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LACK OF SPECIFICITY OF MELITTIN AS A PROBE FOR INSULIN RELEASE MEDIATED BY ENDOGENOUS PHOSPHOLIPASE A_2 OR LIPOXYGENASE

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Abstract—The effects of the basic polypeptide melittin on islet phospholipid degradation and insulin release were studied in static incubations of intact rat islets as a possible model of endogenous phospholipase A_2 (PLA_2) activation. Melittin (2 μg/ml) increased [^{3}H]-arachidonic acid ([^{3}H]-AA) release from prelabeled islets (at 1.7 mM glucose) to 371% of basal values. Concomitantly, melittin induced degradation of islet phospholipids labeled with [^{3}H]-AA or [^{14}C]-stearic acid, and led to the accumulation of stearate-labeled (but not AA-labeled) lysophosphatidylcholine (LPC, 605% of control). These findings suggested, for the first time in intact rat islets, the presence and activation of a PLA_2. Under identical conditions, melittin initiated insulin secretion (at 1.7 mM glucose) in a manner that represented stimulation of physiologic exocytosis—that is, it was dose-dependent, reversible (albeit slowly), unassociated with impairment of other physiologic islet processes (i.e. the response to 16.7 mM glucose after removal of the drug) and inhibitable by reduced ambient temperature. The effect of melittin seemed to be independent of extracellular Ca^{2+} influx or mobilization of intracellular Ca^{2+} stores but was blocked by nickel or lanthanum, indirectly suggesting that the effects of this cationic amphiphile might involve a superficial pool of Ca^{2+}. Unexpectedly, melittin-induced insulin release (at least at low glucose concentrations) was not greatly or consistently altered by a battery of inhibitors of endogenous PLA_2 or of enzymes affecting AA oxygenation. Furthermore, significant contamination by bee venom PLA_2 of the commercially-available melittin preparation was found, and insulin release could be induced by pure bee venom PLA_2, probably through the generation of lysophospholipids. Although estimates of the amount of PLA_2 in the melittin preparation suggested that such contamination was insufficient to explain at least some of the islet phospholipid hydrolysis and insulin release caused by melittin, we conclude that this agent does not serve as a specific probe of the role of endogenous PLA_2 or of AA lipoxygenation in hormone release.

Recently, considerable interest has arisen in the role of phospholipase A_2 (PLA_2) activation in the release of insulin [1–7] and other hormones. PLA_2 cleaves membrane phospholipids into a fatty acid (predominantly arachidonic acid, AA) and lysophospholipids. Most interest has focused on the former, since AA can be converted by an islet 12-lipoxygenase to 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and its derivatives, which appear to play a critical role in glucose-induced insulin release [1–3, 7]. More recently, we have identified, in addition, a putative role for lysophospholipids in the initiation of insulin secretion [4, 8, 9]. To study the respective roles of such lipid mediators, exogenous phospholipase A_2 can be used and will initiate insulin release [5, 9]; however, it would be more physiological to study the specific effects of activation of endogenous PLA_2. Melittin is a cationic, amphipathic peptide which intercalates into phospholipid-containing membranes and can lyse cells [10, 11]; at lower concentrations, however, it can specifically activate endogenous PLA_2 [12, 13], and release lipoxygenase-mediated metabolites of AA [13], probably by a Ca^{2+}-dependent mechanism [13, 14]. We report herein data suggesting that melittin can indeed activate a phospholipase A_2 in intact rat islets, and concomitantly initiate physiologic exocytotic insulin release; however, the latter effect seems to be independent (at least to a considerable degree) of endogenous PLA_2 or lipoxygenase activation at low glucose concentrations. Rather, it may primarily reflect the observed contamination of commercial melittin preparations by bee venom PLA_2, possibly in conjunction with direct Ca^{2+} mobilization induced by melittin.

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MATERIALS AND METHODS

Islet isolation and incubation. Intact rat islets were isolated after pancreatic digestion using collagenase (type IV, Worthington Biochemicals) as previously described [15] with the following minor modifications. After being cleaned of obvious blood vessels, connective tissue and lymph nodes, the whole pancreases were placed in siliconized scintillation vials containing 5 mg/ml of collagenase and 10 μg/ml of DNase (Sigma, St. Louis, MO) in Hanks'

buffered salt solution containing 20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 0.15% bovine serum albumin, and the vials were shaken for 20 min in a water bath. Collagenase action was stopped by the addition of cold medium, and the tissue was allowed to settle on ice before removing the supernatant fraction. To the remaining tissue was added fresh medium containing 1.87 mg/ml collagenase, and the vial was shaken for 5 min. The latter procedure was repeated 2–4 more times. Following this pancreatic digestion, islets were concentrated on a Ficoll gradient. Ten islets of equal size were then transferred using stereomicroscopy, into specially designed 10 × 75 mm inner test tubes [15] whose bottom surfaces had been removed and replaced with a polyethylene mesh filter of 62 micron pore size (Tetko, Elmsford, NY) welded to the glass using the flame from a Bunsen burner. These inner tubes were placed into standard 16 × 100 mm glass outer test tubes containing 1 ml of Krebs–Ringer bicarbonate (KRB) buffer containing 143 mM Na^+, 5.9 mM K^+, 1.2 mM Mg^{2+}, 1.2 mM phosphate, 128 mM Cl^-, 1.2 mM sulfate, 25 mM bicarbonate and a measured, ionized Ca^{2+} concentration of 2.1 mM, gassed with 95% O_2, 5% CO_2, and maintained at pH 7.4. Islets were then preincubated for 30 min at 1.7 mM glucose and 37°. After this preincubation period, the inner tubes containing the islets were removed allowing complete drainage of medium; they were placed into new outer tubes containing 1 ml of fresh KRB medium for a 30-min static incubation period. Since our previous studies demonstrated that the effects of lipoxygenase inhibitors on insulin release were reduced markedly by the presence of albumin, incubations containing these inhibitors were carried out in 750 μl of albumin-free medium, which was brought to a final concentration of 0.5% BSA (for adequate recovery and measurement of secreted insulin) by addition of 250 μl of 2% BSA at the end of the 30-min incubation. Test chemicals, such as melittin (Sigma), were generally added during the incubation period only. However, 8-(*N*-*N*-diethylamino) octyl 3,4,5-trimethoxybenzoate HCl (TMB-8, Sigma), BW755c, ETYA, mepacrine (quinacrine, Sigma), bromophenacyl bromide (Sigma), tetracaine (Sigma), nordihydroguaiaretic acid (Sigma), butylated hydroxytoluene (Sigma) or antimycin A (Sigma) were present in both preincubation and incubation periods. For addition of $LaCl_3$ (Sigma), the routine KRB medium was replaced with a bicarbonate-, sulfate-, and phosphate-free medium (supplemented with 25 mM HEPES buffer) in order to avoid any precipitation of the La^{3+} by the anions. Calcium-"free" medium was made by excluding $CaCl_2$ and replacing it isotonically with NaCl. All other chemicals were from Sigma except ETYA (from Dr. W. B. Scott, Hoffmann-LaRoche, Nutley, NJ) and BW755c, which was a gift of Dr. P. J. McHale of the Wellcome Research Laboratories, Kent, England.

For studies of the reversibility of melittin, inner tubes containing islets were removed from the outer tubes containing the melittin-supplemented medium after the first 30-min incubation period and were transferred into new outer tubes containing fresh KRB buffer with 1.7 mM glucose for 20 min. Following this wash step, islets were again transferred to new medium containing 1.7 or 16.7 mM glucose in order to test the reversibility of the melittin effect and to examine the effect of a prior exposure to melittin (during the first incubation period) on the insulin response to 16.7 mM glucose during the second incubation period.

The percent reversibility was calculated using the following formula:

$$100 - \left[\frac{\text{(Insulin release in second incubation, after previous exposure to melittin)} - \text{(Mean insulin release in second incubation, control)}}{\text{(Mean insulin release in first incubation in response to melittin)} - \text{(Mean insulin release in first incubation, control)}} \right]$$

Studies of arachidonic acid release and islet phospholipid metabolism. Seventy-five to one hundred islets were transferred into each siliconized, mesh-bottomed tube and incubated for 90 min with 2 μCi/ml of octatritiated arachidonic acid ([^{3}H]-AA; 60–100 Ci/mmole; New England Nuclear, Boston, MA) in 1 ml of KRB buffer containing 16.7 mM *d*-glucose, as previously described [15], in order to label preferentially the *sn*-2 position of islet phospholipids. Studies with [^{14}C]-stearic acid (to label the *sn*-1 position preferentially) were carried out similarly except that islets were incubated overnight for 18 hr in the presence of 0.02% fatty acid free–bovine serum albumin (FAF-BSA) and 5 μCi/ml [1-^{14}C]-stearic acid (40–60 mCi/mmole; New England Nuclear) in a single culture dish and then were transferred into individual test tubes the next morning.

For studies of [^{3}H]AA release, prelabeled islets were washed three times in 1% FAF–BSA to remove unesterified [^{3}H]AA, preincubated for 30 min in KRB buffer containing 1.7 mM glucose and 0.5 to 1.0% FAF–BSA, and then incubated for 30 min in 1.7 mM glucose (with 0.5% FAF–BSA as a trap for released [^{3}H]-AA) in the presence or absence of melittin. Release of [^{3}H]AA was quantitated by measuring the disintegrations per minute in the supernatant fraction and those remaining in the islets (after sonification in 10% Triton-X) after washing off loosely adherent, unesterified [^{3}H]-AA by three rinses in 1% FAF–BSA. The counts measured in the supernatant fraction and islets were corrected for background values reflecting non-specific adsorption of [^{3}H]-AA to the mesh filter and/or test tube by including islet-free controls in each study and subtracting these values from all measurements. Release was expressed as percent of total (supernatant/supernatant + islets) to normalize variation in total labeling with [^{3}H]-AA between replicate tubes.

For studies of islet phospholipid metabolism, islets were treated similarly except that in some studies, where indicated, *p*-hydroxymercuribenzoic acid

(200 μM, Sigma) was present during the incubation period (in addition to 0.5% FAF–BSA) to assure that any lysophospholipids formed would not be rapidly re-acylated with fatty acid [4, 8]. Following the 30-min incubation in the presence or absence of melittin, incubations were rapidly terminated by submersion of the mesh filters containing the islets into 3 ml of ice-cold methanol, in a conical centrifuge tube. Islets were left overnight in the freezer in these tubes, to which had been added 2.5 μg of fluorescein as internal standard. Total lipid extracts were obtained by warming the tubes to room temperature, adding 3 ml chloroform, vortexing, and removing the filters. The solvent was removed to a silanized pear-shaped flask. Any residual phospholipid was removed from the original centrifuge tube (or extracted from islet fragments remaining in it) by sequential extractions into 6 ml methanol–chloroform (1:1), 6 ml chloroform–methylene chloride–isopropanol (2:1:1) and 3 ml methanol–chloroform (1:2). Acidification or alkalinization of samples was strictly avoided to minimize artifactual hydrolysis of phospholipids. All rinses/extracts were pooled, rotary evaporated to dryness, and injected onto the high performance liquid chromatograph in 10 μl of chloroform–methanol (1:1). Recoveries of fluorescein averaged 78 ± 2% (N = 59) after extraction and chromatography; values for individual phospholipids were corrected for the peak height (by u.v. detection at 203 nm) of the fluorescein internal standard in each sample. Analysis of lipids in aqueous samples (i.e. for studies of lysophosphatidylcholine released into the medium) was complicated by the fact that extractions of buffer necessitated a two-phase system. Fluorescein proved to be an inadequate internal standard under these conditions since some of it remained in the aqueous phase. Therefore, in these studies ultraviolet-detectable amounts of unlabeled phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) were added to the medium at the end of the 30-min islet incubations in order to monitor recovery of these lipids from the medium. The samples were then extracted sequentially with chloroform–methanol (1:1) and chloroform–methylene chloride–isopropanol (2:1:1). The pooled organic phases were then treated similarly to the total lipid extracts as described above.

Chromatography was carried out isocratically on previously described [15] Waters HPLC equipment but using a silica "Guard-pak" (Waters Assoc.) guard column and a Beckman Ultrasphere-Si column (5 μm particle size; 25 cm × 4.6 mm column size) purchased from Altex. The mobile phase consisted of acetonitrile/methanol/phosphoric acid (130/1.5/1.5) generally pumped at 1 ml/min; however, flow programming was used to maximize peak separation where needed. (This system is quite sensitive to very small variations in pH or methanol content in the mobile phase.) Elution of standards (phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; and LPC from soybean; phosphatidylserine, PS, from bovine brain; lyso-PE from bovine liver; all purchased from Sigma and containing some unsaturated fatty acid residues; lysophosphatidylinositol was from Avanti Polar Lipids, Birmingham, AL) was monitored by u.v.

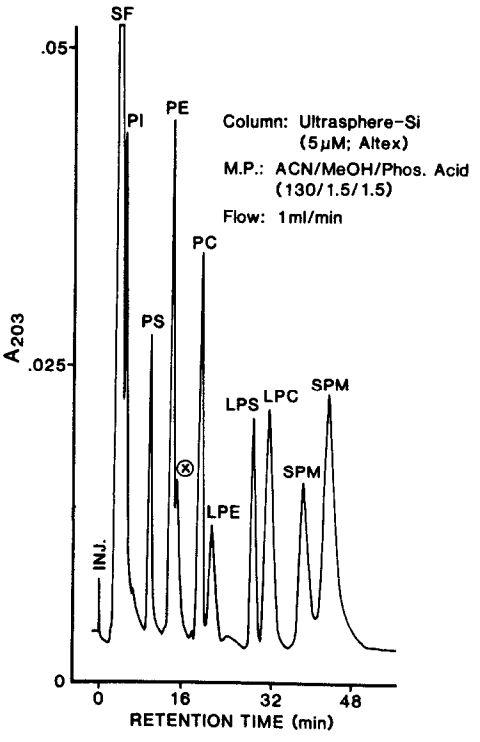

Fig. 1. High performance liquid chromatography (HPLC) method to separate phospholipid and lysophospholipid standards and radiolabeled samples. Microgram amounts of lipids were injected in 5 μl of chloroform. Chromatographic conditions are listed on the figure. Abbreviations: SF, solvent front; (L)PC, (L)PE, (L)PS, PI = (lyso)phosphatidyl-choline, -ethanolamine, -serine, -inositol (elution of lyso-PI occurs between PI and PS); SPM, sphingomyelin; M.P., mobile phase. ⊗ = unidentified contaminant in standards. Fluorescein elutes between PE and PC. The acidic phospholipids (PI and phosphatidic acid) were not adequately separated from the solvent front by this system.

absorbance at 203 nm, as illustrated in Fig. 1. PI was not adequately separated from the solvent front containing free fatty acids and neutral lipids by this HPLC system and therefore was not quantified in the current study.

Measurements and data analysis. Insulin was measured by radioimmunoassay using an antibody donated by Dr. O. Kolterman (San Diego, CA) and rat insulin standards (Novo Research Institute, Bagsvaerd, Denmark). Melittin (2 μg/ml), when added to the insulin standards, had no effect on the recovery of insulin in the radioimmunoassay. Melittin also did not alter the recovery of total [^{14}C]radioactivity in lipids during phospholipid extraction procedures. However, as indicated under Results, melittin contained significant activity to hydrolyze [^{14}C-*dipalmitoyl*]-phosphatidylcholine to LPC and free fatty acids, probably due to contamination by bee venom phospholipase A_2.

Insulin data are presented as mean ($\bar{x}$) (± S.E.M.) of insulin secreted (μU/10 islets/30 min). Changes in insulin levels were analyzed by non-paired *t*-testing.

Phospholipid data are expressed either as $\bar{x}$ (± S.E.M.) for dpm/80 or 100 islets (as indicated)

and analyzed by non-paired *t*-testing, or as percent of the appropriate control values from the same day's experiment (= 100%) by ANOVA followed by a single sample *t*-test. The ratio of lysophosphatidylcholine to phosphatidylcholine (LPC/PC) was also calculated; this ratio not only takes into account a rise in LPC and a concomitant degradation of PC, but also normalizes counts for any possible differences in the degree of labeling of islets ([^{3}H]AA or [^{14}C]stearate incorporation) and/or recovery of lipids between replicate tubes.

RESULTS

Effect of melittin on islet phospholipase A_2 activity. Islets incubated for 30 min at 1.7 mM glucose in the presence of 2 μg/ml melittin released 19 ± 3% ($\bar{x}$ ± S.E.M.; N = 10) of the total [^{3}H]-AA incorporated (averaging 153186 dpm/100 islets) compared to 6 ± 1% (N = 12) in the absence of melittin (df = 20; $P < 0.001$). This represents an increase to 371 ± 46% of basal in paired comparisons.

Since this release could reflect the end-result of several lipolytic cascades, any of which culminates in AA release, further studies of phospholipid metabolism were carried out. Degradation of phospholipids (i.e. loss of label) from prelabeled islets, if accompanied by concomitant formation of labeled lysophospholipids, is an index of phospholipase A activity. During a 30-min incubation with melittin, 2 μg/ml, [^{3}H-AA]-phosphatidylcholine (PC) and more variably phosphatidylserine (PS) and phosphatidylethanolamine (PE) were degraded (Table 1). Expressed in relative terms, the changes were: −38 ± 4% for PC; −24 ± 2% for PS, and −25 ± 7% for PE, all N = 8, and all changes were all highly significant ($P < 0.01$ to 0.001; df = 7). However, an increase in [^{3}H-AA]-lysophosphatidylcholine (LPC) was not observed (Table 1), suggesting that melittin did not activate a phospholipase A_1. (The greater basal formation of [^{3}H-AA]-labeled LPC compared to that labeled with [^{14}C]-stearate does, however, suggest the possible presence of basal phospholipase A_1 activity; Table 1.) Melittin also led to similar degradation of [^{14}C-stearate]-labeled phospholipids (Fig. 2): PS = −39 ± 9%, PE = −26 ± 7%, PC = −22 ± 4%, all $P < 0.05$ to 0.01, df = 4). In addition, melittin caused a significant accumulation of 1-stearoyl-LPC, whether the data are expressed in absolute terms (as dpm) or in relative terms (as the LPC/PC ratio) (Table 1). These LPC findings remained remarkably constant when the number of studies was extended further to N = 9 (the addition of *p*-hydroxymercuribenzoic acid during the last four studies did not alter the results): LPC in islet membrane rose to 248 ± 5% of control values. In preliminary additional studies (Table 1; Fig. 2), melittin was found to induce the release of considerable amounts of 1-stearoyl-LPC into the BSA-containing medium; the net melittin effect on LPC generation (medium plus cells) reached 605 ± 21% of basal values (df = 4; $P < 0.001$) when this release is taken into account.

Melittin-induced changes in phospholipids were not occurring in small amounts of acinar tissue or platelets possibly contaminating the islets, since organ culture of the islets for 6 days in RPMI 1640 medium containing 10% fetal calf serum (to permit disintegration of any such contaminating tissue) did not alter the findings (Fig. 2). Additionally, labeling

Table 1. Effect of melittin on islet phospholipids

	Radioactivity (dpm × 10^{-2}/80 or 100 islets)				
	PS	PE	PC	LPC*	LPC/PC ratio
A. [^{14}C-Stearate]-labeled islets (N = 80 islets/tube)					
Control (N = 4)	137.8 ± 20.2	221.1 ± 26.0	1136 ± 101	22.33 ± 0.99	(1.99 ± 0.12%)
Melittin, 2 μg/ml (N = 5)	93.12 ± 10.0 ($P < 0.10$)	161.1 ± 12.9 ($P < 0.10$)	861.5 ± 23.3 ($P < 0.05$)	60.78 ± 4.78 ($P < 0.001$)	(7.06 ± 0.53%) ($P < 0.001$)
B. [^{3}H-Arachidonate]-labeled islets (N = 100 islets/tube)					
Control (N = 4)†	58.18 ± 7.64	78.46 ± 12.8	1355 ± 120	63.46 ± 6.03	(4.78 ± 0.59%)
Melittin, 2 μg/ml (N = 8)†	44.54 ± 0.89 ($P < 0.05$)	59.54 ± 3.17 ($P < 0.10$)	855.6 ± 40.5 ($P < 0.001$)	51.36 ± 2.90 ($P < 0.10$)	(6.06 ± 0.37%) ($P < 0.10$)

Glucose = 1.7 mM. All incubations were for 30 min and carried out in the presence of 0.5% fatty acid free–bovine serum albumin. Values are $\bar{x}$ ± S.E.M. Abbreviations: PS, phosphatidylserine; PE, phosphatidylethanolamine; and LPC, lysophosphatidylcholine.

* In additional preliminary studies, media were also extracted and found to contain inconsequential amounts of intact phospholipids. [^{3}H-AA]-labeled islets released small amounts (about 35% of that in membranes) of LPC; while this amount appeared to be slightly increased by melittin, the total of (medium + cellular) [^{3}H-AA]-LPC was still not increased by melittin. In contrast, 2,477 ± 303 dpm of [^{14}C-stearate]-LPC (N = 2) was released by control islets rising to 14,997 ± 510 ($P < 0.001$) in melittin-treated islets (N = 3). Thus, the net effect of melittin (the sum of medium + cells) was 605 ± 21% of basal (N = 3). In these studies, the increase of medium + cellular [^{14}C-stearate]-LPC (12,520 ± 510 dpm) in melittin-treated islets was only 52 ± 2% of the mean decrement in [^{14}C-stearate]-PC (24,094 dpm). This discrepancy persisted when islets were labeled in the presence of 5 μM unlabeled AA, to increase the specificity of the [^{14}C]-stearate for the *sn*-1 position. Thus, it can be suggested but not shown with certainty that some lysophospholipase-like activity exists in the islets, i.e. that there is less than stoichiometric formation of LPC from degraded PC.

† Two control tubes and five experimental tubes also contained 200 μM *p*-hydroxymercuribenzoic acid (PHMB) to assure that released fatty acids were not re-esterified [4, 8]. The presence of PHMB in the control or experimental tubes did not alter results; data are therefore pooled.

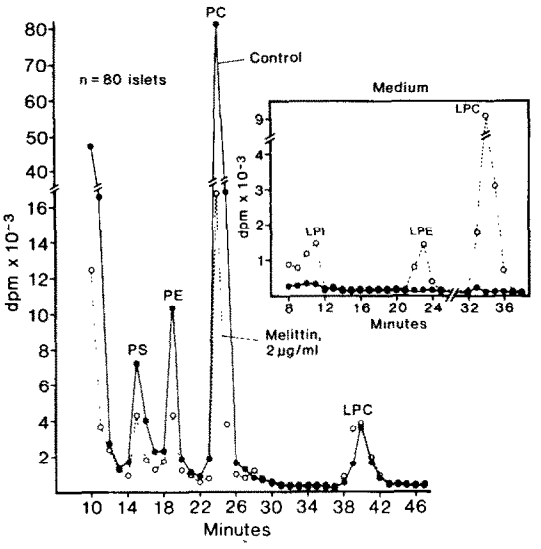

Fig. 2. Chromatogram of extracts of 80 islets, or of the corresponding medium (inset), after a 30-min incubation in the presence (dotted lines) or absence (solid lines) of melittin (2 μg/ml). Islets were cultured for 6 days and then labeled for 18 hr with [^{14}C]stearic acid in the presence of 5 μM unlabeled arachidonic acid. Values are uncorrected for the recoveries of internal standards (cells, control = 80.3%; cells, melittin = 75.5%; supernatant, control = 74.8%; supernatant, melittin = 79%). Abbreviations: LPE, lysophosphatidylethanolamine; LPI, lysophosphatidylinositol (identification provisional); and LPC, lysophosphatidylcholine.

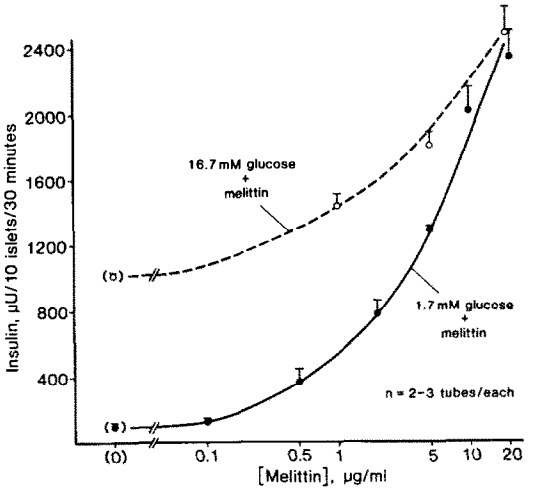

Fig. 3. Effect of various concentrations of melittin on insulin release at 1.7 or 16.7 mM glucose during a static, 30-min batch-type incubation.

the islets with [^{14}C]-stearate in the presence of 5 μM unlabeled AA (to saturate the *sn*-2 position and increase selectivity of the [^{14}C]-stearate for the *sn*-1 position) reduced islet phospholipid labeling by about one-third (data not shown) but otherwise did not alter results (Fig. 2). For example, in Fig. 2, control values for LPC in islet membranes and in the corresponding supernatant fraction were 8,814 and 334 dpm/80 islets respectively; these values rose to 11,201 and 18,142 dpm in samples from islets treated with melittin (2 μg/ml). Correspondingly, there was degradation of PS, PE and PC in islet membranes and also the appearance of some lyso-PI and lyso-PE in the medium. Thus, the addition of melittin resulted in the effects expected for a phospholipase of the A_2 type.

Melittin-induced insulin release. Melittin evoked a concentration-dependent release of insulin at a substimulatory glucose concentration of 1.7 mM (Fig. 3); at 16.7 mM glucose, the effects of the two agonists were incompletely additive and insulin release at 16.7 mM glucose in the presence of 20 μg/ml melittin was no greater than the effect of the drug alone at 1.7 mM glucose (Fig. 3). Melittin-treated islets looked morphologically normal under light microscopic examination (200×). If, after a 30-min incubation at 1.7 mM glucose with melittin at 0.5, 2 or 5 μg/ml, islets were washed in buffer containing 1.7 mM glucose in the absence of drug for 20 min and then reincubated in 1.7 mM glucose, the stimulatory effect of melittin was reversed by 61 ± 2, 51 ± 3 and 55 ± 9% respectively (Table 2). (Longer or more frequent washings were not studied.) If the same islets were then exposed to a stimulatory glucose

Table 2. General characteristics of melittin-induced insulin release*
Dose-dependency, reversibility and lack of effect on subsequent responses to glucose

Treatment (1st incubation)	Insulin (μU/10 islets/30 min)	Treatment (2nd incubation)	Insulin (μU/10 islets/30 min)
Control (1.7 mM glucose)	61 ± 7† (8)	1.7 mM glucose (G)	16 ± 5† (3)
		16.7 mM G	611 ± 11 (4)
Control + Melittin, 0.5 μg/ml	209 ± 27 (6)	1.7 mM G	73 ± 2 (2)
		16.7 mM G	725 ± 6 (3)
Control + Melittin, 2.0 μg/ml	733 ± 37 (12)	1.7 mM G	346 ± 18 (6)
		16.7 mM G	846 ± 50 (6)
Control + Melittin, 5.0 μg/ml	1574 ± 138 (6)	1.7 mM G	699 ± 143 (3)
		16.7 mM G	1416 ± 69 (3)

* 1st incubation (INC_1, 30 min) → (20-min wash) → 2nd incubation (INC_2; 30 min).
† Values are $\bar{x}$ ± S.E.M.; numbers in parentheses = number of tubes/condition.

concentration (16.7 mM) during the second incubation, it could be seen that the previous treatment with melittin did not alter the glucose responsivity of the islets (Table 2). The insulin release induced by melittin reflected physiologic exocytosis since it was prevented by reduced ambient temperature (Table 3). The insulin release seemed to represent true, energy-independent initiation of secretion since it not only occurred at substimulatory glucose levels, but it also was resistant to antimycin A, 10 μM (Table 3); in contrast, antimycin A reduced glucose-induced release by 96 ± 2% (df = 6; $P < 0.001$). In fact, antimycin A potentiated the effect of melittin, presumably by reducing ATP formation and thereby interfering with Ca^{2+} extrusion or sequestration mechanisms and/or preventing the ATP-dependent reacylation of any lysophospholipids generated.

Three PLA_2 inhibitors were examined for their effect on melittin-induced insulin release at 1.7 mM glucose. Two of these (bromophenacyl bromide, 25 μM, or tetracaine, 1 mM) were ineffective (Table 4), even though the concentrations used markedly impaired glucose-induced insulin release (by 94–100%, not shown). Higher concentrations of bromophenacyl bromide (100 or 250 μM) were also ineffective (Table 4) even against a lower melittin concentration of 0.5 μg/ml (data not shown). A third PLA_2 inhibitor, mepacrine (200 μM), was only partly effective against melittin (Table 4). Of four inhibitors of islet lipoxygenase tested, each of which inhibits glucose-induced insulin release [1–5, 7, 8], three (BW755c, nordihydroguaiaretic acid, and butylated hydroxytoluene) were ineffective or at most weakly inhibitory (Table 4). A fourth, ETYA (eico-

Table 3. Effects of various inhibitors on melittin-induced insulin release

Condition (1.7 mM glucose)	Insulin (μU/10 islets/30 min)		
Experiment 1			
a. Control (37°)	43 ± 12	(2)	
b. Control (16°)	27 ± 7	(3)	incremental response
c. Melittin, 2 μg/ml (37°)	1007 ± 47	(6)	reduced by
d. Melittin, 2 μg/ml (16°)	205 ± 18	(6)	81 ± 2% ($P < 0.001$, df = 10)
Experiment 2			
a. Control	164 ± 40	(3)	
b. Control (+ antimycin A, 10 μM)	135 ± 35	(3)	
c. Melittin, 2 μg/ml	1141 ± 95	(5)	potentiation =
d. Melittin + antimycin A	1718 ± 86	(5)	$P < 0.01$ (df = 8)
Experiment 3			
a. Control	81 ± 11	(3)	
b. Control (+ $LaCl_3$, 2 mM)	80 ± 18	(3)	
c. Melittin, 0.5 μg/ml	311 ± 48	(3)	
Melittin, 2.0 μg/ml	803 ± 60	(6)	
Melittin, 10.0 μg/ml	1711 ± 40	(3)	
d. Melittin, 0.5 μg/ml + $LaCl_3$	139 ± 28	(3)	incremental responses
Melittin, 2.0 μg/ml + $LaCl_3$	199 ± 16	(6)	reduced by 78 ± 11% ($P < 0.05$, df = 4),
Melittin, 10 μg/ml + $LaCl_3$	359 ± 43	(5)	84 ± 2% ($P < 0.001$, df = 10, and 83 ± 3% ($P < 0.001$, df = 6) respectively
Experiment 4			
a. Control	63 ± 11	(3)	
b. Control (+ $NiCl_2$, 2 mM)	22 ± 5	(3)	incremental response
c. Melittin, 2 μg/ml	400 ± 34	(5)	reduced by 100%
d. Melittin + $NiCl_2$	21 ± 1	(6)	($P < 0.001$, df = 9)
Experiment 5			
a. Control	63 ± 11	(3)	
b. Control (+ Ca^{2+}-free medium, + EGTA 0.2 mM)	58 ± 12	(3)	
c. Melittin, 2 μg/ml	400 ± 34	(5)	
d. Melittin (+ Ca^{2+}-free medium, + EGTA)	400 ± 39	(6)	
Experiment 6			
a. Control	63 ± 11	(3)	
b. Control (+ TMB-8, 100 μM)	107 ± 7	(2)	
c. Melittin, 2 μg/ml	400 ± 34	(5)	
d. Melittin + TMB-8	433 ± 76	(3)	
Experiment 7			
a. Control	164 ± 40	(3)	
b. Melittin, 2 μg/ml	1141 ± 95	(5)	
c. Melittin + epinephrine, 10 μM	1428 ± 79	(5)	NS

Values are $\bar{x}$ ± S.E.M.; numbers in parentheses = number of tubes/condition. NS = not significant.

Table 4. Effect on melittin-induced insulin release of inhibitors of phospholipases, lipoxygenase, cyclo-oxygenase or epoxygenase

Condition	Insulin (μU/10 islets/30 min)		
Experiment 1			
1. Control	87 ± 22	(3)	
2. Control + bromphenacyl bromide (BPB), 25 μM	114 ± 57	(2)	
Control + mepacrine, 200 μM	158 ± 44	(3)	incremental response reduced by 60 ± 4% (P < 0.001, df = 10)
Control + tetracaine, 1 mM	169 ± 19	(3)	
3. Melittin, 2 μg/ml	1072 ± 87	(6)	
4. Melittin + BPB	1007 ± 87	(6)	
Melittin + mepacrine	554 ± 34	(6)	
Melittin + tetracaine	1100 ± 196	(3)	
Experiment 2			
1. Control	84 ± 13	(3)	
2. Control + BPB, 25 μM	134 ± 56	(2)	
Control + butylated hydroxytoluene (BHT), 25 μM	152 ± 46	(3)	
3. Melittin, 2 μg/ml	960 ± 116	(4)	
4. Melittin + BPB	1314 ± 187	(6)	
Melittin + BHT	961 ± 65	(3)	
Melittin + ETYA, 33 μM	578 ± 41	(3)—(response reduced by 43 ± 5%; P < 0.05, df = 5)	
Experiment 3			
1. Control	116 ± 22	(3)	
2. Control + BPB, 100 μM	130 ± 16	(3)	
Control + BPB, 250 μM	370 ± 15	(2)	
3. Melittin, 2 μg/ml	1245 ± 84	(3)	
4. Melittin, 2 μg/ml + BPB, 100 μM	1577 ± 91	(6)	
Melittin, 2 μg/ml + BPB, 250 μM	1593 ± 74	(6)	
Experiment 4			
1. Control	91 ± 9	(3)	
2. Control + nordihydroguaiaretic acid (NDGA), 50 μM	96 ± 17	(3)	
Control + BW755c, 500 μM	78 ± 10	(3)	
3. Melittin, 2 μg/ml	779 ± 53	(4)	
4. Melittin + NDGA	789 ± 73	(6)	
Melittin + BW755c	1393 ± 48	(6)	
Melittin + ETYA, 33 μM	447 ± 41	(6)—(response reduced by 48 ± 6%; P < 0.01, df = 8)	
Experiment 5			
1. Control	77 ± 7	(3)	
2. Control + indomethacin, 2 μg/ml	98 ± 16	(3)	
Control + ibuprofen, 10 μg/ml	96 ± 11	(2)	
3. Melittin, 2 μg/ml	1112 ± 90	(6)	
4. Melittin + indomethacin	1175 ± 49	(6)	
Melittin + ibuprofen	1098 ± 99	(6)	
Melittin + metyrapone, 100 μM	1255 ± 93	(6)	

Values are $\bar{x}$ ± S.E.M.; numbers in parentheses = number of tubes/condition.

satetraynoic acid, the acetylenic analog of AA) was only partially inhibitory (Table 4); on one of three occasions tested, nordihydroguaiaretic acid also had a modest effect (−42 ± 11%; N = 6). Inhibitors of islet cyclo-oxygenase (ibuprofen, indomethacin), or an inhibitor of cytochrome P450-dependent "epoxygenases" [16] in many systems (i.e. metyrapone) also were ineffective (Table 4).

However, the effect of melittin was reduced markedly by co-incubation with 2 mM $NiCl_2$ or 2 mM $LaCl_3$ (Table 3). This effect presumably did not represent the effect of Ni^{2+} or La^{3+} to reduce Ca^{2+} influx (through voltage-dependent "slow" Ca^{2+} channels), since it was not reproduced (Table 3) by incubation in Ca^{2+}-"free" medium in the presence of 0.2 mM ethyleneglycolbis(aminoethylether)-tetra-acetate (EGTA) (which markedly blunted glucose-induced release; not shown). Furthermore, the anion-poor buffer used in the La^{3+} study itself reduces Ca^{2+} influx [17], but did not seem to reduce the control response to melittin (Table 3). TMB-8 (100 μM), a putative inhibitor of the release of intracellular Ca^{2+} stores [18], was ineffective in blocking the action of melittin (Table 3), again in contradistinction to the corresponding effects on 16.7 mM glucose (−96 ± 1%, df = 7, P < 0.001).

Table 5. Effects of melittin or bee venom phospholipase A_2 (BV-PLA_2) on phosphatidylcholine (PC) hydrolysis, accumulation of lysophosphatidylcholine (LPC), and insulin release

Condition	Islet-free	Islets				
	[^{14}C-dipalmitoyl]PC hydrolysis* (%)	Insulin release, (μU/10 islets/30 min)	PC (% of control)	LPC (cells) (% of control)	LPC (cells + supernatant) (% of control)	LPC/PC (cells) (%)
A. Control	13 ± 4 (3)	130 ± 16 (7)	(100)	(100)	(100)	2.4 ± 0.15 (3)
B. Melittin, 2 μg/ml	62 ± 7 (5)	1268 ± 70 (6)	70 ± 5 (3)	222 ± 42 (3)	487 ± 43 (3)	7.9 ± 1.2 (3)
C. BV-PLA_2, 20 mU/ml	17.8 (1)	525 ± 48 (3)	109 (1)	135 (1)		3.4 (1)
BV-PLA_2, 50 mU/ml	48.2 (1)	591 ± 43 (6)	110 (1)	163 (1)	203 (1)	3.85 (1)
BV-PLA_2, 200 mU/ml	56.7 (1)	1105 ± 12 (3)	124 (1)	112 (1)		2.34 (1)
BV-PLA_2, 500 mU/ml	95.1 (1)	1065 ± 66 (3)	92 ± 5 (3)	206 ± 21 (3)	259 ± 21 (3)	5.57 ± 0.3 (3)
BV-PLA_2, 1000 mU/ml		1395 ± 53 (2)				
BV-PLA_2, 2500 mU/ml			86 (1)	256 (1)	196 (1)	6.28 (1)

* Percent hydrolysis/30 min of tracer amounts of PC, labeled in both *sn*-1 and *sn*-2 positions with [^{14}C]palmitic acid and calculated as:

$$\frac{(\text{dpm in fatty acid}) + (\text{dpm in LPC})}{\text{fatty acid} + \text{LPC} + \text{intact PC})}$$

All incubations were for 30 min, and were performed in 1.7 mM glucose. Values are $\bar{x}$ ± S.F.M.; numbers in parentheses = numbers of replicates/condition.

Similarly, epinephrine, 10 μM, abrogated the effect of 16.7 mM glucose (−99%) but not at all that of melittin (Table 3).

Possible contamination of commercially-available melittin by bee venom phospholipase A_2. Although the manufacturer of the melittin preparation (which is isolated from bee venom) claimed it to be devoid of bee venom PLA_2, the vendor (Sigma) reports in their catalog that it contains enzyme activity equivalent to 22–40 mU/ml (up to 20 units/mg) (Biochemical and Organic Compounds for Research, Sigma Chemical Co., St. Louis, MO). To examine further the possibility of contamination of melittin with exogenous PLA_2, we incubated [^{14}C-*dipalmitoyl*]phosphatidylcholine (80–120 mCi/mmole; New England Nuclear; 250–650,000 dpm/tube) in the absence of islets but under identical conditions as those used for islet incubations. Unequivocal contamination with PLA_2 (as indicated by the nearly-stoichiometric generation of labeled palmitic acid and lysophosphatidylcholine, in conjunction with the loss of intact PC) was found ($P < 0.01$ vs control incubations; df = 6; Table 5). The quantities of PLA_2 estimated to be present were up to five times the maximum amount of contamination estimated by the supplier (Table 5). Melittin did not alter the total amount of radioactivity recovered (data not shown), indicating lack of irreversible binding of lipids by melittin.

Pure bee venom PLA_2 could initiate insulin release at 1.7 mM glucose in a dose-dependent manner ($P < 0.001$, ANOVA; Table 5). However, 3–4 times as much pure bee venom PLA_2 as that estimated to be in the melittin preparation (Table 5) appeared to be required to induce secretion comparable to that induced by melittin (Table 5). Furthermore, even as much as 10–12 times the amount of exogenous PLA_2 (2500 mU/ml) as that estimated to be in the melittin preparation (*ca.* 200 mU/ml) induced very little if any degradation of islet PC (Table 5) or of islet PE or PS (not shown), and induced only a limited generation of islet lysophosphatidylcholine (Table 5), which was still less than that caused by melittin. Thus, it seems unlikely that the contamination by exogenous PLA_2 explains (at least totally) the observed biological effects of melittin.

DISCUSSION

Melittin (2 μg/ml) led to an increased release from prelabeled islets of tritiated arachidonic acid ([^{3}H]-AA, to 371% of basal values) and induced the degradation of [^{3}H-AA]-labeled or [^{14}C-stearate]-labeled phospholipids (especially of PC but also by other phospholipids; PI was not quantified). Others have observed that melittin, which may facilitate PLA_2 activity by altering phospholipid domains through its interaction with phospholipids [11, 19], is relatively non-selective in the phospholipid degradation induced [12]. Melittin also led to accumulation (both in membranes and in the medium) of 1-[^{14}C-stearate]-2-lysophosphatidylcholine (LPC) but not of 2-[^{3}H-AA]-LPC, suggesting the presence (for the first time in *intact* islets) of a phospholipase A_2 able to remove labeled or unlabeled AA which had been preferentially esterified in the *sn*-2 position of

phospholipids. The possible presence, in addition, of basal islet phospholipase A_1 activity was suggested by the higher basal accumulation of [^{3}H-AA]-LPC as compared to [^{14}C-stearate]-LPC (Table 1). Although no stimulation of a phospholipase A_1 activity by melittin was detected, it is possible that any [^{3}H-AA]-LPC formed was rapidly degraded (to glycerophosphorylcholine) by an islet lysophospholipase. Indeed, phospholipase A_1 often contains intrinsic lysophospholipase activity [20]. Compatible with that possibility was the finding that the accumulation of [^{14}C-stearate]-LPC seemed to be less than stoichiometric with the degradation of PC (cf. Fig. 2 and footnote to Table 1), suggesting the presence in the islet either of a lysophospholipid-degrading enzyme or of a second type of phospholipase acting on PC.

Melittin also induced insulin release. This secretion had characteristics of physiologic, exocytotic hormone secretion rather than those of a toxic or lytic effect, i.e. it was dose-dependent, inhibitable pharmacologically ($NiCl_2$, $LaCl_3$, mepacrine) or by reduced temperature, and unassociated with impairment of the subsequent physiologic functioning of the islets (glucose-induced insulin release). While the effect on basal insulin release was only 50–60% reversed after a single, 20-min wash, this presumably reflects the ability of melittin to intercalate avidly into membranes [10, 11]. Morgan and Montague [21] reported several results similar to ours and added, in addition, a rapid onset of action (<2 min) and eventual total (albeit slow) reversibility of drug effect. The effect of melittin seemed to reflect true *initiation* of insulin release (as opposed to *potentiation* of stimulus-induced release) since it occurred at substimulatory glucose levels and despite inhibition of mitochondrial energy flux using antimycin A. Interestingly, 16.7 mM glucose became increasingly less effective in the face of increasing concentrations of melittin until, at 20 μg/ml of drug, glucose had no additional stimulatory effect. Tanaka *et al.* [22] and Morgan and Montague [21] also observed that high concentrations of melittin and maximally effective concentrations of glucose were not fully additive. This probably reflects a shared mechanism of action, presumptively mobilization of a common pool of Ca^{2+} (see below); however, the ability of cationic amphiphiles like melittin to inhibit Ca^{2+}- and calmodulin- or phospholipid-dependent protein kinases in other cell systems [23] may be relevant, in view of the possible role of this kinase in glucose-induced insulin release [24]. Contamination by bee venom phospholipase A_2 may also contribute to this finding since the effects of glucose and of exogenous PLA_2 on insulin release are also submaximally additive [9]. A direct activation of a phospholipid-dependent protein kinase [25] by melittin at 1.7 mM glucose seems not to have mediated the associated insulin secretion, since the latter was not reduced by antimycin A, which inhibits the effects of C kinase activators [26].

Dunlop *et al.* [27] reported that melittin-induced insulin release (at an unspecified glucose concentration) is inhibited by the phospholipase inhibitor bromophenacyl bromide or by any of several lipoxygenase inhibitors in dispersed, cultured neonatal rat islet cells. Our results with intact, adult rat islets, at 1.7 mM glucose suggest, in contrast, that the mechanism of the effect of melittin to initiate insulin release probably did not involve in a major way the activation of endogenous PLA_2 or lipoxygenase, although it seemed to be able to activate PLA_2 in the current study and possibly in that of Dunlop *et al.* [27]. Thus, coincubation with two of three phospholipase inhibitors (including bromophenacyl bromide) had no effect on the insulin release; the partial inhibition induced by mepacrine may therefore represent its known inhibitory interaction with Ca^{2+} stores [28, 29] and membrane phospholipids [30], with which melittin also interacts [10–12].

In addition, no evidence was found for mediation of melittin-induced insulin release via the one hydrolytic by-product of PLA_2 action, arachidonic acid (AA). Blockers of AA lipoxygenation, cyclo-oxygenation or epoxygenation had only partial and inconsistent inhibitory effects and thus our data suggest only a small, if any, contribution of AA release and oxygenation to melittin-induced insulin release. While unoxygenated AA might be a potential stimulator of hormone release in some endocrine cell systems [31], we have been unable to find effects of exogenous AA (or of endogenously-generated lipoxygenase-derived metabolites) at 1.7 mM glucose [4, 8, 15, 32] in intact rat islets, as opposed to our findings in neonatal rat islet cells [33]. This discrepancy between cultured neonatal islet cells and intact adult rat islets with regard to the effect of AA or its metabolites at substimulatory glucose concentrations appears to explain the difference between our current data and those of Dunlop *et al.* [27]. Recently, Morgan and colleagues [34] reported that melittin-induced insulin release is inhibited in intact adult rat islets by a single lipoxygenase inhibitor (nordihydroguaiaretic acid) as well as by extracellular Ca^{2+} depletion, two findings not reproduced in the current study. We cannot definitively resolve these disparities; the same supplier of melittin was used. However, it should be noted that a potentiating concentration of glucose (4 mM) was used in that study and thus it is not clear whether the direct effects of melittin were inhibited, or whether it was a modulatory effect of the glucose which was being suppressed. As indicated above, the response of intact adult islets to lipoxygenase pathway products appears to be quite dependent on extracellular glucose concentration. Also, prolonged incubations in high concentrations (4 mM) of EGTA were used in that communication; such treatment, like that with La^{3+} in the current study, will also deplete superficial, membrane-bound Ca^{2+} stores [35] which appear to be critical to the action of melittin. Together these findings reinforce the conclusion that the effects on insulin release of PLA_2 activity, and the concomitant release of arachidonic acid and its metabolites, may depend critically both on the cells studied and especially on the ambient glucose concentration.

It is likely, however, that there was a contribution to the observed insulin release by lysophospholipid generated by the endogenous PLA_2 and/or the contaminating bee venom PLA_2 *(vide infra)*. The initiation of insulin release by exogenous bee venom phos-

pholipase A_2 supports our prior observations using the enzyme from porcine pancreas [9, 32] and those of Zawalich and Zawalich [5] who used the enzyme from *Naja naja* venom. Both laboratories concur that such secretion is lipoxygenase-independent. We have suggested [9, 32] that it is at least in part explicable by the generation of endogenous lysophosphatidylcholine; such lysophosphatidylcholine is primarily unlabeled since it is derived from PC localized in the outer membrane leaflet, which is known to take up very little radiolabeled fatty acid [36]. Thus, bee venom PLA_2 did cause formation of some radiolabeled LPC but this was modest and out of proportion to the concomitant insulin release.

Melittin-induced insulin release was blocked by $LaCl_3$ or $NiCl_2$ (but not by extracellular Ca^{2+} depletion). In addition to impeding Ca^{2+} influx, these agents inhibit, respectively, the release of Ca^{2+} from superficial, membrane-bound stores [37, 38] and its intracellular actions, probably at or near the exocytotic machinery [39]. Thus, as a cationic, membrane-active peptide, melittin (like many drugs such as 48/80, mepacrine, tetracaine or trifluoperazine) may directly or indirectly release, via electrostatic forces, a membrane pool of Ca^{2+} (cf. Refs. 28 and 40), most likely located on anionic binding sites such as acidic phospholipids, allowing its translocation to the cell interior, where it can contribute to the initiation of secretion. The observed generation of lysophosphatidylcholine (LPC) by "melittin" (or the bee venom PLA_2 contained in the preparation of melittin used) may have contributed in an important way to the observed insulin release, since lysophosphatidylcholine is also a cationic amphiphile which releases insulin probably by a similar mechanism [4, 8, 9, 32]. However, we emphasize that we have not measured mobilization of specific pools of Ca^{2+} or intracellular Ca^{2+} levels. Thus, this formulation (based largely on the use of pharmacologic probes such as La^{3+}) remains speculative, and the participation of other possible cellular actions of melittin in the insulin release is not yet excluded. One other such possibility is suggested by the observation that melittin induces fusion of secretory granules in rat anterior pituitary cells [41]; lysophospholipids have long been postulated to have this same action [42].

In addition to possible direct effects of melittin on Ca^{2+} flux [14], which cast doubt on the use of melittin as a specific probe of islet phospholipase A_2- and lipoxygenase-dependent insulin release, we found significant contamination by exogenous bee venom phospholipase A_2. Although such contamination seemed to be insufficient, when quantified, to explain fully the effects of "melittin" on either phospholipid degradation or insulin release, it does confound further the use of this reagent as a selective probe. Our melittin had the same source as that used by other investigators using this compound as if it were a selective activator of endogenous islet phospholipase A_2 [21, 27, 34]. In keeping with the studies of Schier [12] and Habermann [11], but apparently not those of Kojima *et al.* [43], pure bee venom PLA_2, a poorly penetrating phospholipase, led to very little degradation of (radiolabeled) phospholipids in intact cells. This presumably reflects the fact that exogenous bee venom PLA_2 can only hydrolyze the outer membrane leaflet [44–47] and thus spares much PC and most of PE and PS (which are preferentially localized to the inner membrane leaflet of most cells; Refs. 45–47). In contrast, *endogenous* PLA_2 is localized to the inner membrane leaflet [48] and can hydrolyze the phospholipid there which selectively takes up radiolabeled fatty acids [36]. Therefore, the marked hydrolysis of PC, PE and PS induced by "melittin" reinforces the conclusion that this reagent probably does induce to some degree the activation of the *endogenous* islet PLA_2. However, it should be noted that melittin potentiates the action of exogenous phospholipase A_2 from bee venom selectively [49, 50] further complicating quantitative analysis of the relative effects of exogenous versus endogenous PLA_2, and thus additional studies will be needed to demonstrate conclusively the presence of endogenous PLA_2 activity in intact islets.

In summary, the biologic effects of melittin probably reflect the summation of the activation of endogenous PLA_2, the content of exogenous PLA_2 and possibly the direct release of membranous Ca^{2+} stores. Therefore, under the conditions of this study (adult rat islets studied at low ambient glucose concentrations), and using the commercially available melittin preparation we employed, this reagent seems not to be a specific agent with which to probe the role of endogenous PLA_2 or lipoxygenase in the control of insulin secretion.

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