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UNMASKING OF ARACHIDONATE-INDUCED INSULIN RELEASE BY REMOVAL OF EXTRACELLULAR CALCIUM

Arachidonic Acid Mobilizes Cellular Calcium in Rat Islets of Langerhans

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SUMMARY: Exogenous arachidonic acid does not stimulate insulin release in Ca^{++}-containing medium, but a potent effect was unmasked by extracellular Ca^{++} depletion. This secretion met several criteria of exocytotic release. It did not require the oxygenation of arachidonate or its esterification into islet membranes, but was potentiated by the presence of 16.7mM glucose such that 33μM arachidonate could reverse the inhibitory effects of extracellular Ca^{++} removal on glucose-induced insulin secretion. Arachidonic acid alone stimulated a rise in intracellular Ca^{++} concentrations in dispersed islet cells (measured by the fura-2 technique) equal to that induced by 16.7mM glucose in normal medium. Arachidonic acid may be a critical coupling signal in normal islets.

Inhibitors of 12-lipoxygenase (LPX) in rat pancreatic islets block glucose- or ketoisocaproate-induced insulin (I) release (1-4); in addition, such fuels stimulate the release of arachidonic acid (AA) and/or its LPX-derived metabolites (2,4-6). These findings have suggested a role for the release and oxidative metabolism of AA in nutrient-induced I secretion. However, exogenous provision of AA or its 12-LPX-derived metabolites has little or no effect on I secretion from intact, adult islets under a variety of conditions (2,4,6,7,8). To examine this issue further, we tested the effects of exogenous AA in medium deprived of extracellular Ca^{++}; we reasoned that, under these

ABBREVIATIONS

AA = arachidonic acid; I = insulin; LPX = lipoxygenase; Ca^{++}_i = intracellular calcium; HETE = hydroxyeicosatetraenoic acid (hydroxy-AA); HPETE = hydroperoxyeicosatetraenoic acid (hydroperoxy-AA); EGTA = ethyleneglycol-bis-(aminoethylether)-N,N'-tetra-acetic acid; TMB-8 = 8-(N,N-diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride; BW755c = 3-amino-1-(trifluoromethyl-phenyl)-2-pyrazoline; DTPA = diethylenetriaminepenta-acetic acid; F = fluorescence.

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conditions, intracellular Ca^{++} stores would become critical to I secretion and a role for AA might be unmasked.

MATERIALS AND METHODS

Isolation of Rat Islets and Assessment of Insulin Release

The methods for the isolation and incubation of intact rat islets and for the measurement of insulin by RIA have been reported in detail (2,5). Small aliquots (2-5 µl) of stock solutions of AA (Nu-Chek Prep, Elysian, MN), or its diluent (50% ethanol in 1% Na_2CO_3), were added to 750 of Krebs Ringer Bicarbonate buffer (with a composition as described in refs. 2,5) and vortexed into solution. BSA was excluded in order not to impede rapid uptake of AA into the islets. The sources of chemicals are provided in refs. 2,5. AA (100µM) had no effect on I recovery in the immunoassay.

Measurements of Free Cytosolic Calcium Concentration, $[Ca^{++}_i]$

Details of the fura-2 method have been described in detail previously (9,10). One thousand to 3000 islets were dispersed into individual cells in 2ml of Krebs/HEPES buffer, containing 5mg/ml BSA and 1.7mM glucose. Cell viability was over 85%, as determined by trypan blue exclusion. Calibration of fluorescence spectra for the indicator, fura-2 and its loading into the cells, was carried out as described by Grzynkiewicz et al. (9). To introduce fura-2 intracellularly, 5ul of 1.249mM fura-2 AM (Behring Diagnostics; San Diego, CA) dissolved in DMSO was added to 2ml of cell suspension to yield a fura-2 concentration of 3.11µM, and unincorporated probe was removed by centrifugation at 500xg (4°C). The fluorescence of control and fura-2 loaded islet cells ($1\text{-}3x10^5$ cells) was measured at excitation wavelengths of 347nm and 387nm and at an emission wavelength of 478nm using a Turner Model 430 spectro-fluorometer (fitted with a magnetic stirrer and a thermostatted cuvette holder). The ratio of the fluorescence (F_2) at 347/387 nM was then determined. The concentration of free intracellular Ca^{2+} was calculated from a previously published equation (9,10) (Method 1). Autofluorescence of buffer or cells was 1/8 - 1/10 that of fura-2 loaded cells and was unchanged by experimental perturbations (the addition of AA). The diluent used for the AA had no effect on $[Ca^{++}_i]$.

Because of concerns that islet cell viscosity may alter the values of cytosolic free calcium concentration obtained with this method (11), we performed additional experiments in which F_{max} and F_{min} were estimated as previously reported for quin-2 (12). In these experiments, the cells were lysed with 0.04% Triton X-100 and the F_{max} was measured in the presence of 1mM $CaCl_2$. The F_{min} was then measured in the presence of 2mM EGTA and 50mM Tris base (pH > 8.3). The fluorescence of the extracellular fura-2 was estimated by adding $MnCl_2$ (50uM) which quenches extracellular fura-2. $MnCl_2$ was then chelated by the addition of 100uM DTPA. The estimate of extracellular fura-2 was made both prior to and after stimulation of the cells with secretagogues. The results obtained with this method (method II) are compared with the results obtained by the ratio method (method I) in Table 2, with the results being qualitatively similar but varying between the two by a relatively constant ratio, as observed previously in PtK_1 cells (11).

RESULTS AND DISCUSSION

Effects of Exogenous AA on Insulin (I) Release

AA potently triggered I release at a substimulatory glucose concentration (1.7mM) in Ca^{++}-free medium (no $CaCl_2$ added; 200µM of EGTA was present) (Fig. 1 and Table 1). The threshold effect of AA was generally between 25 and 33µm and saturation was reached by 262µM AA (Fig. 1). The addition of EGTA was not required to demonstrate a stimulatory effect of AA, although EGTA magnified the effect of omission of added

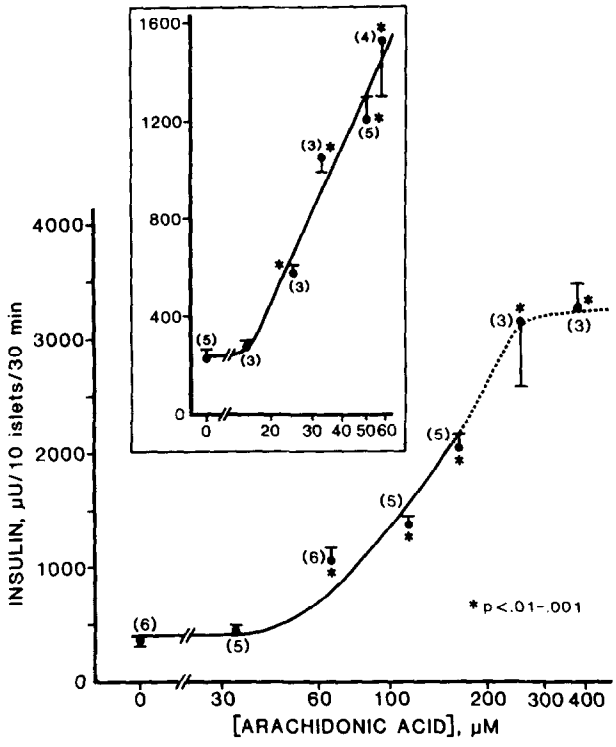

Figure 1. Concentration-response curves for AA-induced I release at 1.7mM glucose. In the main panel, the data using higher concentrations (to demonstrate saturability of effect) are shown as a dashed line since they were obtained from studies on a separate day. Data in the inset provide detailed data at lower AA concentrations to provide a better estimation of the threshold concentration inducing I release. Numbers in parentheses = number of replicate tubes/condition. *indicates p < .01 - .001 by non-paired t testing.

Ca^{++} alone. AA was synergistic with a high concentration of glucose (16.7mM, still in Ca^{++}-free medium) at AA concentrations between 1.4 and 49μM (e.g., Table 1; Experiments 1 and 2). In fact, AA was now able to augment I release at concentrations at least as low as 11μM, and exogenous AA (33μM) could reverse the inhibition of glucose-induced I release induced by Ca^{++}-free medium (Table 1, Experiment 2). Extracellular Ca^{++} removal is associated with inhibition of glucose-stimulated release of endogenous AA from islets (13) and a concomitant reduction of the I release induced by 16.7mM glucose by 70-85%; exogenous AA returned I release to levels as least as high as those normally seen at 16.7mM glucose in Ca^{++}-containing media (glucose 1.7mM = 96±7, n=63; glucose, 16.7mM = 1076±31, n=79). Concentrations of AA of 49μM or higher were only submaximally additive to the effects of the 16.7mM glucose.

We have demonstrated (14) that 200μM p-hydroxymercuribenzoic acid (PHMB) blocks the esterification of exogenous AA into the phospholipids of intact islets and

Table 1. Effect of AA on Insulin Release in the Absence of Extracellular Ca^{++} and in the Presence or Absence of Various Potential Inhibitors

	INSULIN	
EXPERIMENT ONE		
a. control (1.7mM glucose)	201±41	(4)
b. control + AA, 11μM	152±22	(4)--p = n.s. vs. (a)
c. control (16.7mM glucose)	369±35	(5)
d. control + AA, 11μM	481±28	(5)--p < .05 vs. (c)
EXPERIMENT TWO		
a. control (1.7mM glucose)	109±23	(4)
b. control + AA, 33μM	537±25	(3) increment = 428
c. control (16.7mM glucose)	316±46	(3)
d. control + AA, 33μM	1374±192	(4) increment = 1058; p < .05 vs. increment at 1.7mM glucose
EXPERIMENT THREE		
a. control (1.7mM glucose)	343±47	(4)
b. AA, 16μM	239±13	(4)--p = n.s. vs. (a)
c. AA, 25μM	418±58	(4)--p = n.s. vs. (a)
d. AA, 33μM	606±167	(3)--p = n.s. vs. (a)
e. p-hydroxymercuribenzoic acid (PHMB, 200μM)	1164±34	(4)
f. PHMB + AA, 16μM	1417±149	(5)--p = n.s. vs. (e)
g. PHMB + AA, 25μM	1892±210	(4)--p < .02 vs. (e)
h. PHMB + AA, 33μM	2611±263	(4)--p < .01 vs. (e)
EXPERIMENT FOUR		
a. control (37°C)	303±8	(3)
b. control (16°C)	149±21	(7)
c. AA, 82μM (37°C)	1354±91	(6)
d. AA, 82μM (16°C)	237±22	(7)--p < .001 vs. (c)
EXPERIMENT FIVE		
a. control (1.7mM glucose)	103±16	(6)
b. control + dantrolene, 100μM	99±20	(6)
c. AA, 66 μM	922±217	(5)
d. AA + dantrolene	390±23	(5)--p < .05 vs. (c)
EXPERIMENT SIX		
a. control (1.7mM glucose)	257±78	(2)
b. control + trifluoperazine, 50μM	309±70	(2)
c. AA, 66μM	1319±161	(5)
d. AA + trifluoperazine	367±67	(5)--p < .001 vs. (c)
EXPERIMENT SEVEN		
a. control (NaCl present)	173±22	(4)
b. control (-118mMNaCl; +118mM choline chloride)	157±26	(5)
c. AA, 66μM (NaCl present)	948±154	(6)
d. AA, 66μM (-NaCl; + choline chloride)	1281±127	(6)

Insulin release was determined during static, batch-type incubations over a 30-min period except where indicated. Glucose = 1.7mM except where indicated. Values are means ± SEM and are expressed as μU insulin/10 islets/incubation. The numbers in parentheses indicate the number of replicate tubes/condition. Statistical analysis is by non-paired t test. AA = arachidonic acid.

thereby triggers I secretion. Pretreatment of islets with PHMB potentiated the releasing action of AA (Table 1; Experiment 3), reducing the threshold at which AA was effective to between 16 and 25μm. Thus, during coincubation with PHMB or in the presence of

16.7mM glucose, the effective concentrations of AA in intact islets approach those used by Wolf et al. (6) to release Ca^{++} from the endoplasmic reticulum or mitochondria of permeabilized islets. Furthermore, those authors have estimated (6) that 28mM glucose may produce endogenous concentrations of unesterified arachidonate in intact islets several times higher than concentrations which were effective in the current study. Thus the concentrations of AA used may be physiologically relevant. These data also suggest that AA was acting intracellularly and only after it bypassed the membrane barrier of intact islets.

In addition, the effect of AA seemed to involve stimulation of exocytosis, and not a a non-specific, detergent effect, since it: 1) was saturable (Fig. 1); 2) was inhibited by reduced ambient temperature (Table 1; Exp. 4), which blocks exocytotic I release late in the cascade of stimulus-secretion coupling (15); 3) did not affect islet ability to exclude trypan blue; 4) does not lead to a generalized increase in islet permeability (6) or induce permeation of fura-2 (a molecule of lower molecular weight than insulin) (vide infra); and 5) was not reproduced by another cis-unsaturated fatty acid, oleic acid, even when used at higher concentrations of 106μM or 150μM.

Dantrolene (which inhibits the release of endoplasmic reticular Ca^{++} pools; ref. 16) did blunt (by 65±3%) I release elicited by AA (Table 1, Experiment 5). Pretreatment of the islets with 1mM ouabain to maximally block (Na^+-K^+)-ATPase did not reduce the subsequent releasing action of AA (data not shown), suggesting that AA did not act by inhibiting islet (Na^+-K^+)-ATPase. AA may activate protein kinase C in several cell types (17) and may interact with that kinase in the islet (8). Interestingly, trifluoperazine, an inhibitor of calmodulin- and phospholipid-dependent protein kinases (18), blunted the effect of AA by 93±6% (Table 1, Experiment 6).

Effects of AA on Cytosolic Free Ca^{++} Concentration, $[Ca^{++}_i]$

AA also led to a dose-responsive increase in $[Ca^{++}_i]$, in Ca^{++}-free medium, at 1.7mM glucose, in dispersed islet cells; these responses were comparable to those elicited by 16.7mM glucose alone in the presence of extracellular Ca^{++} (Table 2). AA (up to 162μM) did not cause fura-2 to leak from the islet cells, as assessed in two ways: 1) failure of $MnCl_2$, which inhibits extracellular Ca^{++}-fura-2 complexing (19), to reduce fluorescence; 2) failure to detect increased fluorescence (compared to control incuba-

Table 2. Effect of Arachidonic Acid, Oleic Acid, Potassium and Glucose on Cytosolic Free Ca^{++} Concentrations, $[Ca^{++}_i]$

	$[Ca^{++}_i]$			
	Method One		Method Two	
EXPERIMENT ONE (Ca^{++}-free medium; EGTA = 1mM; glucose = 1.7mM)				
a. basal	50±5	(5)		
b. arachidonic acid, 90μM	93±6*	(5)		
c. arachidonic acid, 180μM	114±6*	(5)		
a. basal			54±8	(4)
b. arachidonic acid, 82μM			262±9**	(4)
EXPERIMENT TWO (Ca^{++} = 1mM; No EGTA)				
a. basal (1.7mM glucose)	44±2	(20)		
b. 16.7mM glucose	118±5**	(20)		
a. basal (1.7mM glucose)			105±16	(3)
b. 16.7mM glucose			263±18**	(3)

Cystolic free Ca^{++} concentrations, $[Ca^{++}_i]$, were determined by the fura-2 method as described in "Methods". Calibration of the signal was by either of two techniques, as described under "Methods". Basal $[Ca^{++}_i]$ was determined and then an aliquot of a concentrated stock solution of the test agent was added to the cuvette to achieve the reported concentration for a 10 min incubation. Values are mean ± SEM, with statistical comparison by t testing. The numbers in parentheses = the number of experiments performed. *$p < .01$, **$p < .001$ vs. basal.

tions) after spinning down the islets and examining the supernatant. In preliminary studies, oleic acid was observed to have a modest, saturable effect but this was clearly much less than that of AA: basal = 40±2nM, n=4; oleate, 75μM = 52±2nM, n=2; oleate, 145μM = 54±0.2nM, n=2 (cf. Table 2, Method 1).

Independence of AA-induced Effects from its Oxygenation

The effect of AA on I release was totally resistant to blockade by any of three islet LPX inhibitors (1-4): nordihydroguaiaretic acid, 50μM, butylated hydroxytoluene, 25μM, or BW755c, 500μM, as well as to blockade of cyclo-oxygenase by ibuprofen, 20μg/ml or indomethacin, 2μg/ml (data not shown). Two inhibitors of cytochrome P450-dependent "epoxygenases" (20)--metyrapone (200,500 or 2000μM) or SKF525a (50,250 or 500μM)--also failed to show consistent inhibition. Nordihydroguaiaretic acid (50μM) had no effect on the rise in $[Ca^{++}_i]$ elicited by AA (data not shown). It is possible that AA induced secretion via its contamination by auto-oxidation products. However, we assessed the

content of hydroperoxy- or hydroxy-AA (HPETE or HETE) in our stock solutions of AA, using an HPLC system previously described by us (5). Such studies revealed absolutely no correlation between the minor content of oxygenated products (as determined by UV peak height in comparison to standards of authentic 5-, 12-or 15-HETE) and the I release assessed on the same day in parallel studies.

The mechanism of the finding that depletion of extracellular Ca^{++} unmasks the ability of exogenous AA to promote I secretion at low glucose concentrations is not fully clarified by these studies. One interpretation is that, under these conditions, cytoplasmic Ca^{++} concentrations are maintained by fluxes from intracellular stores and that AA promotes such fluxes. Extracellular Ca^{++} could also non-specifically impede the action of exogenous AA by blocking its access to critical cellular sites, as has been reported for other biologic systems (21,22). Such blockade of the effects of AA by extracellular Ca^{++} (or by other cations such as Ni^{++} or Ba^{++}; data not shown) might be attributed to cross-linking of carboxylic fatty acids by such cations, leading to formation of films, dimers or aggregates (23-26) which are excluded from critical compartments of the beta cell. Lastly, AA could, via a membrane action, open ionic micropores (27) in the membrane, leading to Na^{+} influx, with consequent Na/Ca^{++} exchange (28) at intracellular sites (leading to a rise in free cytoplasmic Ca^{++} concentrations). Under certain conditions, extracellular Ca^{++} can stabilize membranes and impede such Na^{+} influx (27,29). However, removal of NaCl from the buffer and replacing it with choline chloride failed to similarly reduce arachidonate-induced I release (Table 1, Experiment 7).

Whatever the exact mechanism for this phenomenon, the observation that exogenous AA acutely stimulates I release only in Ca^{++}-free medium may have considerable importance to our understanding of the role of lipoxygenase in I release. While virtually all investigators agree that LPX inhibitors abrogate glucose-induced I release, no study has convincingly identified the active LPX-derived metabolite of AA in intact adult rat islets, using authentic metabolites provided exogenously in Ca^{++}-containing medium (reviewed in ref. 7). Clearly these studies need to be re-examined in light of the current findings.

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