxic insulin release at concentrations >66 μM [2], whereas AA-induced Ca^{2+} mobilization has essentially reached a plateau by that concentration*. Furthermore, we have observed recently (unpublished observations) that AA (or TPA) promoted

* S. Metz, manuscript submitted for publication.

insulin release in digitonin-permeabilized islets where the ambient cytosolic Ca^{2+} concentration can be fixed through the use of Ca^{2+}–EGTA buffers; dantrolene potently inhibited such secretion whether initiated by AA or TPA. Furthermore, arachidonate has been shown to bind to, and activate, protein kinase C in many cells [35–38]. The near-total blockade of AA-induced insulin release [2] by a concentration of trifluoperazine (50 μM) which inhibits protein kinase C in the islet and elsewhere [39, 40] is in accord with such an event in the islet. In fact, cationic amphiphilic drugs such as TFP may inhibit protein kinase C more potentially when it has been activated by AA than when activated by phospholipid [36]. In addition, we previously presented [19] data which we interpreted as suggesting that AA may compete with TPA for binding to a common cellular receptor; such an effect has been attributed to activators of protein kinase C, such as oleoylacetylglycerol [41], diolein [42, 43] and indeed, in other cells, arachidonate [37, 44]. Further evidence that fatty acids have a second, dantrolene-sensitive mechanism of action can be seen in the current studies in which the insulin release induced by exogenous phospholipase A_2 or PHMB was blocked by dantrolene without any inhibition of $^{45}Ca^{2+}$ efflux. Thus, whatever the exact source of this $^{45}Ca^{2+}$ (presumably in part the co-generation of lysophospholipids), it is clear that dantrolene-induced inhibition of insulin release cannot be fully explained by changes in Ca^{2+} fluxes (at least insofar as they are reflected in Ca^{2+} efflux) and must involve a second mechanism. We cannot totally exclude the possibility that phospholipase A_2, PHMB and PLC displace a "trigger pool" of $^{45}Ca^{2+}$ in the plasma membrane which, via $^{45}Ca^{2+}$–$^{45}Ca^{2+}$ exchange or Ca^{2+}–Ca^{2+} release [9, 10, 45–47], leads to efflux of a small, compartmentalized "pool" of $^{45}Ca^{2+}$ in the endoplasmic reticulum which is tightly coupled to exocytosis. No, or little, inhibition of *net* $^{45}Ca^{2+}$ efflux would be detectable under these conditions. However, at least in other cells, Ca^{2+}–Ca^{2+} release is poorly, or not at all, inhibited by dantrolene [9, 10].

Thus, these data together suggest that AA elicits insulin release by (at least) two mechanisms—Ca^{2+} mobilization at lower concentrations, and an additional unidentified effect (perhaps at higher concentrations). Dantrolene may inhibit both processes [2]; however, the data using PLA_2 and PHMB suggest that the latter mechanism may be the more important site of dantrolene action under the conditions of these studies. There is precedent for an effect of dantrolene on hormone release apart from effects on Ca^{2+} fluxes. In zona glomerulosa cells, dantrolene inhibits aldosterone release stimulated by angiotensin II without inhibiting IP_3-induced Ca^{2+} mobilization (even at a 200 μM concentration of the drug) [8]. Dantrolene putatively could inhibit either the activation of protein kinase C, or its effects after stimulation by fatty acids, TPA or diacylglycerol. However, since the $^{45}Ca^{2+}$ efflux stimulated by TPA or PLC was not reduced by dantrolene, it is likely that dantrolene inhibits the *effects* of this enzyme on exocytosis, *not* the activation of the enzyme itself, at least to the extent that these probes have some specificity in activating protein kinase C. Further studies involving the measurements of protein kinase C activity will be required to validate or refute these tentative conclusions. Whatever the exact mechanism(s) of dantrolene's inhibitory effects, the current data nonetheless suggest that it may still serve as a relatively selective probe of fatty acid effects. This is in contradistinction to TMB-8 ([8-diethylamino]-octyl-3,4,5 trimethoxybenzoate hydrochloride), another putative probe of cellular Ca^{2+} mobilization, which blocks the effects of K^+, IBMX or Ba^{2+} on insulin release but not those of phospholipase A_2, PHMB and similar probes [14].

Lastly, these observations may have relevance to the syndrome of Malignant Hyperthermia [48]. This potentially lethal syndrome can be specifically treated by dantrolene [48]. Dantrolene selectively blocks the Ca^{2+} mobilized from sarcoplasmic reticulum by a provocative agent (halothane) in an animal model for this syndrome [10]. Recently, it has been proposed that Malignant Hyperthermia is associated with activation of phospholipase A_2 in muscle [49] and increases in cytosolic Ca^{2+} concentration [50]. These observations raise the intriguing possibility that the therapeutic effects of dantrolene may be mediated via inhibition of the effects of free fatty acids accumulating in muscle. Further studies seem warranted to test this hypothesis.

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