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BLOCKADE BY LIPOXYGENASE INHIBITORS OF Ca^{2+}-DEPENDENT INSULIN SECRETION FROM PERMEABILIZED RAT ISLETS

A MOLECULAR MECHANISM DISTINCT FROM THAT OF α_2-ADRENERGIC AGONISTS

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Abstract—To evaluate the regulation and effects of pancreatic islet lipoxygenase, adult rat islets were permeabilized, using digitonin or staphylococcal α-toxin, and then were studied in a medium simulating an intracellular milieu at fixed ambient concentrations of Ca^{2+}. Permeabilized islets retained 12-lipoxygenase activity, as indicated by conversion of tritiated arachidonic acid to a predominant peak of [^{3}H]12-hydroxyeicosatetraenoic acid (12-HETE); this activity was inhibited (89–98%) by the lipoxygenase blockers nordihydroguaiaretic acid (35 μM), BW755c (250 μM) or ETYA (35 μM). Lesser amounts of compounds coeluting with 15- and 11-HETE (but little or no 5-HETE) were formed; however, 11-HETE (and possibly some 15-HETE) was probably synthesized (at least in part) via cyclooxygenase, as suggested by the partial synthesis blockade induced by 50 μM ibuprofen. The production of 12-HETE did not require the presence of Ca^{2+}, Mg^{2+} or ATP; it also was not stimulated by addition of cyclic AMP, a phorbol ester, or calmodulin. However, it was augmented modestly by provision of a basal cytosolic free Ca^{2+} concentration of 60–80 nM, with no further increase at physiologically elevated levels of 260–530 nM. Elevations in cytosolic free Ca^{2+} concentrations induced insulin release which was inhibited by cooling, epinephrine or protein kinase inhibitors and, therefore, was exocytotic in nature. Lipoxygenase inhibitors blocked this insulinotropic effect of calcium at submaximal or saturating Ca^{2+} concentrations (with or without its potentiation by 12-*O*-tetradecanoylphorbol-13-acetate, an activator of protein kinase C) by 53–82%. However, they did not reduce the Ca^{2+}-independent secretory effects (at subnanomolar Ca^{2+} concentrations) of the phorbol ester alone. Similar results were seen using dibutyryl cyclic AMP to activate protein kinase A. The α_2-adrenergic agonists epinephrine or clonidine inhibited Ca^{2+}-, TPA- or cyclic AMP-induced insulin release without reducing HETE formation. We conclude that (1) islet lipoxygenase is constitutively expressed and is not physiologically regulated by α_2-adrenergic agonism, Ca^{2+} or protein kinases; (2) lipoxygenase modulates insulin release; HETE production is not merely an epiphenomenon reflecting the activation (or inhibition) of exocytotic secretion; (3) islet lipoxygenase inhibitors reduce insulin secretion, at least in part, by blocking the direct effects of Ca^{2+} on exocytosis and/or its synergism with Ca^{2+}-binding proteins such as protein kinase C; and (4) these same inhibitors do not directly poison protein kinase C or A, or the exocytotic apparatus. Furthermore, the retention of significant activity and biological effects of lipoxygenase in leaky islets suggests that this enzyme may be associated with, and act upon, cell membranes in islets.

Alpha$_2$-adrenergic agonists (such as epinephrine or clonidine), and inhibitors of pancreatic islet lipoxygenase (LPX†), represent two apparently dissimilar classes of compounds which are of considerable interest since they both inhibit the insulin release which is induced by a wide variety of beta cell secretagogues. For example, using intact rat islets, we observed [1, 2] that either class of compound reduces insulin secretion induced by the influx of intracellular Ca^{2+}, the mobilization of intracellular Ca^{2+} stores or the activation of protein kinase C (PKC). Since such actions on insulin release occurred in the absence of detectable effects on Ca^{2+} fluxes [1, 2], we speculated that these agents may block the effects of Ca^{2+} on the exocytotic effector system, rather than reducing cytosolic free Ca^{2+} concentrations, $[Ca^{2+}]_i$. A direct test of this hypothesis would be to examine their effects in permeabilized islets, where the plasma membrane has been rendered permeable to Ca^{2+}-chelators such as EGTA which can be used to "clamp" $[Ca^{2+}]_i$. This same approach can also be used to examine the identity and regulation of islet LPX, without the methodologic constraints both of

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† Abbreviations: LPX, lipoxygenase; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; BW755c, 3-amino-1-(trifluoromethyl-phenyl)-2-pyrazoline; ETYA, eicosa-5,8,11,14-tetrynoic acid; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; PKC, protein kinase C; $G_{pp}(NH)_p$, guanyl-5′-yl imidodiphosphate; GTPγS, guanosine-5′-(3-*O*-thiotripnosphate); AMP, adenosine monophosphate; GMP, guanosine monophosphate; EGTA, ethyleneglycolbis-(amino-ethylether)tetra-acetate; BSA, bovine serum albumin; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

prelabeling various membrane lipid pools with substrate (labeled arachidonic acid; AA) and then of selectively releasing AA (via phospholipase activation) in a fashion coupled to the islet LPX. This approach was taken for the current studies for the first time in an endocrine cell system.

METHODS

Permeabilization of rat islets for studies of insulin release or hydroxyeicosatetraenoic acid (HETE) production. Islets were obtained via collagenase digestion of rat pancreata, followed by isolation on a Ficoll gradient, as previously described [1, 2]. To permeabilize islets, they were exposed to digitonin (20 μg/ml × 20 min) in a medium simulating an intracellular milieu consisting of 20 mM sodium glutamate, 100 mM potassium glutamate, 3.5 mM $MgCl_2$, 0.5% BSA, 2 mM disodium ATP, 1.7 mM glucose, 1.0 mM EGTA, 20 mM HEPES, with no added Ca^{2+}, at pH 6.8. In some experiments, digitonin was omitted and islets were permeabilized using staphylococcal α-toxin (15–50 μg/ml × 20–30 min, as indicated in the text). In some studies in which a $[Ca^{2+}]_i$ of $\leq 12\ \mu M$ was used to stimulate insulin release, disodium ATP and $MgCl_2$ were omitted and were replaced by 2 mM MgATP (see Results).

For studies of insulin release, 10 islets/tube were then transferred into medium of similar composition (except that BSA and digitonin were omitted) for a 30-min incubation period at pH 7.2. This period constitutes the experimental period for assessments of static insulin release. $CaCl_2$ was added in various amounts in the presence of 1 mM EGTA in a fashion designed to yield desired ambient (cytosolic) free Ca^{2+} concentrations, $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was estimated using an iterative program [3], supplied by Dr Dermot Cooper, and run using GWBASIC on an Epson Equity II Computer. The association constants used were those of Ahlijanian and Cooper [3] except that the stability constant for calcium-EGTA was the preferred value cited by Fabiato and Fabiato, corrected for temperature [4]. Permeabilization of islets which persisted through the incubation period was ascertained by at least one of the following: (1) trypan blue retention in the vast majority of islet cells (>80–90%) when the dye was added during the incubation period; or (2) an increase in insulin release when intracellular free Ca^{2+} concentrations were raised into the micromolar range by adding 1.0 to 1.8 mM $CaCl_2$ to the 1 mM EGTA. The latter finding is in contradistinction to intact islets, where basal insulin release is actually higher in the absence of extracellular Ca^{2+} [5, 6]. Furthermore, in preliminary studies, either GTPγS or $G_{pp}(NH)_p$, which are impermeant to intact cells, generally promoted insulin secretion from α-toxin- or digitonin-treated islets. In addition, it has been documented previously that an identical treatment of islets with digitonin renders them permeable to large molecules such as lactate dehydrogenase [7].

Assessments of islet lipoxygenase activity. The activity of islet lipoxygenase (LPX) was assessed by the direct conversion of substrate ([^{3}H]arachidonic acid; [^{3}H]AA) to [^{3}H]hydroxyeicosatetraenoic acid (HETE). Previous studies had documented that intact islets synthesize 12-HETE and perhaps much smaller amounts of 15-HETE [8, 9] but only arguably 5-HETE (summarized in Ref. 10); however, in these earlier studies, HETE production reflected both the release of AA and its subsequent lipoxygenation, thereby making the regulation of LPX alone impossible to assess.

After permeabilization of islets (N = 250/tube) as described above, islets were transferred to 1 ml of a similar "intracellular" medium as that described above except that (1) in initial studies, $[Ca^{2+}]_i$ was fixed at very low levels (*ca.* 10^{-9} M), since we previously determined that Ca^{2+} can bind AA, forming soaps, aggregates and films and thus reducing its cellular bioavailability for islet studies [11–13]; and (2) Mg^{2+} initially was also omitted, since Mg^{2+}, like Ca^{2+}, can bind to AA and reduce its availability [11, 12]. This approach seemed reasonable since studies of other 12-lipoxygenases suggested that it is probably not a Ca^{2+}-activated enzyme [14]. To the incubation medium was added 0.13 μM unlabeled AA as carrier and 1–4 μCi/ml [^{3}H]AA (100 Ci/mmol) (total AA = 0.15 to 0.17 μM); the pH was 7.2. The tritiated arachidonate was purified by reverse phase HPLC prior to use. Reduced glutathione (GSH) was not added to promote the reduction of HPETE to HETE via glutathione peroxidase, since preliminary studies suggested that the provision of 0.5 mM GSH lowered the total production of lipoxygenase pathway products, presumably by removing the small amount of HPETE known to be needed as product activator of lipoxygenases [15].

In subsequent studies, the concentrations of unlabeled AA, Ca^{2+}, Mg^{2+} and ATP were independently varied. In preliminary studies, it was observed that increasing the concentration of exogenous substrate (AA) from 0.17 μM through 6.7 μM progressively increased the "velocity" of each islet LPX. However, at exogenous AA concentrations of 6.7 μM or greater, the quantitation of enzymatic HETE synthesis became inaccurate due to the relatively greater contribution of HETEs formed via auto-oxidation (in the face of a marked reduction in the specific activity of the [^{3}H]AA available for enzymatic conversion via lipoxygenase). Therefore, exogenous substrate concentrations of 0.17 to 2 μM only were used for the studies reported in the text; "substrate concentration" refers to the amount of exogenous AA added (the true concentration being somewhat higher due to the likely presence of some unquantified endogenous AA).

Incubations were continued for 45 min and then were terminated by addition of 7 ml of ice-cold methanol. Islets were sonified briefly (×15 sec) in their respective media to assure that all unesterified HETEs were freely accessible to the extracellular space. Media were then extracted and assessed by HPLC as previously described in detail [8]. Values were corrected for the recovery of exogenous, UV-detectable amounts of 15-, 12- and 5-HETE (200 ng/ml, added prior to extraction and HPLC), as well as for ^{3}H-dpms eluting with HETEs in samples incubated identically except for the omission of islet tissue (controls for the auto-oxidation of [^{3}H]AA).

The reverse phase HPLC system used does not differentiate hydroxyeicosatetraenoic acids (HETEs) from hydroperoxyeicosatetraenoic acids (HPETEs, their labile precursors); therefore, quantitation of HETEs in the text actually refers to the sum of HETE plus any unreduced HPETE in the sample. Results are expressed as dpm/250 islets/45 min (mean ± SE) where (N) = the number of determinations.

Prolonged pre-exposure of islets to phorbol esters to deplete protein kinase C. To test the effects of the depletion of islet protein kinase C, islets were cultured overnight (×18 hr), as previously described [11], in bicarbonate-buffered RPMI 1640 medium containing 10% fetal calf serum, in the presence of a phorbol ester which activates protein kinase C (12-*O*-tetradecanoylphorbol-13-acetate; TPA, 1 μM) or its diluent. Islets were then picked from the culture plates into Krebs–Ringer bicarbonate (KRB) buffer in individual test tubes containing no TPA. They were then studied for insulin release (starting 2.5 hr after the removal of TPA) after permeabilization of the islets as described above. Such treatment virtually obliterates protein kinase C activity from islets [11, 16, 17], whereas pretreatment with an inactive phorbol (4α-phorbol12,13-didecanoate) has no effects [11, 17].

Data presentation. Data are expressed as the mean (± SE) where (N) generally represents the number of observations (i.e. batches of islets) from a population of islets derived from the pancreata of four to eight male, fed Sprague–Dawley rats, except where stated otherwise. Because of variability between batches of permeabilized islets, statistical comparisons for [^{3}H]HETE production were generally only made on data obtained on one such day and confirmed on at least one separate occasion. However, where indicated in the text, data expressing the effect of LPX inhibitors on insulin release reflect the mean of (N) separate experiments on different days, each comprised of multiple, replicate control and experimental tubes; control and experimental data were analyzed by paired *t*-test in only these studies. Otherwise, statistical analyses were by non-paired *t*-testing; $P < 0.05$ was considered significant.

Materials. BW755c was a gift of the Wellcome Laboratories (Kent, England). Staphylococcal α-toxin, calmidazolium, W-7, calmodulin and staurosporine were purchased from CalBiochem (San Diego, CA). ETYA and HETE standards were purchased from Biomol Research Laboratories (Philadelphia, PA). Unlabeled arachidonic acid was from Nu-Chek Prep (Elysian, MN); [^{3}H]AA was purchased from New England Nuclear (Boston, MA). Clonidine was a gift of Boehringer-Ingelheim (Ridgefield, CT). Other chemicals were from Sigma (St Louis, MO). Where present in studies of 12-HETE production or insulin release, LPX inhibitors were generally present in both preincubation (i.e. permeabilization) and incubation periods; epinephrine or clonidine were added during the incubation periods only.

RESULTS

Assessments of islet lipoxygenase (LPX) activity in permeabilized islets. Digitonin-permeabilized islets synthesized a predominant compound which co-eluted with authentic 12-HETE (Fig. 1). However, since digitonin-permeabilized islets are "leaky" to molecules having a M_r of 140,000 [7] and mammalian lipoxygenases generally have a $M_r < 80{,}000$ [18, 19], it seemed necessary to validate that permeabilized islets had not lost most of their lipoxygenase. Therefore, islets were also studied after permeabilization with staphylococcal α-toxin, an agent which creates smaller pores than digitonin [20, 21]. The chromatograms obtained were qualitatively identical to those from digitonin-permeabilized islets, with peak areas for 12-HETE from the latter being only slightly and variably smaller (−32 ± 18%; N = 3 pairs) than those from α-toxin-permeabilized islets in paired analyses on the same days (cf. Fig. 1). Further evidence that a large amount of LPX activity had not been lost during permeabilization was seen in preliminary studies, in which the addition of [^{3}H]AA during the permeabilization with digitonin (which should assess both intracellular and released, extracellular LPX activity) led to HETE formation comparable to that seen when [^{3}H]AA was added to the α-toxin- or digitonin-pretreated islets during a subsequent incubation period (data not shown).

In agreement with studies of intact islets [8, 9, 22], the peak of 12-HETE in digitonin- or α-toxin-permeabilized cells was inhibited by known islet 12-LPX inhibitors: −89 ± 7% (N = 3) by BW755c, 250–300 μM; −96 ± 2% (N = 5) by 35–40 μM NDGA (cf. Fig. 1), and −98 ± 2% (N = 4) by 35 μM ETYA, when studied at low substrate concentrations (0.17 μM AA) and the absence of $Ca^{2+}{}_i$. At a substrate concentration of 2.2 μM and in the presence of 150 nM $[Ca^{2+}]_i$, these agents also inhibited 12-HETE formation (−80 ± 10%; N = 8). The 12-HETE peak was not reduced by the cyclooxygenase inhibitor ibuprofen (50 μM) at low arachidonate concentrations (data not shown) or at higher (1 μM) concentrations: ibuprofen = 92 ± 14% of control (N = 4 each; P = NS).

In addition, smaller amounts of compounds co-migrating with authentic 15-HETE and with 11-HETE were also seen (Fig. 1). These two peaks were not always adequately separable for radiometric quantitation, since the presumed 11-HETE peak sometimes merged with that for 15-HETE or formed a shoulder with 12-HETE (Fig. 1) when samples were collected for radioactivity only every minute. However, as substrate concentrations were increased, the 15-HETE (as well as the 11-HETE) peak increased relative to that for 12-HETE such that the ratio of 15-HETE to 12-HETE increased from 0.13 ± 0.02 ($\bar{x}$ of 5 studies at ≤ 0.15 μM AA) to 0.32 ± 0.05 (N = 9 studies; $P < 0.05$) at 1.0 to 2.2 μM arachidonate. The 15-HETE (± some 11-HETE) peak formed at low substrate concentrations and in the absence of $Ca^{2+}{}_i$ was also inhibited (by 89 ± 7, 44 ± 11 and 78 ± 5%, respectively) by the three LPX inhibitors listed above; similarly at 2.2 μM AA and 150 nM $[Ca^{2+}]_i$, 15-HETE was inhibited 57 ± 10% (N = 8) and 11-HETE was inhibited 70 ± 3% (N = 8) by the same inhibitors. In addition, however, 50 μM ibuprofen (a cyclooxygenase inhibitor) also reduced the (15- plus 11-) HETE peak

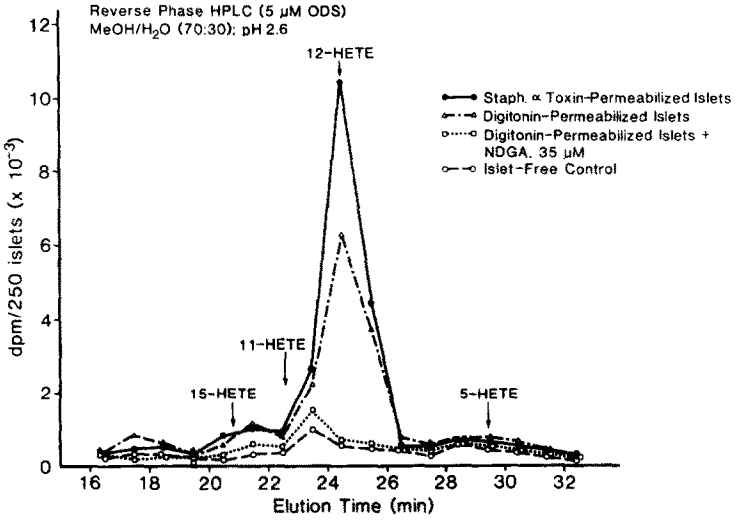

Fig. 1. Reverse phase HPLC analysis of islets exposed to [^{3}H]AA (1 μCi/ml, in the presence of unlabeled AA, 65 nM) after permeabilization by staphylococcal α-toxin (50 μg/ml × 30 min) or digitonin (20 μg/ml × 20 min). $[Ca^{2+}]_i$ was $\leq 10^{-9}$ M; Mg^{2+}_i was absent; [ATP] was 2 mM. 35 μM NDGA reduced 15-, 11- and especially 12-HETE to levels close to autooxidation (islet-free) control levels. Data points are uncorrected for recoveries, for each curve, which were within 6% of each other.

by 44% from 2965 ± 315 dpm to 1648 ± 824 dpm (N = 4 each; $P < 0.05$) in α-toxin permeabilized islets (substrate concentration = 1 μM). Such inhibition seemed to involve primarily the 11-HETE component (with a consistent inhibition of 39–64%), whereas 15-HETE seemed to be less consistently and convincingly blocked (inhibition of 0–36%), a distinction reinforced when eluant samples were collected every 18 sec to maximize peak separation. Indomethacin (10 μM) or ibuprofen (50 μM) also markedly reduced the (11 + 15)-HETE peak at an arachidonate concentration of 0.15 μM (not shown). Thus, as in other cell types [23–26], the 11-HETE and possibly the 15-HETE may have been derived, at least in part, via cyclooxygenase.

A few dpms co-eluted near or with authentic 5-HETE (cf. Fig. 1); however, this peak was only inconsistently greater then auto-oxidation controls and, furthermore, it was not consistently inhibitable by 250 μM BW755c (not shown). Other data have been presented indicating that this unidentified peak may not be authentic 5-HETE [9].

The possible requirements for Ca^{2+}, Mg^{2+} or ATP in HETE formation were then studied by using an iterative computer program to fix concentrations of each variable selectively. The presence of Ca^{2+} was not required for the lipoxygenation of AA (Fig. 1). However, providing a $[Ca^{2+}]_i$ of 150 nM (i.e. close to that of resting islet cells; Refs. 5 and 13) did increase HETE production somewhat (Table 1, Expt. 1). Thus, in islets permeabilized by α-toxin (50 μg/ml × 30 min), 12-HETE formation increased to 220% of Ca^{2+}-free samples (Table 1, Expt. 1); however, there was no further increment (167% of Ca^{2+}-free control) at a $[Ca^{2+}]_i$ of 530 nM (Table 1, Expt. 1), which is similar to that achievable by β cell stimulation [5, 13]. 11-HETE (Table 1, Expt. 1) and "5-HETE" (209 ± 28%; df 6; $P < 0.05$) but not 15-HETE (Table 1, Expt. 1) also seemed to be greater in the presence of some Ca^{2+}_i. A similar effect on 12-HETE production was seen when increasing $[Ca^{2+}]_i$ from 0 to 150 nM islets permeabilized with a lower concentration of α-toxin (15 μg/ml × 20 min: 180 ± 16% of Ca^{2+}_i-free samples; df 5; $P < 0.02$) or with digitonin (138 ± 8% of Ca^{2+}_i-free samples; df 4; $P < 0.05$). To ascertain further whether this effect of Ca^{2+} might be physiologically relevant, HETE production at a basal $[Ca^{2+}]_i$ of 60 nM was compared to that at a level of 260 nM which is similar to that of glucose-stimulated islets [5, 13]. There was no significant effect of the higher $[Ca^{2+}]_i$, with 12-HETE formation being 109 ± 12% of values at the lower $[Ca^{2+}]_i$. Similar results were seen going from 80 to 265 nM (Table 1, Expt. 2). A $[Ca^{2+}]_i$ higher than 530 nM could not be studied, since this leads to the formation of prohibitive amounts of Ca^{2+}-arachidonate soaps and thereby reduces the bioavailability and permeation of AA [11–13] especially at the low substrate concentrations used. In addition, higher $[Ca^{2+}]_i$ might activate phospholipases, releasing endogenous (unlabeled) AA and reducing the specific activity of [^{3}H]AA in the medium (see footnote to Table 1). Indeed, in preliminary studies at a $[Ca^{2+}]_i$ of 3.4 μM, HETE formation (assessed as dpms) showed an apparent, but probably artifactual, decline (not shown).

In preliminary studies at 2 μM arachidonate, there was no apparent difference in 15-HETE or 12-HETE formation in the absence or presence of Mg^{2+}; for example, at a calculated cytosolic free Mg^{2+} concentration of 8 μM, 12-HETE was 112 ± 7% of that in the absence of Mg^{2+} (df 3; P = NS). Likewise, the presence of 2 mM disodium ATP during the permeabilization and incubation periods did not increase the formation of any of the HETEs; in fact, it consistently tended to reduce 12-HETE formation (by 57%), albeit to a variable degree (ATP present = 1658 ± 190; ATP absent = 3813 ± 1218 dpm/250

Table 1. Effects of Ca^{2+} on lipoxygenase activity in staphylococcal α-toxin-permeabilized islets,* as assessed by the formation of hydroxyeicosatetraenoic acids (HETE)†

$[Ca^{2+}]_i$	15-HETE‡	11-HETE‡ (dpm/250 islets/45 min)	12-HETE
Expt. 1			
a. 0 (N = 3)	1261 ± 33	920 ± 75	3235 ± 514
b. 150 nM (N = 2)	1452 ± 351	1405 ± 297	7123 ± 720
c. 530 nM§ (N = 3)	1129 ± 53	1760 ± 272	5410 ± 286
Statistical analysis (no Ca^{2+} vs combined data for Ca^{2+} present):			
15-HETE:	P = NS (df 6)		
11-HETE:	P < 0.05 (df 6)		
12-HETE:	P < 0.01 (df 6)		
Expt. 2			
a. 80 nM (N = 4)	1100 ± 158	1683 ± 346	6260 ± 866
b. 265 nM§ (N = 5)	1099 ± 136	1646 ± 138	6064 ± 511
Statistical analysis: all differences are NS (df 7)			

* Islets were permeabilized using staphylococcal α-toxin (50 μg/ml × 25 min). During the subsequent incubation period, Mg^{2+} was absent; 2 mM disodium ATP was present; pH was 7.2. The substrate concentration was 2.0 μM.

† Data are means ± SE. Values are corrected for the specific recoveries of UV-detectable quantities of exogenous 15- and 12-HETE as well as for specific autoxidation controls (islet-free samples) at each $[Ca^{2+}]_i$.

‡ Identifications of 15-HETE and 11-HETE are tentative, being based on HPLC elution times and inhibitability by semi-selective blockers of arachidonate oxygenation.

§ It should be noted that the binding of Ca^{2+} to arachidonic acid (AA) may lead to the formation of calcium–AA soaps, films, and aggregates, which may decrease the concentration of substrate (AA) available to the lipoxygenase [12, 13]. The theoretical maximum for this complex formation is one Ca^{2+} bound to the carboxylate moiety of two molecules of AA. Therefore in Expt. 1, an increase of $[Ca^{2+}]_i$ of 380 nM (i.e. from 150 to 530 nM) potentially could decrease the available substrate concentration by 760 nM, or 40%, thereby totally accounting for the apparent decrease in 12-HETE formation at the highest $[Ca^{2+}]_i$. Conversely, in Expt. 2, an increase in $[Ca^{2+}]_i$ of 185 nM (80–265 nM) could reduce the concentration of AA by no more than 20%; thus, no major increase in 12-HETE could be obscured by this artifact.

islets/45 min; df 7). This apparent "inhibitory" effect of ATP presumably represents the ATP-dependence of arachidonate's esterification into membrane phospholipids, an effect which reduces the concentrations of unesterified fatty acid available to the lipoxygenase. The following compounds had no significant effect on islet lipoxygenase activity: TPA, dibutyryl cyclic AMP, GTPγS, $G_{pp}(NH)_p$, calmodulin and staurosporine (Table 2).

By using data such as that in Table 1 and calculating the specific activity of the medium (and assuming the absence of any significant isotope effect), one can estimate that rat islets produced about 4.5 fmole/islet/45 min of 12-HETE. Thus, average concentrations of 12-HETE across the islet could potentially reach 1.5 μM based on an average islet volume of 3 nl, and assuming the absence of diffusion of 12-HETE from the islet, or any significant degradation, as suggested by Ref. 8. This estimate is, however, only a minimal figure, since any release of unlabeled endogenous AA would dilute the specific activity of the medium, leading to an underestimation of 12-HETE production rates.

Effects of LPX inhibitors on insulin release from digitonin-permeabilized islets. To ascertain whether LPX inhibitors can reduce insulin secretion in permeabilized and "Ca^{2+}-clamped" islets, insulin release initially was stimulated near-maximally by a marked rise in $[Ca^{2+}]_i$ to a saturating level of 225 μM, in the presence of 2 μM TPA to activate protein kinase C and potentiate the effects of $Ca^{2+}{}_i$. Such high concentrations of $[Ca^{2+}]_i$ were initially employed to avoid the trivial possibility that micromolar concentrations of LPX inhibitors might merely chelate Ca^{2+}. Such secretion was inhibited 81 ± 3% and 59 ± 3% by NDGA (35 μM) or BW755c (250 μM) respectively (Fig. 2); these are concentrations of the drugs which markedly block the 12-lipoxygenase of permeabilized islets (*vide supra*). Similar inhibition was also seen when 2 μM TPA was added to more physiologic cytosolic free Ca^{2+} concentrations (200–650 nM); the synergistic effects of $Ca^{2+}{}_i$ and TPA were inhibited 71 ± 14% ($P < 0.05$; N = 4 experiments) and 50 ± 7% ($P < 0.02$; N = 4 experiments) by NDGA, 35 μM, or BW755c, 250 μM, respectively (cf. Table 3, Expt. 1 and 2).

To determine whether this effect of the LPX inhibitors was due to blocking the effect of a rise in $[Ca^{2+}]_i$ or of the activation of protein kinase C, the two agonists were provided separately. BW755c and NDGA failed to inhibit the calcium- "independent" effect of TPA (2 μM) at very low $[Ca^{2+}]_i$ (Fig. 3, inset; Table 3, Expt. 1 and 2). TPA is acting specifically via the stimulation of protein kinase C under these conditions, since the insulinotropic effects of

Table 2. Effects of various test agents on 12-HETE synthesis by permeabilized rat islets*

Experimental agent	12-HETE (% of control)	Number of pairs
1. a. TPA, 0.5–1 μM (Mg^{2+} absent)†‡	101 ± 6§	9
b. TPA, 1 μM (Mg^{2+} present)‖†	105 ± 11	3
2. a. Dibutyryl cyclic AMP, 0.5 mM (Mg^{2+} absent)†‡	112 ± 12	4
b. Dibutyryl cyclic AMP, 0.5 mM (Mg^{2+} present)‖†	94 ± 3	3
3. a. GTPγS, 50 μM†	95 ± 8	2
b. $G_{pp}(NH)_p$, 100 μM†‖	91 ± 6	11
4. Calmodulin, 2 μM‡	99 ± 1	2
5. Staurosporine, 1 μm†	98 ± 8	3

* Except where indicated, [^{3}H]AA = 3–4 μCi/ml; unlabeled AA = 1.5–2 μM; $[Ca^{2+}]_i$ = 150 nM; disodium ATP = 2 mM; Mg^{2+} was absent in the incubation period. Test agents (or their respective diluents) were present during both the permeabilization and incubation periods.

† α-Toxin-permeabilized islets.

‡ Digitonin-permeabilized islets.

§ Values are means ± SE. The mean absolute control value for 12-HETE synthesis in these studies was 5591 dpm/250 islets.

‖ In these studies, disodium ATP was omitted and was replaced by 1 mM MgATP (to provide the preferred form of the nucleotide for protein kinases and to provide Mg^{2+} as a cofactor for G protein activation); the free Mg^{2+} concentration was 54 μM, and the free Ca^{2+} concentration was 7 nM.

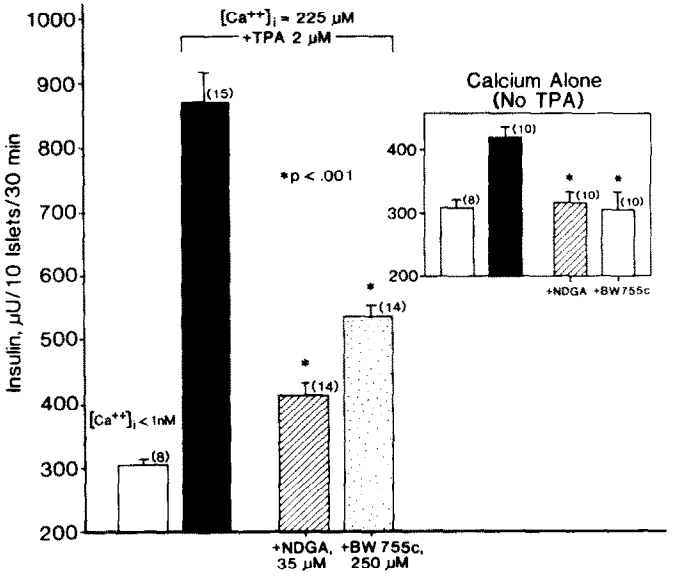

Fig. 2. Effect of nordihydroguaiaretic acid (NDGA, 35 μM) or BW755c (250 μM) on insulin release from digitonin-permeabilized islets stimulated by a high $[Ca^{2+}]_i$ of 225 μM in the presence of 2 μM TPA. The numbers of observations are shown in parentheses; data are from two separate experiments on different days yielding essentially identical data. The inset shows the effects of these inhibitors on the stimulation induced by Ca^{2+} alone (three bars on right are at a $[Ca^{2+}]_i$ of 225 μM; control bar on left is at ≤ 1 nM $[Ca^{2+}]_i$.

TPA in the absence (or the presence) of $Ca^{2+}{}_i$ were obliterated by the depletion of protein kinase C achieved by a prolonged pre-exposure of islets to a phorbol ester (−100%; df 8); they were also blocked by two protein kinase inhibitors [27–29]: 1 μM staurosporine (−85 ± 8%, df 10, $P < 0.001$) or 2 mM spermine (−71 ± 11%; df 6; $P < 0.01$; see also Ref. 11).

In contrast, the LPX inhibitors did block the "direct" effect of high $[Ca^{2+}]_i$ alone in the absence of TPA (Fig. 2, inset; Fig. 3). In this digitonin-permeabilized preparation, Ca^{2+} has two con-

Table 3. Effects of nordihydroguaiaretic acid (NDGA, 35 μM) or BW755c (250 μM) on insulin release from digitonin-permeabilized islets at basal- and physiologically-elevated $[Ca^{2+}]_i$ in the presence and absence of TPA (2 μM)

	$[Ca^{2+}]_i$ (nM)	Insulin* (μU/10 islets/30 min)	
Expt. 1			
a. Control	<1	232 ± 13 (3)	
b. Control + NDGA, 35 μM	<1	252 ± 24 (3)	
c. Control + TPA, 2 μM	<1	332 ± 13 (7)	
d. Control + TPA, 2 μM, + NDGA, 35 μM	<1	293 ± 11 (7)	P = NS vs (c)
e. Control (physiologic $[Ca^{2+}]_i$)	200	257 ± 30 (3)	
f. Control (physiologic $[Ca^{2+}]_i$) + NDGA	200	274 ± 17 (3)	
g. Physiologic $[Ca^{2+}]_i$ + TPA	200	657 ± 51 (7)	P < 0.001 vs (c or e)
h. Physiologic $[Ca^{2+}]_i$ + TPA + NDGA, 35 μM	200	382 ± 26 (7)	P < 0.001 vs (g)
Expt. 2			
a. Control (physiologic $[Ca^{2+}]_i$)	600	340 ± 26 (3)	
b. Control + NDGA, 35 μM	600	426 ± 20 (3)	
c. Control + BW755c, 250 μM	600	349 ± 32 (3)	
d. Control + TPA, 2 μM	<1	576 ± 11 (3)	
e. Control + TPA + NDGA	<1	577 ± 24 (4)	
f. Control + TPA + BW755c	<1	600 ± 39 (3)	
g. Physiologic $[Ca^{2+}]_i$ + TPA, 2 μM	600	638 ± 26 (7)	
h. Physiologic $[Ca^{2+}]_i$ + TPA + NDGA	600	500 ± 21 (6)	P < 0.01 vs (g)
i. Physiologic $[Ca^{2+}]_i$ + TPA + BW755c	600	498 ± 17 (6)	P < 0.01 vs (g)

* Data are means ± SE; numbers in parentheses equal the number of observations.

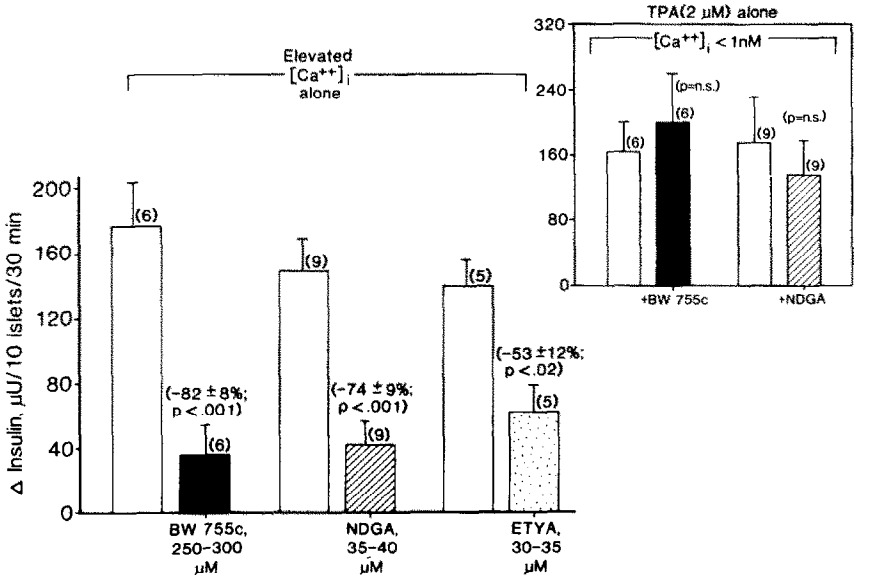

Fig. 3. Effects of LPX inhibitors on insulin release stimulated by high concentrations of $[Ca^{2+}]_i$ (≥ 225 μM). Numbers in parentheses represent the number of separate experiments carried out on different days, with each single experimental value representing the mean of multiple replicate tubes. Values are expressed as incremental insulin release above the relevant basal values for each experiment. The inset shows the effect of BW755c or NDGA on the Ca^{2+}-"independent" effect of 2 μM TPA at a $[Ca^{2+}]_i$ of ≤ 1 nM.

centration-dependent effects on insulin release—one beginning at $< 1\ \mu M$ and peaking by 10–15 μM, followed by a second increase above about 20 μM, which does not reach saturation until greater than 100 μM. The effect of such high $[Ca^{2+}]_i$ represents exocytosis, not a toxic effect on the islets, since it was totally inhibited by cooling the islets to 16° (Table 4, Expt. 1), by epinephrine or clonidine (*vide infra*), as well as by staurosporine or spermine (Table 4, Expt. 2) which, at the concentrations used, inhibit several protein kinases non-selectively [27–29]. The non-selective protein kinase inhibitor trifluoperazine (5 μM) also inhibited Ca^{2+}-activated secretion by an average of 81% in two experiments (data not shown). However, the calmodulin inhibitors calmidazolium (1 μM) or W-7 (15 μM) had no inhibitory effects ($+11 \pm 16\%$; df 11, and $-7 \pm 15\%$, df 10, respectively) and the addition of 2 μM calmodulin had no stimulatory effect (e.g. at a saturating $[Ca^{2+}]_i$, calmodulin yielded insulin release rates $102 \pm 18\%$ of control).

LPX inhibitors reduced the effects of both submaximal and saturating concentrations of Ca^{2+}. Thus, BW755c, NDGA and ETYA each inhibited the effect of saturating concentrations of cytosolic free Ca^{2+} by $82 \pm 8\%$ ($N = 6$ separate experiments; $P < 0.001$), $74 \pm 9\%$ ($N = 9$ experiments; $P < 0.001$) and $53 \pm 12\%$ ($N = 5$ experiments; $P < 0.02$), respectively (Fig. 3). The effects of a lower $[Ca^{2+}]_i$ of 11.5 μM were also studied. For these studies, the amount of $MgCl_2$ added was reduced to 2 mM (free $Mg^{2+} = 175\ \mu M$) since excess Mg^{2+} selectively inhibits Ca^{2+}-induced exocytosis at $[Ca^{2+}]_i < 20\ \mu M$ [30]. Under these conditions, the effect of lower $[Ca^{2+}]_i$ was also inhibited by BW755c ($-58 \pm 14\%$; $N = 5$ experiments; $P < 0.05$) or NDGA ($-56 \pm 8\%$; $N = 5$ experiments; $P < 0.05$); in absolute terms, the incremental response to low micromolar $[Ca^{2+}]_i$ was reduced from $101 \pm 13\ \mu U/10$ islets/30 min to 41 ± 8 (df 9; $P < 0.001$) by the inhibitors.

When Ca^{2+} was replaced with very high concentrations of Ba^{2+} (1–2 mM), insulin release was again stimulated. This was probably not due to displacement of Ca^{2+} from tissue binding sites by Ba^{2+}, since such release was seen even when no Ca^{2+} was added (and 1 mM EGTA was present). Nordihydroguaiaretic acid (30–35 μM) or BW755c (250 μM) modestly inhibited Ba^{2+}-induced release, by $36 \pm 8\%$, in 11 paired experiments (control = $172 \pm 16\ \mu U/10$ islets/30 min; lipoxygenase inhibition = 105 ± 13; $N = 11$; $P < 0.001$).

When $[Ca^{2+}]_i$ is considerably elevated, it is possible that protein kinase C could be recruited, either directly due to the rise in $[Ca^{2+}]_i$ or secondary to a Ca^{2+}-induced activation of phospholipase C [31], which generates diacylglycerol. This possibility was specifically examined by a prolonged pre-exposure of islets to 1 μM TPA, a procedure which depletes islets of protein kinase C [11, 16, 17]. This maneuver did "down-regulate" PKC, as indicated by the loss of the subsequent response to a dissimilar PKC activator, mezerein; however, it did not inhibit the secretory response to even a $[Ca^{2+}]_i$ of 350 μM (Table 4; Expt. 3) or of 175 μM (data not shown).

To determine whether LPX inhibition also reduces the synergistic effect of cyclic AMP on Ca^{2+}-activated insulin release, digitonin-permeabilized islets were treated with dibutyryl cyclic AMP (dbcAMP, 0.5 mM) during the permeabilization as well as incubation periods. The addition of dbcAMP to islets at $[Ca^{2+}]_i$ of 2 nM, 1 μM or 250 μM led to significant increases in insulin release, especially at the highest Ca^{2+} concentration (Fig. 4, main panel). In the presence of micromolar levels of Ca^{2+} and cyclic AMP, BW755c still reduced insulin release, while having no effect at very low $[Ca^{2+}]_i$ (Fig. 4, main panel). Furthermore, at the highest $[Ca^{2+}]_i$, BW755c did not totally prevent an incremental response to the addition of dbcAMP, despite markedly reducing the direct effect of Ca^{2+} alone (in the absence of dbcAMP) (Fig. 4, inset). The first two bars of the inset indicate that BW755c inhibited Ca^{2+}-activated release in the absence of cAMP; the third and fourth bars show that an inhibitory effect still occurred despite the addition of dbcAMP (as seen in the main panel, when micromolar Ca^{2+} concentrations were present). A comparison of the second and fourth bars of the inset confirms that an effect of dbcAMP persisted even when the direct effects of high Ca^{2+} were abrogated. Thus, BW755c does not inhibit the direct effects of cyclic AMP but only its synergism with Ca^{2+}. Furthermore, the provision of cyclic AMP does not prevent or bypass an inhibitory effect of lipoxygenase inhibition on insulin secretion.

In contrast, dibutyryl cyclic GMP (1 mM) had no effect by itself on insulin release at low, intermediate, or saturating $[Ca^{2+}]_i$, and also failed to reverse the inhibitory effects of BW755c (data not shown).

Effects of α_2 agonists on insulin release and LPX activity in permeabilized islets. Previously we observed that LPX inhibitors and α_2-adrenergic agonists such as epinephrine are similar in that either can reduce insulin release without a concomitant inhibition of Ca^{2+} fluxes. The possibility was excluded that LPX inhibitors interact with α_2 receptors; however, it is possible that α_2 agonists may inhibit LPX activity, as they do in other tissues, at least at very high concentrations [32, 33]. Therefore, we examined the effects of epinephrine, or of clonidine (an α_2 agonist which inhibits insulin release [34]) in permeabilized islets.

Either α_2 agonist markedly reduced insulin release induced by Ca^{2+} alone, by Ca^{2+} plus TPA, or by TPA alone, from islets permeabilized by digitonin or α-toxin (Table 4; Expt. 4–6). Similarly, 1 μM epinephrine inhibited the incremental effects of 0.5 mM dibutyryl cyclic AMP at a $[Ca^{2+}]_i$ of 2 nM (by $100 \pm 13\%$; df 11; $P < 0.001$) or at a saturating $[Ca^{2+}]_i$ (by $95 \pm 4\%$; df 6; $P < 0.001$). This blockade of the Ca^{2+}-"independent" effect of TPA or dibutyryl cyclic AMP is in contradistinction to the lack of inhibition by LPX inhibitors of TPA- or cyclic AMP-induced secretion at very low $[Ca^{2+}]_i$. In further contradistinction, α_2 agonists did not reduce LPX activity, at least under the conditions of our assay. In digitonin-permeabilized islets at low substrate concentrations (0.17 μM) and in the absence of $Ca^{2+}{}_i$, epinephrine (1 μM) did not reduce significantly 12-HETE (EPI = $87 \pm 9\%$ control; $N = 4$ pairs; P = NS) or 15-HETE; the 11-HETE peak tended to increase. In islets permeabilized by α-toxin

Table 4. Effects of cooling, inhibitors of protein kinases, or α_2-adrenergic agonists on insulin release induced by Ca^{2+} and/or TPA from permeabilized islets*

Condition	$[Ca^{2+}]_i$	Insulin release† (μU/10 islets/30 min)	
Expt. 1			
a. Low $[Ca^{2+}]_i$, 37°	25 nM	170 ± 11 (5)	
b. High $[Ca^{2+}]_i$, 37°	350 μM	323 ± 29 (9)	P < 0.001 vs (a)
c. Low $[Ca^{2+}]_i$, 16°	25 nM	130 ± 23 (4)	P = NS vs (a)
d. High $[Ca^{2+}]_i$, 16°	350 μM	129 ± 8 (6)	P = NS vs (c)
Expt. 2			
a. Low $[Ca^{2+}]_i$	2 nM	205 ± 21 (9)	
b. Low $[Ca^{2+}]_i$ + spermine, 2 mM		208 ± 26 (10)	
c. Low $[Ca^{2+}]_i$ + staurosporine, 1 μM		203 ± 23 (10)	
d. High $[Ca^{2+}]_i$	275 μM	409 ± 18 (16)	
e. High $[Ca^{2+}]_i$ + spermine		154 ± 12 (16)	P < 0.001 vs (d)
f. High $[Ca^{2+}]_i$ + staurosporine		198 ± 18 (16)	P < 0.001 vs (d)
Expt. 3			
a. Low $[Ca^{2+}]_i$	47 nM	273 ± 31 (5)	
b. High $[Ca^{2+}]_i$	350 μM	562 ± 21 (9)	P < 0.001 vs (a)
c. Low $[Ca^{2+}]_i$ + mezerein, 2 μM	47 nM	693 ± 31 (5)	P < 0.001 vs (a)
d. Low $[Ca^{2+}]_i$ (PKC depleted)	47 nM	311 ± 16 (5)	
e. High $[Ca^{2+}]_i$ (PKC depleted)	350 μM	544 ± 28 (9)	P < 0.001 vs (d) P = NS vs (b)
f. Low $[Ca^{2+}]_i$ + mezerein, 2 μM (PKC depleted)	47 nM	258 ± 21 (5)	P = NS vs (d)
Expt. 4			
a. Low $[Ca^{2+}]_i$	5 nM	176 ± 17 (4)	
b. Low $[Ca^{2+}]_i$ + epinephrine, 1μM		177 ± 22 (4)	
c. Low $[Ca^{2+}]_i$ + TPA, 2 μM	5 nM	385 ± 26 (5)	
d. Low $[Ca^{2+}]_i$ + TPA, 2 μM + epinephrine		281 ± 16 (5)	P < 0.01 vs (c)
e. High $[Ca^{2+}]_i$	350 μM	307 ± 11 (7)	
f. High $[Ca^{2+}]_i$ + epinephrine		219 ± 27 (5)	P < 0.01 vs (e)
g. High $[Ca^{2+}]_i$ + TPA	350 μM	700 ± 53 (5)	
h. High $[Ca^{2+}]_i$ + TPA + epinephrine		252 ± 23 (4)	P < 0.001 vs (g)
Expt. 5			
a. Low $[Ca^{2+}]_i$	<1 nM	247 ± 9 (3)	
b. Low $[Ca^{2+}]_i$ + clonidine, 1 μM		258 ± 16 (4)	
c. Low $[Ca^{2+}]_i$ + TPA, 2 μM	<1 nM	388 ± 44 (5)	
d. Low $[Ca^{2+}]_i$ + TPA, 2 μM + clonidine		196 ± 17 (5)	P < 0.01 vs (c)
e. High $[Ca^{2+}]_i$	350 μM	394 ± 28 (7)	
f. High $[Ca^{2+}]_i$ + clonidine		253 ± 13 (6)	P < 0.01 vs (e)
g. High $[Ca^{2+}]_i$ + TPA	350 μM	722 ± 26 (5)	
h. High $[Ca^{2+}]_i$ + TPA + clonidine		355 ± 85 (4)	P < 0.01 vs (g)
Expt. 6			
a. Low $[Ca^{2+}]_i$	2 nM	199 ± 15 (4)	
b. Low $[Ca^{2+}]_i$ + epinephrine, 1 μM		175 ± 4 (4)	
c. Low $[Ca^{2+}]_i$ + TPA, 2 μM	2 nM	638 ± 39 (5)	
d. Low $[Ca^{2+}]_i$ + TPA, 2 μM + epinephrine		197 ± 8 (5)	P < 0.001 vs (c)
e. High $[Ca^{2+}]_i$	250 μM	376 ± 18 (7)	
f. High $[Ca^{2+}]_i$ + epinephrine		129 ± 10 (5)	P < 0.001 vs (e)
g. High $[Ca^{2+}]_i$ + TPA	250 μM	868 ± 43 (8)	
h. High $[Ca^{2+}]_i$ + TPA + epinephrine		321 ± 23 (5)	P < 0.001 vs (g)

* Islets in Expt. 1–5 were permeabilized using digitonin (20 μg/ml × 20 min). In Expt. 6, staphylococcal α-toxin (15 μg/ml × 20 min) was used.

† Data are means ± SE, the number of observations is given in parentheses.

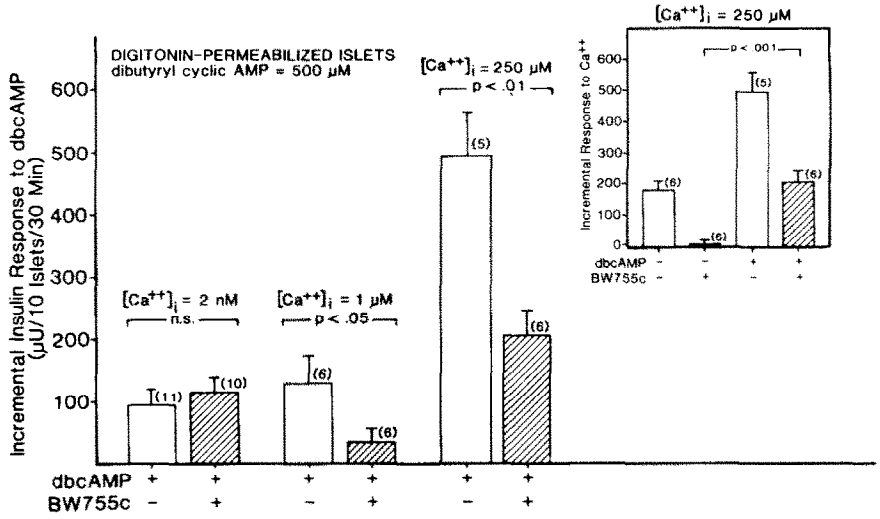

Fig. 4. Effect of BW755c (250 μM) on insulin release induced in the presence of dibutyryl cyclic AMP (dbcAMP, 0.5 mM) at various $[Ca^{2+}]_i$. All data are expressed as insulin release increments above basal control levels (no drug, or BW755c alone, at 2 nM $[Ca^{2+}]_i$ in the absence of dbcAMP). The inset depicts the effect of a $[Ca^{2+}]_i$ of 250 μM in the presence or absence of BW755c and/or dbcAMP. These results were seen in three separate experiments.

(50 μg/ml × 30 min), even 10 μM epinephrine did not reduce 12-HETE (99 ± 20% of control; N = 4 pairs), 11-HETE (137 ± 28%) or 15-HETE (101 ± 72% of control); likewise, in such islets, 1 μM clonidine did not reduce 12-HETE (100 ± 16% of control; N = 4 pairs) or 15-HETE (103 ± 36% of control) while tending to increase 11-HETE. Even in the presence of Ca^{2+} ($[Ca^{2+}]_i$ = 150 nM) and a higher substrate concentration (1.1 μM), clonidine (1 μM) did not reduce significantly 15-HETE (129 ± 9%), 11-HETE (75 ± 2%), or 12-HETE (75 ± 11% of control; all = 4 pairs each; all = NS) in digitonin-permeabilized islets; likewise, epinephrine (1 μM) did not reduce 15-HETE, 11-HETE or 12-HETE (117 ± 5% of control) formation in islets permeabilized using a lower concentration of α-toxin (15 μg/ml × 20 min).

DISCUSSION

The current studies were predicated upon our earlier studies using intact islets which demonstrated that islet LPX inhibitors [1] or α-adrenergic agonists [2] reduce insulin secretion induced by 50 mM K^+ (Ca^{2+} influx), 2 mM Ba^{2+} (mobilization of intracellular Ca^{2+}), 1 mM isobutylmethylxanthine (mobilization of both intracellular and extracellular Ca^{2+}) or 2 μM TPA (which potentiates the effects of Ca^{2+} without major changes in Ca^{2+} fluxes). During these studies, Ca^{2+} mobilization was assayed by measurements of $^{45}Ca^{2+}$ efflux; neither group of inhibitors systematically reduced Ca^{2+} fluxes. We inferred from such data that LPX inhibitors and α_2 agonists might reduce the sensitivity of the exocytotic apparatus to Ca^{2+}. The permeabilized islet is a more direct model with which to test such hypotheses, since $[Ca^{2+}]_i$ can be fixed through the use of EGTA in the medium which reaches the cytosol through pores in the plasma membrane. These are the first studies, to my knowledge, which assess the effects of LPX inhibitors in such "Ca^{2+}-clamped" cells in any cell system. However, similar data regarding the effect of α_2 agonists on insulin release have been obtained by others using permeabilized islet cells [35].

These studies confirm reports [8–10, 22, 36] showing that a 12-lipoxygenase exists in intact or homogenized islets. Assuming the absence of significant isotope effects or HETE degradation, a production rate of 4.5 fmol/islet/45 min was calculated, which is only modestly more than the synthesis (1.2 fmol/islet/30 min) estimated for intact human islets by mass spectrometric measurements [37]. The 12-HETE production was not due to auto-oxidation (as shown by its blockade using enzymic inhibitors and the use of islet-free auto-oxidation controls). Such 12-HETE synthesis has been shown previously to derive from islet cells, not from contaminating platelets or acinar cells, and its identity has been confirmed by gas chromatography–mass spectrometry [38, 39]. Since TPA or dibutyryl cyclic AMP stimulated, and α_2 agonists or protein kinase inhibitors reduced, insulin release but none of these altered HETE production, it is unlikely that 12-lipoxygenase activity is merely secondary to the release of secretory granules through the plasma membrane or the endocytosis of granule-associated fragments from the plasma membrane (i.e. membrane recycling). In contrast, unequivocal 5-lipoxygenase activity was not seen even when potential activating cofactors for 5-lipoxygenases such as Ca^{2+} and ATP [40, 41] were present. Others have observed small peaks eluting near 5-HETE and concluded, as did we, that such peaks probably do not represent authentic 5-HETE [9]. It is unlikely that higher levels of $[Ca^{2+}]_i$ would unmask more 5-HETE production since cytosolic free Ca^{2+} levels of 260 nM, 530 nM and 3.3 μM did

not further increase the sizes of the peaks eluting with 5-HETE compared to those at resting levels of 60–150 nM.

The islet 12-LPX did not seem to be regulated by physiologic increments in $[Ca^{2+}]_i$ but rather seemed to be maximal at a $[Ca^{2+}]_i$ near that of resting cells, with no apparent further rise at a $[Ca^{2+}]_i$ similar to that achievable in stimulated beta cells [13]. However, these results should be regarded with caution, since increasing cytosolic free Ca^{2+} concentrations may also decrease the bioavailability of intracellular AA (thereby tending to reduce artifactually the formation of metabolites of AA at higher $[Ca^{2+}]_i$, Refs. 11–13). It is also conceivable that saturating amounts of Ca^{2+} remained bound to the islet LPX despite the 65–75 min in a Ca^{2+}-free intracellular milieu during the permeabilization and incubation periods. Furthermore, studies using leaky cells cannot exclude effects of Ca^{2+} to partially reseal the islets [21, 42], to translocate enzymes like lipoxygenases to membranes [40] or induce their dimerization, thereby reducing their loss during the permeabilization procedure. Nonetheless, the limited studies thus far do not suggest that cytosolic Ca^{2+} concentration is a physiologically important regulator of islet 12-LPX activity. Indeed, the 12-lipoxygenases of bovine leukocytes [14], human platelets [43] or guinea pig epidermis [44] are largely Ca^{2+}-independent; only the 12-lipoxygenases of rat basophilic leukemia cells or uterine cervix have been demonstrated to be activated by Ca^{2+} [45, 46]. In these studies, we did not formally exclude the possibility that physiologic elevations in $[Ca^{2+}]_i$ may have qualitative effects on the disposition of 12-hydroperoxyeicosatetraenoic acid (12-HPETE), the labile precursor of 12-HETE. For example, it has been suggested that Ca^{2+} may impede the reduction of HPETE to HETE, favoring its degradation to more polar products [45] such as epoxyhydroxyeicosatrienoic acids and trihydroxyeicosatrienoic acids; it is also possible that HPETE was metabolized to more polar derivatives such as lipoxins, dihydroxy-fatty acids [47] or leukotriene analogs, as we previously suggested [48]. However, in preliminary studies, we have also failed to note any obvious stimulatory effect of physiologic levels of $[Ca^{2+}]_i$ on the more polar peaks eluting in the region expected of these metabolites (S. Metz, unpublished data). The islet 12-LPX also does not require the presence of ATP, as is the case with other 12-lipoxygenases [14], but in contradistinction to some 5-lipoxygenases, which are activated by ATP [49]. In addition, a phorbol ester, cyclic AMP, calmodulin, $G_{pp}(NH)_p$ or GTPγS failed to augment HETE production, and a protein kinase inhibitor did not reduce it. Thus, islet lipoxygenase activity seems to be constitutive, albeit presumably still dependent on substrate availability and tissue redox tone [15]. The apparently constitutive nature of the islet LPX adds more support to the formulation that the fuel-induced increase in [^{3}H]12-HETE synthesis in intact islets prelabeled with [^{3}H]arachidonate [8, 22] reflects an increase in the release, rather than in the lipoxygenation, of arachidonic acid.

Especially at higher substrate concentrations, other peaks, which co-eluted with 15-HETE and 11-HETE, seemed to become more prominent, an observation which may reflect the relatively low K_m for AA of 12-LPX [19, 43, 50] compared to those of other lipoxygenases [49, 50]. Interestingly, while earlier studies using intact islets prelabeled with [^{3}H]AA suggested that only a minute amount of 15-HETE is released by islets [8, 9], the current studies, as well as a study of islet homogenates [36], detected a more impressive peak co-migrating with 15-HETE when AA was directly provided to islets via permeabilization. Perhaps AA released by phospholipases in intact cells is poorly coupled to the enzyme converting it to 15-HETE, or possibly the endogenous level of AA in intact islets favors the formation of 12-HETE over other HETEs (*vide supra*). Indeed, a "cryptic" 15-lipoxygenase has been described in many cell types [51]. Alternatively, the putative 15-LPX might primarily peroxidize AA still esterified in the phospholipids of intact cells [10, 52]; we have postulated previously [10] that the effect of LPX action on insulin release may be due (at least in part) to such lipoxygenation of membrane phospholipids, rather than of "free" AA (which is conventionally thought of as the substrate for lipoxygenases). In keeping with such postulated membrane-association of the enzymes generating 15- or 12-HETE is the observation that very little LPX seemed to leak from digitonin- or α-toxin permeabilized cells, even though mammalian lipoxygenases have an M_r of <80 kDa [18, 19], and cytosolic proteins larger than that are known to leak from islet cells permeabilized by digitonin [7]. Others have suggested previously that 12-lipoxygenases, while largely cytosolic, may be in part associated with intracellular membranes [43, 44, 46, 53]; it is tempting to speculate that the secretory granule of islet cells may represent one such "binding" site. Further studies will be needed to substantiate or refute a membrane association or site of action for islet LPX.

The apparent reduction in 11-HETE (and possibly some 15-HETE) production by selective inhibitors of cyclooxygenase suggests that, as in other cells [23–26], a portion of this activity may have been cyclooxygenase-derived. Since both 15-HETE and 11-HETE can inhibit insulin release [1, 9, 36, 54], it is possible that these compounds mediate a part of the negative modulatory role of the cyclooxygenase pathway in insulin release (reviewed in Refs. 12 and 38). However, since the primary object of HETE measurements in the current studies was to ascertain whether permeabilized islets retained significant 12-lipoxygenase activity (which was inhibitable by specific test agents), we did not assess rigorously the identity or source of these two peaks further.

Three LPX inhibitors reduced both HETE formation and Ca^{2+}-dependent insulin release. Two of these (NDGA, BW755c) are anti-oxidants/peroxide scavengers but one (ETYA) is not; they also are structurally dissimilar. Thus, it seems reasonable to conclude that these drugs inhibit insulin release specifically by inhibiting LPX (as previously concluded from detailed studies of intact islets; Ref. 1) and not by an unforeseen non-specific effect. However, that possibility cannot be discounted totally. We stimulated insulin release at times with marked elevations in $[Ca^{2+}]_i$ in order to avoid any possibility that the drugs might merely chelate Ca^{2+};

we also wished to achieve an evaluable secretory response in digitonin-permeabilized cells whose response to Ca^{2+} is sluggish (probably due in part to leakage from the cell of important, potentiating cytosolic factors; Ref. 55). However, at such high micromolar concentrations it is possible that other cellular mediators were recruited, e.g. that islet phospholipase C was activated [31], inducing the accumulation of diacylglycerol. If this were true, it might not be possible to conclude that the LPX blockers were acting to blunt specifically the effects of Ca^{2+} on the exocytotic apparatus. Indeed, TPA (which substitutes for diacylglycerol as a direct activator of protein kinase C) was able to potentiate the effect of very high concentrations of Ca^{2+}, an interaction blunted by LPX inhibitors. However, the effect of high $[Ca^{2+}]_i$ alone was resistant to blockade by the specific depletion of protein kinase C; this finding indicates that, while protein kinase C may potentiate the effects of $Ca^{2+}{}_i$, its activation probably had not been recruited by the high cytosolic free Ca^{2+} concentrations used. Furthermore, NDGA does not inhibit islet phospholipase C [31] and so would not be expected to reduce the production of diacylglycerol, the endogenous activator of protein kinase C. Together, these data suggest that the effect of high $[Ca^{2+}]_i$ in these islets was not dependent on, or secondary to, activation of phospholipase C. Since LPX inhibitors did not reduce the effect of TPA at very low $[Ca^{2+}]_i$, it is also clear that they do not directly poison protein kinase C or exocytosis in general (i.e. Ca^{2+}-"independent" secretion was not blocked). Further evidence that LPX inhibitors do not directly block protein kinase C or globally inhibit exocytosis can be found in our earlier observations that the insulin release stimulated by unesterified fatty acids from intact or permeabilized islets (and which is dependent on the activity of protein kinase C) is not reduced by such inhibitors [11]. Furthermore, they do not block the insulin release induced by a number of other secretagogues in intact islets [1].

Another potential amplifier of Ca^{2+}-induced insulin release is cyclic AMP [56]. It is conceivable that LPX inhibitors reduce cyclic AMP accumulation, as we previously reported in neonatal islet cells [57]. However, the provision of 0.5 mM dibutyryl cyclic AMP did not prevent or reverse the ability of a LPX inhibitor to impede Ca^{2+}-activated secretion (Fig. 4). Dibutyryl cyclic AMP did promote insulin release in "Ca^{2+}-clamped", permeabilized cells as observed by others [56]; this effect was not blunted by LPX inhibitors in the near absence of $Ca^{2+}{}_i$ but it was reduced in the presence of micromolar levels of Ca^{2+}. Thus (as with protein kinase C) it appears that lipoxygenase blockers do not inhibit protein kinase A directly, but only its synergism with Ca^{2+}.

Our data using chemically-permeabilized islets are similar to those of others who studied digitonin-permeabilized islets [56], digitonin- or α-toxin-permeabilized chromaffin cells [21, 30] or digitonin-permeabilized neutrophils [42], in that only modest secretion is elicited by $[Ca^{2+}]_i < 10\,\mu M$ ($K_m \sim 2$–$3\,\mu M$); however, a second inflection in the Ca^{2+} dose–response curve occurs above $10\,\mu M$ [30] and is followed by progressive increases in secretion. Since this phenomenon is not seen when using cells permeabilized by high voltage [58], the limited secretion from digitonin-permeabilized cells at $[Ca^{2+}]_i < 10\,\mu M$ may reflect a loss of soluble factors (which are important to exocytosis) via the larger pores of chemically-permeabilized cells [55]. The secretion which is seen at cytosolic free Ca^{2+} concentrations above $10\,\mu M$ in this setting is not toxic, since it was inhibited by cooling (current data and [30], by α_2-adrenergic agonists, and by protein kinase inhibitors, and it is not accompanied (in adrenal medullary cells) by the release of cytoplasmic markers [30]. Lipoxygenase inhibitors reduced the effects of Ca^{2+} at both lower and high $[Ca^{2+}]_i$. The protein kinase inhibitors spermine, trifluoperazine or staurosporine (when provided in concentrations which are not selective for any one kinase) also obliterated such Ca^{2+}-induced secretion. Since LPX inhibitors reduced Ca^{2+}-dependent secretion but did not seem to directly block cAMP- or phospholipid-dependent protein kinases A and C, it is possible that they inhibit an unidentified protein kinase. A Ca^{2+}- and calmodulin-activated protein kinase is present in digitonin-permeabilized islets [7]; interestingly, the K_m for free Ca^{2+} of this protein kinase is about $2\,\mu M$, with saturation at greater than $100\,\mu M$ [59], levels which are similar to the concentrations of $Ca^{2+}{}_i$ inducing submaximal and maximal insulin release in our permeabilized islet preparation. However, exogenous calmodulin failed to potentiate, and calmodulin inhibitors did not reduce, Ca^{2+}-activated secretion. Fatty acids or their hydroperoxides can augment cyclic GMP production [60] and could activate a cyclic GMP-dependent protein kinase; however, exogenous cyclic GMP had no effect on insulin secretion. Thus, any protein kinase involved remains unidentified. Alternatively, Ca^{2+}-induced secretion could reflect the effects of divalent cations to promote the fusion of secretory granule membranes [61], possibly by neutralizing negative charges on secretory granules [62], or to the promotion of granule movement or aggregation. In fact, many Ca^{2+}- and phospholipid-binding proteins postulated to be important to exocytosis (such as synexin, lipocortin and calelectrin) promote aggregation of secretory granules half-maximally only at Ca^{2+} concentrations of 140–280 μM [63], the range of $[Ca^{2+}]_i$ used in part of the current study. Whatever the exact process involved, these findings together permit the formulation that LPX inhibitors antagonize the direct effects of Ca^{2+} (i.e. are "Ca^{2+} antagonists") and/or its interaction (synergism) with Ca^{2+}-activated processes (such as protein kinases). Further study will be needed to define their exact molecular mechanism of action.

Note added in proof. Recently, in preliminary studies, we ascertained that Ca^{2+}-induced insulin release from permeabilized islets is inhibited nearly totally by diethylcarbamazine (2.5–5.0 mM), similar to its inhibitory effect in intact islets [48]. Although putatively a selective leukotriene synthesis inhibitor, this drug was found to reduce 12-HETE formation by $40 \pm 9\%$ ($N = 5$) in permeabilized islets (S. Metz, unpublished data) and thus is a general 12-lipoxygenase blocker. A fifth putative lipoxygenase inhibitor, butylated hydroxytoluene, also reduces insulin release

in intact [1] or permeabilized islets (S. Metz, unpublished data).

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