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The Cyclic AMP–Protein Kinase A Pathway Restrains Islet Phospholipase A_2 Activation

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Although phospholipase A_2 (PLA_2) is of importance for insulin secretion, it is not established how it relates to other signalling mechanisms. This study examined the crosstalk between PLA_2 and the cyclic AMP (cAMP)–protein kinase A (PKA) pathway in isolated rat islets. Forskolin, IBMX, and dbcAMP reduced [^{3}H]arachidonic acid ([^{3}H]AA) efflux from prelabelled islets during PLA_2 activation by mellitin or cholecystokinin (CCK-8), while efflux induced by carbachol was unaffected. The PKA inhibitor myrPKI_{14-22} prevented this reduction of CCK-8-induced efflux. Glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP), and vasoactive intestinal polypeptide (VIP) diminished CCK-8-induced efflux. Also in the absence of Ca^{2+}, forskolin/IBMX and dbcAMP reduced CCK-8-induced efflux. In parallel with effects on [^{3}H]AA, the expected additive insulin secretion induced by mellitin or CCK-8 in combination with forskolin or GLP-1, respectively, was reduced. In conclusion, the cAMP–PKA pathway restrains both Ca^{2+}-dependent and Ca^{2+}-independent PLA_2 activation, indicating a regulating crosstalk between these two pathways. © 2000 Academic Press

Key Words: **phospholipase A_2; arachidonic acid; cyclic AMP; protein kinase A; crosstalk; interaction, islet; β-cell; insulin secretion.**

Phospholipase A_2 (PLA_2) activation with subsequent formation of arachidonic acid (AA) is an important signalling mechanism mediating a variety of regulatory influences in different cell systems, where both extracellular and cytosolic PLA_2 forms are known to exist (1, 2). The presence of the cytosolic PLA_2 ($cPLA_2$) in pancreatic islet β-cells is evident by demonstrations of (i) $cPLA_2$ immunoreactivity (3, 4), (ii) $cPLA_2$ activity (5), and (iii) accumulation of arachidonic acid or lysophosphatidylcholine, markers for $cPLA_2$ activation (6–8). In fact, in the β-cell, both Ca^{2+}-*dependent* (5, 9) and Ca^{2+}-*independent* ($iPLA_2$; 10, 11) $cPLA_2$ forms exist. Furthermore, $cPLA_2$ is of functional importance for glucose-induced insulin secretion since (i) islet $cPLA_2$ activity accompanies increased glucose metabolism (9, 12), (ii) pharmacological $cPLA_2$ inhibition markedly blocks glucose-induced insulin secretion (13, 14), and (iii) exogenously provided $cPLA_2$ promotes the release of insulin (15).

In rat islets, $cPLA_2$ is activated also by the cholinergic agonist carbachol and the C-terminal octapeptide of cholecystokinin (CCK-8; 7, 8, 16). Whereas carbachol activates only the Ca^{2+}-*dependent* $cPLA_2$, CCK-8 stimulates both the Ca^{2+}-*dependent* and the Ca^{2+}-*independent* forms (8). The role of $cPLA_2$ in insulin secretion is, however, largely unknown. One significant factor underlying $cPLA_2$ activation in a number of cell systems, including islets, is protein kinase C (PKC; 1, 8, 17, 18). A possible crosstalk also with the cyclic AMP (cAMP)-protein kinase A (PKA) pathway has not been clarified. Such a crosstalk would be of importance for β-cell function due to the central position of these two insulin-releasing mechanisms in the cellular action of neural and hormonal factors (19, 20), and the possibility of using cAMP-raising insulin secretagogues, like glucagon-like peptide-1 (GLP-1), in treatment of type 2 diabetes (21, 22). Therefore, this study investigated the presence of a regulating interaction between the PLA_2-AA and the cAMP-PKA signalling pathways by using isolated rat islets.

RESEARCH DESIGN AND METHODS

Animals. The study was performed on pancreatic islets isolated from normal adult male Sprague-Dawley rats (Möllegaard A/S, Ll. Skensved, Denmark) weighing 200–300 g, with free access to standard pellet diet and tap water.

Isolation and labelling of islets. Islets were isolated by the collagenase digestion technique (23). In brief, after ligation of the papilla Vateri and cannulation of the common bile duct, the pancreas was retrogradely filled through the cannula with 10 ml cold Hanks' Balanced Salt Solution (Sigma Chemical Co., St. Louis, MO), supplemented with 1.0 mg/ml Collagenase P (activity: 1.52 U/mg; Boehringer Mannheim Gmbh, Mannheim, Germany). The pancreas was

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subsequently removed and incubated for 24 min at 37°C. After rinsing in Hanks' solution, islets were hand-picked and incubated overnight, at 37°C in an atmosphere of 95% air and 5% CO_2 (pH 7.4), in 10 ml RPMI 1640 medium, supplemented with 10% heat-inactivated foetal calf serum, 2.05 mM L-glutamin, 2.5 μg/ml amphotericin B (all GIBCO BRL, Paisley, Scotland, UK), 100 IU/ml penicillin, 100 μg/ml streptomycin (both Biological Industries, Beit Haemek, Israel) and 4 μCi of [^{3}H]AA (specific activity: 100 Ci/mmol; DuPont NEN Products, Boston, MA).

Studies on AA efflux. Following overnight incubation, islets were rinsed in a modified HEPES medium (pH 7.4) consisting of 125 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.28 mM $CaCl_2$ and 25 mM HEPES (Boehringer Mannheim), supplemented with 0.1% human serum albumin (HSA; Behringwerke, Marburg, Germany) and 3.3 mM glucose (Fluka Chemie AG, Buchs, Switzerland). The islets were then preincubated for 30 min (37°C; pH 7.4; 5% CO_2) in the HEPES medium, supplemented as above. Thereafter, groups of 25 islets were transferred into separate chambers for 30 min of experimental incubation (37°C; pH 7.4; 5% CO_2) in the HEPES medium supplemented with 0.1% HSA and 5.6 mM glucose, with or without addition of synthetic sulphated CCK-8 (the C-terminal octapeptide of cholecystokinin; $CCK_{26\text{-}33}$; 100 nM; Sigma), carbachol (carbamoyl choline chloride; 100 μM; BDH Chemicals Ltd., Poole, England), GLP-1 (glucagon-like peptide-$1_{(7\text{-}36)}$amide; 100 nM), GIP (gastric inhibitory polypeptide; 100 nM), VIP (vasoactive intestinal polypeptide; 100 nM), forskolin (1 μM), dbcAMP (dibuturyl cyclic AMP; 1 mM), IBMX (3-isobutyl-1-methyl-xanthine; 0.1 mM), mellitin (2 μg/ml; all Sigma) and/or *myr*$PKI_{14\text{-}22}$ (10μM; Biomol, Plymouth Meeting, PA). The studies under Ca^{2+}-free conditions was, both during preincubations and experimental incubations, performed in a $CaCl_2$-deficient medium supplemented with 0.1 mM of the calcium chelator EGTA (ethyleneglycol-bis(β-aminoethyl ether)N,N^1-tetra acetic acid). After incubation, 500 μl of the medium were taken from each chamber and the radioactivity content determined by liquid scintillation counting, using scintillation cocktail Optiphase (Wallac Oy, Turki, Finland). Also the 25 islets were retrieved and their radioactivity content determined. The radioactivity in the medium that was removed was expressed as a percentage of the total islet radioactivity content.

Studies on insulin secretion. Islets were isolated and incubated overnight as above, with the exception that [^{3}H]AA was not added to the medium. Thereafter, the islets were preincubated for 60 min in HEPES with 0.1% HSA and 3.3 mM glucose (37°C, 5% CO_2, pH 7.4), and then transferred in groups of 3 into separate chambers and incubated for 60 min in 200 μl HEPES with 70.1% HSA and 5.6 mM glucose (37°C, 5% CO_2, pH 7.4), with or without mellitin, forskolin, CCK-8 and/or GLP-1. From each chamber, 25 μl were analysed for its content of insulin.

Analysis of insulin. Insulin levels were determined radioimmunochemically by using a guinea pig anti-rat insulin antibody, mono[^{125}I]-labelled human insulin as tracer, and rat insulin as a standard (Linco Research, St. Charles, MO). The separation of free and bound radioactivity was performed with double antibody technique.

Calculations and statistics. All values are presented as the mean ± standard error of the mean. The *net* [^{3}H]AA efflux and insulin secretion induced by each agent is calculated by subtracting the value of control islets, incubated with glucose alone, from the value of islets incubated with glucose and the agent together. Student's *t* test for unpaired data was used for statistical evaluation. A p-value less than 0.05 was considered significant. The abbreviation "ns" means no significant difference, while "n" means number of observations.

RESULTS

Effects of forskolin, dbcAMP, or IBMX on mellitin-induced PLA_2 activation. The PLA_2 stimulator mellitin (2 μg/ml) increased [^{3}H]AA efflux by 334 ± 10% ($p < 0.001$; n = 8). After subtracting values of control islets incubated with 5.6 mM glucose alone from values of islets incubated with glucose and mellitin together, we found that the adenylate cyclase stimulator forskolin (1 μM), the cAMP analogue dbcAMP (1 mM) and the phosphodiesterase inhibitor IBMX (0.1 mM) decreased *net* mellitin-induced efflux by 41 ± 10% ($p = 0.001$; n = 8), 46 ± 10% ($p < 0.001$, n = 8) and 47 ± 11% ($p = 0.001$; n = 8; Fig. 1; upper panel), respectively.

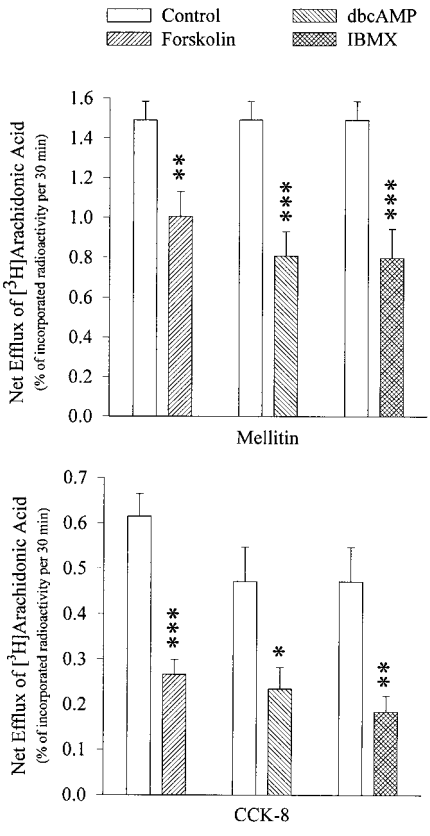

FIG. 1. Fractional efflux of [^{3}H]AA from prelabelled isolated islets incubated in the presence of glucose (5.6 mM), and mellitin (2 μg/ml; upper panel) or CCK-8 (100 nM; lower panel), with or without addition of forskolin (1 μM), dbcAMP (1 mM) or IBMX (0.1 mM), after subtracting controls incubated with glucose alone. Asterisks indicate the probability level of random difference between groups. n = 6–9. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Effects of forskolin, dbcAMP, or IBMX on CCK-8- or carbachol-induced PLA_2 activation. CCK-8 (100 nM) and carbachol (100 μM) both increased efflux of [^{3}H]AA, by 190 ± 13% ($p < 0.001$; n = 21) and 203 ± 16% ($p < 0.001$; n = 24), respectively. The *net* CCK-8-induced efflux of [^{3}H]AA was inhibited by forskolin (1 μM) by 57 ± 10% ($p < 0.001$; n = 8), by dbcAMP (1 mM) by 57 ± 19% ($p = 0.025$; n = 6) and by IBMX (0.1 mM) by 61 ± 18% ($p = 0.006$; n = 6; Fig. 1, lower panel). In contrast, the *net* [^{3}H]AA efflux stimulated by carbachol was unaffected by forskolin, dbcAMP as well as IBMX (ns; n = 5–9).

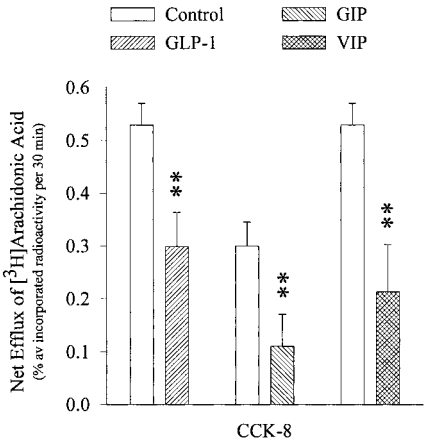

FIG. 2. Fractional efflux of [^{3}H]AA from prelabelled isolated islets incubated in the presence of glucose (5.6 mM) and CCK-8 (100 nM), with or without addition of GLP-1 (100 nM), GIP (100 nM) or VIP (100 nM). Efflux values of control islets incubated with glucose alone are subtracted. Asterisks indicate the probability level of random difference between groups. n = 8 in both panels. $^{**}p < 0.01$.

Effects of GLP-1, GIP, or VIP on PLA_2 activation. The cAMP-PKA pathway-activating insulin secretagogues GLP-1 (100 nM), GIP (100 nM) or VIP (100 nM) were unable to stimulate [^{3}H]AA efflux under baseline conditions (ns; n = 8). In contrast, these three peptides all reduced *net* CCK-8-induced efflux of [^{3}H]AA, by 47 ± 12% ($p = 0.002$; n = 8), 44 ± 14% ($p = 0.009$; n = 8) and 59 ± 15% ($p = 0.002$; n = 8; Fig. 2), respectively.

Effects of PKA inhibition on forskolin/IBMX- or dbcAMP-mediated reduction of CCK-8-induced PLA_2 activation. The *net* CCK-8-induced efflux of [^{3}H]AA was inhibited by a forskolin/IBMX combination (1 μM/0.1 mM) by 58 ± 14% ($p = 0.003$; n = 5), and by dbcAMP (1 mM) by 61 ± 15% ($p = 0.003$; n = 5; Fig. 3). In the presence of the PKA inhibitor *myr*PKI_{14-22} (10 μM), forskolin/IBMX combination ordbcAMP did not affect the *net* CCK-8-induced [^{3}H]AA efflux (ns; n = 5; Fig. 3).

Effects of calcium omission on the actions of forskolin, IBMX/dbcAMP, or GLP-1 on CCK-8-induced PLA_2 activation. In an incubation medium devoid of Ca^{2+} and supplemented with EGTA (0.1 mM), a forskolin/IBMX combination (1 μM/0.1 mM), dbcAMP (1 mM), and GLP-1 (100 nM) did not affect [^{3}H]AA (ns; n = 12) in the sole presence of glucose. However, both forskolin/IBMX and dbcAMP reduced *net* CCK-8-induced efflux of [^{3}H]AA, by 76 ± 36% ($p = 0.049$; n = 12) and 79 ± 37% ($p = 0.042$; n = 12; Fig. 4), respectively, In contrast GLP-1 did not affect the CCK-8-induced efflux of [^{3}H]AA (ns; n = 12; Fig. 4).

Effects of forskolin or GLP-1 on mellitin- or CCK-8-induced insulin secretion. Mellitin-induced (2 μg/ml) and CCK-8 (100 nM)-induced insulin secretion was potentiated by forskolin (1 μM) and GLP-1 (100 nM) by 94 ± 19% ($p < 0.001$; n = 16) and 36 ± 9% ($p =$ 0.007; n = 24), respectively. Likewise, the insulin secretion induced by the sole presence of glucose was potentiated by forskolin and GLP-1 by 447 ± 80% ($p < 0.001$; n = 16) and 145 ± 36% ($p < 0.001$; n = 24), respectively. However, forskolin as well as GLP-1 reduced the *net* mellitin- and CCK-8-induced secretion by

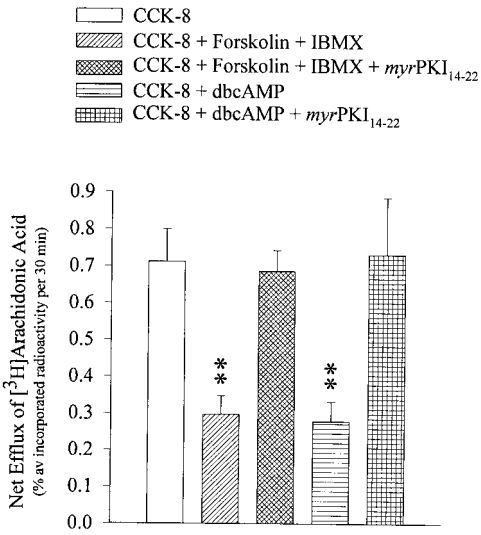

FIG. 3. Fractional efflux of [^{3}H]AA from prelabelled isolated islets incubated with glucose (5.6 mM) and CCK-8 (100 nM), with or without addition of a forskolin/IBMX (1 μM/0.1 mM) combination or dbcAMP (1 mM), in the presence and absence of *myr*PKI_{14-22} (10 μM). The results are shown after subtraction of controls incubated with glucose alone. Asterisks indicate the probability level of random difference between the effects of CCK-8 solely vs CCK-8 and *myr*PKI_{14-22} together. n = 5. $^{**}p < 0.01$, $^{***}p < 0.001$.

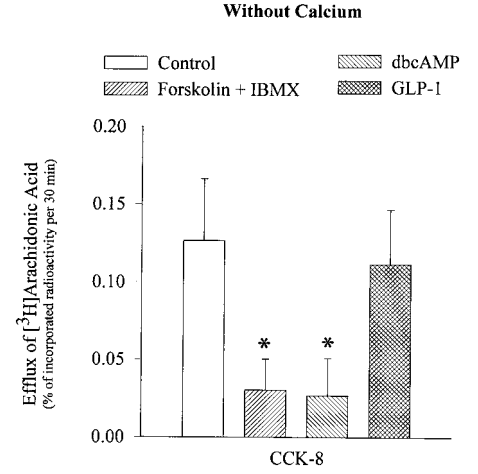

FIG. 4. Fractional efflux of [^{3}H]AA from prelabelled isolated islets incubated in the absence of extracellular Ca^{2+} and after addition of EGTA (0.1 mM). The incubation medium was constituted of glucose (5.6 mM) and CCK-8 (100 nM), with or without the addition of a forskolin/IBMX (1 μM/0.1 mM) combination, dbcAMP (1 mM) or GLP-1 (100 nM). The results are presented after subtracting controls incubated with glucose alone. Asterisks indicate the probability level of random difference between groups. n = 8 in both panels. $^{*}p < 0.05$.

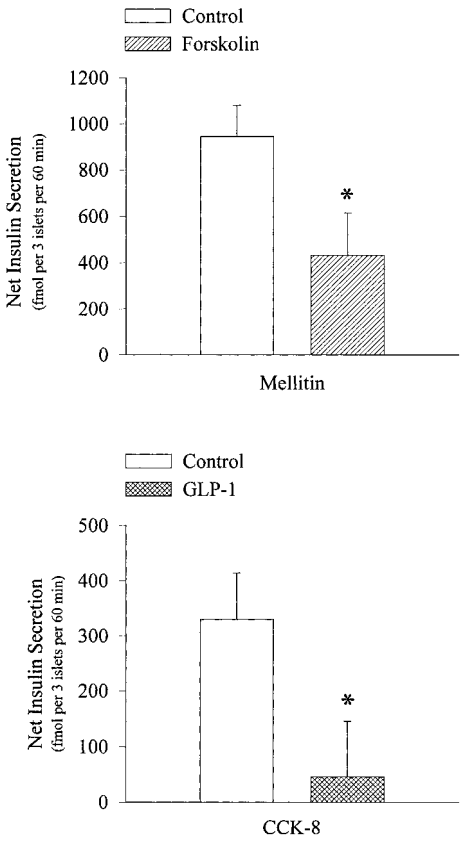

FIG. 5. Upper panel shows insulin secretion from isolated islets incubated in the presence of glucose (5.6 mM) and mellitin (2 μg/ml), with or without the addition of forskolin (1 μM). Lower panel shows insulin secretion in the presence of glucose and CCK-8 (100 nM), with or without GLP-1 (100 nM). The results are presented after subtracting insulin secretion values of control islets incubated with glucose alone. Asterisks indicate the probability level of random difference between groups. n = 16–24. *$p < 0.05$.

54 ± 24% ($p = 0.032$; n = 16; Fig. 5, upper panel) and 86 ± 40% ($p = 0.035$; n = 24; Fig. 5, lower panel), respectively.

DISCUSSION

The use of isolated islets prelabelled with [^{3}H]AA has been shown by Konrad *et al.* to reflect PLA_2 activation (7). Furthermore, we have previously through TLC shown that the radioactivity released from islets during CCK-8 stimulation is [^{3}H]AA, and that the radioactivity in the specific AA band is increased by CCK-8, supporting the validity of this technique (8). In addition, we have also shown that lysophosphatidylcholine accumulation from islets prelabelled with [^{3}H]palmitidic acid, which also reflects PLA_2 activity, is increased by CCK-8, and that pharmacological inhibition of CCK-8-induced PLA_2 activation reduces [^{3}H]AA efflux, showing that also CCK-8, like earlier known for glucose and carbachol (7), stimulates islet PLA_2 (8). In the present study, we examined whether the cAMP/PKA pathway affects PLA_2 activation in islets. In our first series of experiments, we directly stimulated PLA_2 by the honey bee venom constituent mellitin. This is known to cause accumulation of AA that is released from phosphatidylcholine, indicating a specific activation of $cPLA_2$ without involvement of the phospholipase C (PLC)-diacylglyceride lipase pathway (24). Expectedly, therefore, mellitin increased [^{3}H]AA efflux from the labelled islets. The main finding was, however, that the *net* mellitin-induced AA efflux was markedly decreased by three different cAMP-PKA-stimulating approaches, i.e. forskolin, dbcAMP and IBMX. Furthermore, also [^{3}H]AA efflux stimulated by CCK-8 was markedly reduced by these agents. Moreover, CCK-8-induced [^{3}H]AA efflux was inhibited also by GLP-1, GIP and VIP, which are regulatory peptides all known to increase cAMP in islets (25). Finally, the inhibitor of PKA, *myr*PKI_{14-22}, which inactivates the catalytic subunit of PKA (26), prevented forskolin/IBMX and dbcAMP from inhibiting CCK-8-induced [^{3}H]AA efflux. These results therefore indicate that the cAMP-PKA signalling pathway inhibits the PLA_2-AA pathway in islets.

CCK-8 and carbachol activate different subtypes of PLA_2 in islets, in that both agents activate Ca^{2+}-*dependent* PLA_2, whereas only CCK-8 activates Ca^{2+}-*independent* PLA_2 (8). Our findings that forskolin, dbcAMP and IBMX failed to inhibit carbachol-induced PLA_2 activation therefore suggests that the restraining action of cAMP-PKA on PLA_2 involves the Ca^{2+}-*independent* PLA_2 subtype. This is further supported by the finding that forskolin/IBMX and dbcAMP inhibited CCK-8-induced [^{3}H]AA also in the absence of extracellular Ca^{2+}. Ca^{2+}-*independent* PLA_2 ($iPLA_2$) represents approximately half of total PLA_2 activity and has been shown to be activated by glucose metabolism-generated ATP and to amplify glucose-induced insulin secretion (5, 10, 11, 27). In contrast, the restraining effect due to GLP-1, that was seen in the presence of extracellular Ca^{2+}, was abolished when Ca^{2+} was removed. This suggests a dissimilarity between GLP-1, on one hand, and forskolin/IBMX or dbcAMP on the other, regarding the ability to transmit the inhibitory effect of cAMP-PKA to PLA_2. Such a fact might be explained by dual actions of GLP-1 on PLA_2 reversing the restraining effect, or by the possibility that GLP-1 lacks capability to affect $iPLA_2$.

The results of our study is in accordance with findings on smooth muscle cells, showing that both cAMP-dependent protein kinase, through phosphorylation of $cPLA_2$, inhibits $cPLA_2$ activity (28). It is, in this context, important to ponder upon the fact that the efflux of tritiated AA may not only reflect the activation of PLA_2, but also the activation of another AA-generating pathway; the sequential actions of PLC and diacylglyceride lipase (29). In fact, in earlier islet studies, acti-

vation of the cAMP-PKA pathway by forskolin and GIP reduced CCK-8-induced activation of PLC (30). Together with the findings of our study, this suggest that the inhibitory effect of the cAMP-PKA pathway might be directed towards both PLA_2 and PLC. The relevance of the restraint for insulin secretion was studied in incubation experiments during concomitant stimulation by (i) mellitin and forskolin, as well as by (ii) CCK-8 and GLP-1. We found that addition of forskolin and GLP-1 induced a potentiation of mellitin- and CCK-8-stimulated insulin secretion, respectively, which confirms previous studies showing an additive effect on insulin secretion by the combination of GLP-1 and CCK (31). However, by subtracting the impact on insulin secretion induced by the sole action of glucose, we found that the *net* insulin secretion created by the additional influence of mellitin and CCK-8 was significantly diminished by forskolin and GLP-1, respectively. Consequently, stimulation of the cAMP-PKA signalling pathway also results in a reduction of the expected potentiation of insulin secretion. This suggests that the cAMP-PKA-mediated restraint of AA generation is transmitted to a reduction of insulin secretion, which might be regarded as a safe guard preventing inappropriate release of insulin into the circulation, in order to avoid development of acute hypoglycaemia.

In conclusion, the results show that activation of the cAMP-PKA signalling pathway, exerted by several mechanistically different stimulators, reduces PLA_2 activation in islets, and that the main target seems to be the Ca^{2+}-independent PLA_2. The results imply that the restraining effect due to cAMP-PKA activation also reduces an expected potentiation of insulin secretion. Based on this study, we suggest that the PLA_2 and cAMP-PKA pathways interact in the β-cells, which in turn suggests a dual modulating impact on β-cell function for the cAMP/PKA pathway.

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