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Inhibition of Glucose-Induced Insulin Secretion by the Diacylglycerol Lipase Inhibitor RHC 80267 and the Phospholipase A_2 Inhibitor ACA Through Stimulation of K^+ Permeability Without Diminution by Exogenous Arachidonic Acid

Peter Thams and Kirsten Capito*

Department of Medical Biochemistry and Genetics, Biochemistry Laboratory A, The Panum Institute, University of Copenhagen, 3C Blegdamsvej, DK-2200 Copenhagen N, Denmark

ABSTRACT. The effects of the diacylglycerol lipase inhibitor 1,6-bis-(cyclohexyloximinocarbonyl-amino)-hexane (RHC 80267) and the phospholipase A_2 inhibitor N-(p-amylcinnamoyl)anthranilic acid (ACA) on insulin secretion and $^{86}Rb^+$ efflux in mouse pancreatic islets were studied. RHC 80267 (35 μM) and ACA (100 μM) inhibited glucose (16.7 mM)-induced insulin secretion, but did not inhibit insulin secretion induced by K^+ (40 mM) or the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA; 0.16 μM). K^+ (40 mM) or TPA (0.16 μM) potentiated glucose (16.7 mM)-induced insulin secretion, and prevented inhibition of glucose (16.7 mM)-induced insulin secretion by RHC 80267 and ACA. In comparison, potentiation of glucose-induced insulin secretion by albumin-bound arachidonic acid (AA; 200 μM total; 10 μM free unbound) failed to counteract inhibition of glucose-induced insulin secretion by RHC 80267 or ACA, suggesting that inhibition of insulin secretion by these agents was not mediated by a decrease in AA accumulation in islets. Determination of $^{86}Rb^+$ efflux, a marker of K^+ channel activity, revealed that both RHC 80267 and ACA stimulated K^+ efflux from islets. These effects of RHC 80267 and ACA were observed at both 3.3 and 16.7 mM glucose and persisted in Ca^{2+}-free medium, suggesting that they may represent an opening of ATP-sensitive K^+ channels. RHC 80267-mediated stimulation of $^{86}Rb^+$ efflux was not mimicked by the diacylglycerol analog TPA (0.16 μM) and was not prevented by the diacylglycerol kinase inhibitor R 59022 (50 μM), suggesting that stimulation of $^{86}Rb^+$ efflux did not reflect a conditional increase in diacylglycerol or in phosphatidic acid upon inhibition of diacylglycerol lipase. In contrast, TPA (0.16 μM) attenuated RHC 80267 and ACA stimulation of $^{86}Rb^+$ efflux. Addition of AA (200 μM total; 10 μM free unbound) stimulated $^{86}Rb^+$ efflux, suggesting that stimulation of $^{86}Rb^+$ efflux by RHC 80267 and ACA was not due to a decrease in AA accumulation. This stimulation by AA was not dependent on AA metabolism because it persisted in the presence of the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 50 μM) or the cyclooxygenase inhibitor indomethacin (50 μM). In contrast to RHC 80267 and ACA, AA stimulation of $^{86}Rb^+$ efflux was attenuated in Ca^{2+}-free medium, probably implicating Ca^{2+}-sensitive K^+ channels in AA regulation of $^{86}Rb^+$ efflux. Parallel experiments with diazoxide (100 μM) revealed that RHC 80267 and ACA mimicked the effects of diazoxide, a specific activator of ATP-sensitive K^+ channels in islets, on both insulin secretion and $^{86}Rb^+$ efflux. In conclusion, it is suggested that RHC 80267 and ACA, independently of their action on AA release, may inhibit glucose-induced insulin secretion by the opening of ATP-sensitive K^+ channels in islets. BIOCHEM PHARMACOL 53;8:1077–1086, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. insulin secretion; K^+ permeability; RHC 80267; ACA; arachidonic acid; mouse pancreatic islets

* Corresponding author. Dr. Peter Thams, Department of Medical Biochemistry & Genetics, Biochemistry Laboratory A, University of Copenhagen, 3C Blegdamsvej, DK-2200 Copenhagen N, Denmark. Tel. +(45)35327723; FAX +(45)35327701.

† *Abbreviations:* AA, arachidonic acid; ACA, N-(p-amylcinnamoyl)anthranilic acid; HELSS, E-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one; NDGA, nordihydroguaiaretic acid; R 59022, 6-[2-(4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl)ethyl]-7-methyl-5H-thiazolo-[3,2-a]-pyrimidin-5-one; RHC 80267, 1,6-bis-(cyclohexyloximinocarbonylamino)-hexane; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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Considerable evidence suggests that glucose stimulation of Ca^{2+} influx and insulin secretion is initiated by a closure of ATP-sensitive K^+ channels in the plasma membrane leading to depolarization and opening of voltage-sensitive Ca^{2+} channels. ATP is naturally believed to have a critical function in this process, and it is generally accepted that the closure of the channel may be exerted by changes in the concentration of free ATP or the ATP/ADP ratio, at or in the vicinity of the plasma membrane [1, 2]. Conclusive evidence has, however, not been obtained, and recent data

suggest that changes in the intracellular concentration of AA† may be equally potent in stimulating an increase in the cytosolic Ca^{2+} concentration in pancreatic β cells [3–7].

A possible significance of AA in regulation of insulin secretion was inferred by the dual observation that glucose stimulates AA release in islets [3] and that exogenous AA stimulates insulin secretion [4, 5]. Thus, exogenous AA has been suggested to increase cytosolic Ca^{2+} by mechanisms involving closure of ATP-sensitive K^+ channels [6], direct activation of voltage-dependent Ca^{2+} channels [7], and stimulation of Ca^{2+} release from intracellular stores, including the endoplasmic reticulum [3, 5]. In addition, AA has been described to potentiate insulin secretion in response to voltage-dependent Ca^{2+} entry in islet β cells [7].

Despite these effects of exogenous AA, the significance of glucose-induced AA release in regulation of Ca^{2+} influx and insulin release is not known. Until now, the implication of glucose-induced AA release in insulin secretion has been largely based on the ability of distinct lipase inhibitors to cause parallel inhibition of glucose-induced AA release and glucose-induced insulin secretion. Glucose-induced AA release in islets is in part Ca^{2+}-independent and in part Ca^{2+}-dependent [7]. Thus, Ca^{2+}-independent AA release has been implicated in glucose-induced Ca^{2+} influx and insulin release, because HELSS, an inhibitor of an ATP-sensitive, Ca^{2+}-insensitive phospholipase A_2, has been shown to inhibit glucose-induced AA release, Ca^{2+} influx, and insulin secretion [8]. On the other hand, a major portion of glucose-induced AA release in islets is Ca^{2+}-dependent, and activation of Ca^{2+}-dependent phospholipase A_2 may be of equal importance [9]. Thus, glucose may activate Ca^{2+}-dependent phospholipase A_2 [10]. Furthermore, the phospholipase A_2 inhibitor ACA has been described to inhibit both AA release and insulin secretion in response to glucose [11]. In addition, yet other data point to a possible role for diacylglycerol and diacylglycerol lipase in regulation of AA release because RHC 80267, an inhibitor of diacylglycerol lipase, also has been shown to block both glucose-induced AA release and insulin secretion [12, 13].

The specificity of these inhibitors in inhibiting AA release is not known, and the ability of exogenous AA to counteract inhibition of glucose-induced insulin secretion by these agents has not been investigated. The aim of the present study was to study the mechanism of inhibition of glucose-induced insulin release by the diacylglycerol lipase inhibitor RHC 80267 and the phospholipase A_2 inhibitor ACA. It is demonstrated that both agents may inhibit glucose-induced insulin secretion by opening ATP-sensitive K^+ channels. AA, on the other hand, does not appear to close ATP-sensitive K^+ channels or prevent inhibition of glucose-induced insulin secretion by these agents.

METHODS

Materials

Crude bacterial collagenase was obtained from Boehringer, Mannheim, Germany. Human serum albumin was from Behringswerke A.G., Marburg, Germany. [^{125}I]insulin and guinea-pig anti-insulin serum were from Novo Nordisk A/S, Bagsværd, Denmark. [U-^{14}C]glucose (230–350 mCi/mmol), [5-^{3}H]glucose (10–20 Ci/mmol), ^3H_2O (5 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H]AA (150–230 Ci/mmol), and ^{86}RbCl (0.5–10 mCi/mg Rb) were from Amersham Int., Bucks., U.K. RHC 80267 and ACA were from Biomol, Hørsholm, Denmark. TPA, forskolin, AA, indomethacin, NDGA, diazoxide, and R 59022 (diacylglycerol kinase inhibitor 1) were from Sigma Chemical Co., St. Louis, MO. HELSS was from Calbiochem, San Diego, CA. Verapamil was from RBI, Natick, MA. All other chemicals were of analytical grade.

Preparation of Islets

Islets were prepared by collagenase digestion of the pancreas of male albino mice (NMRI) (approx. 18–22 g body wt.) fed ad lib. on a standard laboratory diet. Islets were kept in tissue culture for 22–24 hr in TCM 199 medium (1.26 mM Ca^{2+}, 5.5 mM glucose) supplemented with 10% (v/v) newborn calf serum (Gibco, Paisley, U.K.), 20 mM HEPES, 5 mM $NaHCO_3$, 100 units penicillin/mL, and 100 μg streptomycin/mL.

Insulin Release

In most experiments, insulin release from islets was measured in batch-type incubations. Batches of 5 islets were transferred to test tubes containing 600 μL of Krebs-Ringer medium supplemented with 20 mM HEPES, 5 mM $NaHCO_3$, 2 mg human serum albumin/mL, and 3.3 mM glucose [14]. After preincubation for 45 min at 37°C, the medium was replaced by 600 μL of the same medium also containing test agents as indicated, and the islets were incubated for 1 hr at 37°C. In experiments with HELSS, this agent was only present during the 45 min of preincubation. The rate of insulin release was expressed as ng/hr/5 islets.

When indicated, insulin release from islets was determined by perifusion in a noncirculating system with beads of 0.25 mL Bio-Gel P2 (Bio-Rad Laboratories, Rockville Center, NY) as a supporting medium, as described previously [14]. 25 islets per chamber were perifused at 37°C at a flow rate of 0.26 mL/min. The effluent medium was collected for periods of 5 or 10 min. The rate of insulin release was expressed as ng/min/100 islets.

Insulin was determined by radioimmunoassay using guinea-pig anti-insulin serum, monoiodinated pig insulin as tracer, rat insulin as standard, and ethanol to separate antibody-bound from free insulin.

[^{3}H]AA Release

[^{3}H]AA release was measured essentially as described by Konrad *et al.* [12]. Groups of 600–800 islets were first incubated for 22–24 hr in 3 mL TCM medium containing 30 μCi [^{3}H]AA (150–230 Ci/mmol). After 5 washings in non-radioactive medium, batches of 100 islets were placed in

perifusion chambers in 0.25 mL Bio-Gel P2 matrix, and perifused at 37°C at a flow rate of 0.26 mL/min. The perifusion medium was Krebs-Ringer medium supplemented with 20 mM HEPES, 5 mM $NaHCO_3$, 2 mg human serum albumin/mL and 3.3 mM glucose. Islets were perifused for 45 min with 3.3 mM glucose to obtain a basal release rate and then challenged with 16.7 mM glucose in the absence or presence of RHC 80267 (35 μM), ACA (100 μM), or diazoxide (100 μM) for 45 min as indicated. [^{3}H]AA release at 3.3 mM glucose, expressed as the percentage release of total radioactivity incorporated in 100 islets per 45 min, amounted to 1.67 ± 0.13 (5)%.

Glucose Metabolism

Glucose utilization (formation of ^3H_2O from [5-^{3}H]glucose) and glucose oxidation (formation of $^{14}CO_2$ from [U-^{14}C]glucose) were determined as described previously [15].

$^{86}Rb^+$ Efflux

The $^{86}Rb^+$ efflux (a marker of K^+ permeability) was measured according to the method described by Henquin [16]. Groups of 500–600 islets were first incubated for 22–24 hr in 5 mL TCM medium containing 0.2 mM ^{86}RbCl (40–50 mCi/mmol). After 3 washings in nonradioactive medium, batches of 80–100 islets were placed in perifusion chambers in 0.25 mL Bio-Gel P2 matrix, and perifused at 37°C at a flow rate of 0.26 mL/min. The perifusion medium was Krebs-Ringer medium supplemented with 20 mM HEPES, 5 mM $NaHCO_3$, 2 mg human serum albumin/mL, and 3.3 mM glucose. The radioactivity lost by the islets was measured in effluent fractions collected at 5-min intervals, and the fractional efflux rate was calculated for each period.

Miscellaneous

RHC 80267, ACA, diazoxide, HELSS, R 59022, NDGA, indomethacin, and TPA were added in a small volume of DMSO, final conc. 0.01–0.1%. AA was dissolved in 0.1 M Na_2CO_3/0.15 M NaCl. Appropriate volumes of this solution were then added slowly and during continuous agitation to Krebs-Ringer medium supplemented with 20 mM HEPES, 5 mM $NaHCO_3$, 2 mg human serum albumin/mL, and 3.3 mM glucose. At this stage, the Krebs-Ringer medium did not contain $CaCl_2$, which was only added after the fatty acid. The total concentration of AA was 200 μM, corresponding to a free AA concentration of approx. 10 μM [17, 18]. Results are given as means ± SEM for n experiments. Statistical evaluation of the data was made by ANOVA, followed by the LSD test for multiple comparisons; not significant, $P > 0.05$.

RESULTS

Effects of RHC 80267, ACA and Diazoxide on Insulin Secretion

Both RHC 80267 (35 μM) and ACA (100 μM) inhibited glucose (16.7 mM)-induced insulin secretion (Fig. 1a and b). A similar inhibition by RHC 80267 and ACA was observed upon stimulation by α-KIC (10 mM) and glyceraldehyde (5 mM) (results not shown) which, like glucose, stimulate Ca^{2+} influx through closure of ATP-sensitive K^+ channels [19, 20]. In comparison, RHC 80267 and ACA failed to inhibit insulin secretion induced by K^+ (40 mM), which causes maximum membrane depolarization and Ca^{2+} influx independently of a closure of ATP-sensitive K^+ channels [21]. In addition, RHC 80267 and ACA did not inhibit insulin secretion induced by TPA (0.16 μM), an activator of protein kinase C that may stimulate insulin secretion in the absence of concomitant Ca^{2+} influx [22]. Both K^+ (40 mM) and TPA (0.16 μM) potentiated glu-

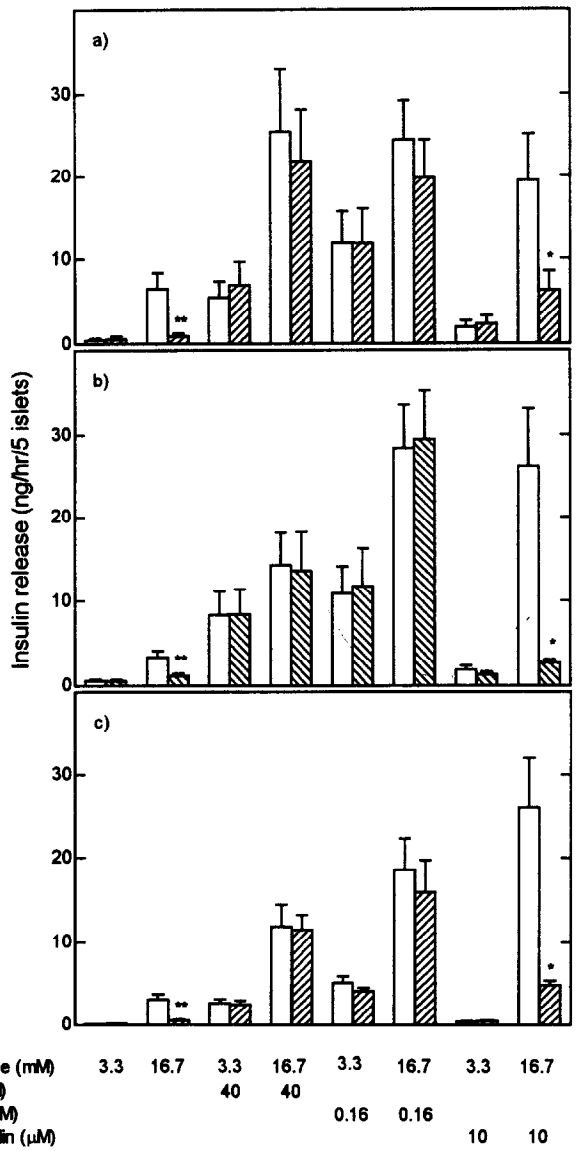

FIG. 1. Effects of RHC 80267, ACA, and diazoxide on insulin secretion. Insulin release was determined as described and with additions as indicated. The striped bars represent insulin release in the presence of (a) RHC 80267 (35 μM); (b) ACA (100 μM); or (c) diazoxide (100 μM). The open bars represent the paired control values in the absence of RHC 80267, ACA, or diazoxide. Results are means ± SEM (n = 3–10). *$P < 0.05$ vs control; **$P < 0.025$ vs control.

cose-induced insulin secretion and prevented inhibition of glucose-induced insulin secretion by RHC 80267 and ACA (Fig. 1a, b). In comparison, potentiation of glucose-induced insulin secretion by forskolin (10 μM), an activator of adenylate cyclase, did not prevent inhibition of glucose-induced insulin secretion by RHC 80267 or ACA (Fig. 1a, b). Parallel experiments with diazoxide, a specific opener of ATP-sensitive K^+ channels in islets [23], revealed that RHC 80267 and ACA in this way mimicked the effects of diazoxide on insulin secretion (Fig. 1c), suggesting that RHC 80267 and ACA may open ATP-sensitive K^+ channels in islets. This inhibition pattern was, however, not mimicked by the ATP-sensitive, Ca^{2+}-insensitive phospholipase A_2 inhibitor HELSS (100 μM), which appeared to inhibit both glucose (16.7 mM)-induced, K^+ (40 mM)-induced, and TPA (0.16 μM)-induced insulin secretion (results not shown).

Although both forskolin and TPA may lower the requirement for extracellular Ca^{2+} in glucose stimulation of insulin secretion, glucose stimulation of insulin secretion in the presence of TPA or forskolin may still be dependent on Ca^{2+} influx from the extracellular medium [24]. An abolition by TPA of the ability of K^+ channel openers to inhibit glucose-induced insulin secretion was, therefore, not to be expected, but was confirmed in perifusion experiments, where inclusion of TPA (0.16 μM) reversed the inhibition by RHC 80267 (35 μM) of glucose-induced insulin secretion (Fig. 2b). In comparison, verapamil (100 μM), an inhibitor of voltage-sensitive Ca^{2+} channels in islets [12, 25], led to inhibition of both glucose and glucose + TPA stimulation of insulin secretion (Fig. 2c).

Effects of RHC 80267, ACA, and Diazoxide on [^{3}H]AA Release

In accordance with previous results with rat islets [12], glucose (16.7 mM) caused a small increase in [^{3}H]AA release from mouse islets. Thus, a change in glucose concentration from 3.3 mM to 16.7 mM during 45 min of incubation led to a 23.80 ± 4.15 (5)% increase in [^{3}H]AA release ($P < 0.025$). As previously demonstrated in rat islets [11, 12], this increase in [^{3}H]AA release was prevented by the lipase inhibitors RHC 80267 (35 μM) and ACA (100 μM) which, at 16.7 mM glucose, inhibited [^{3}H]AA release to 63.60 ± 4.47 (5)% ($P < 0.025$) and to 79.00 ± 3.24 (4)% ($P < 0.025$) of controls in the absence of RHC 80267 and ACA, respectively. In addition, diazoxide (100 μM) inhibited [^{3}H]AA release, suggesting that opening of ATP-sensitive K^+ channels with inhibition of Ca^{2+} influx may lower AA release in islets [12]. Thus, at 16.7 mM glucose, [^{3}H]AA release in the presence of diazoxide (100 μM) amounted to 73.25 ± 3.30 (4)% of controls after 45 min of incubation ($P < 0.025$).

Effects of RHC 80267 on Glucose Metabolism

A previous study from this laboratory suggested that 17.5–70 μM RHC 80267 may lead to a selective stimulation of

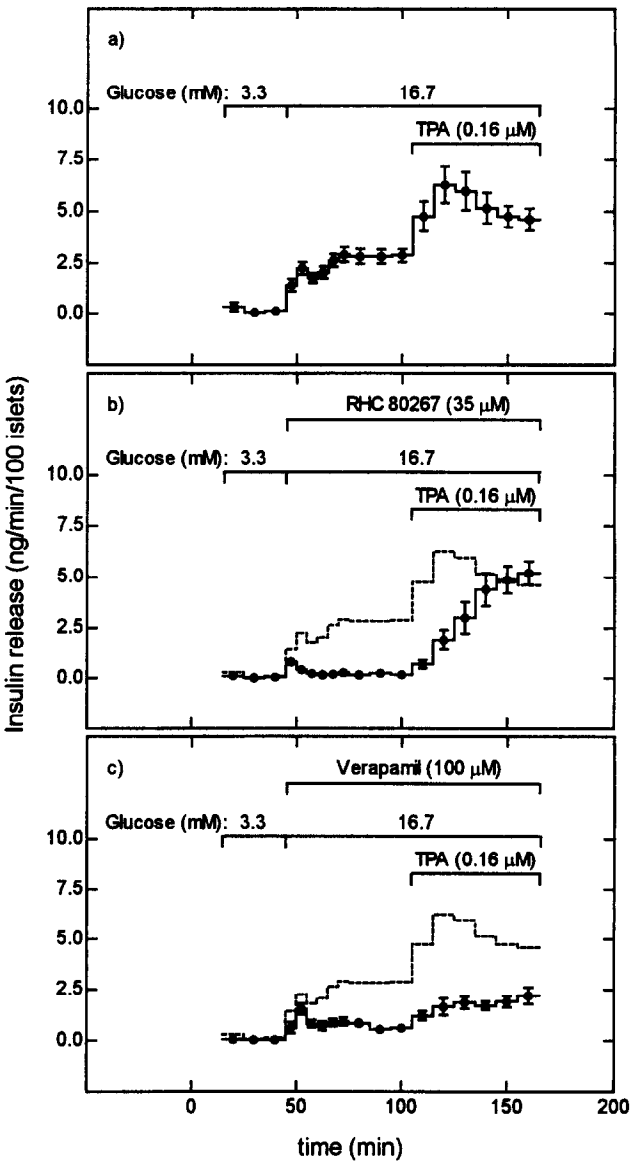

FIG. 2. Effects of RHC 80267 and verapamil on glucose-induced and TPA-induced insulin secretion. The dynamics of insulin release from column-perifused islets in response to glucose (16.7 mM) and glucose (16.7 mM) + TPA (0.16 μM) were determined as described. Islets were perifused in the (a) absence or (b) presence of RHC 80267 (35 μM) or (c) verapamil (100 μM) as indicated. The dotted line represents insulin release in the absence of RHC 80267 or verapamil as determined in (a). Results are means ± SEM (n = 3–9).

glucose utilization without affecting glucose oxidation or other parameters of glucose metabolism [26]. RHC 80267 (35 μM), however, neither affected glucose utilization of [5-^{3}H]glucose to ^3H_2O nor glucose oxidation of [U-^{14}C]glucose to $^{14}CO_2$ in the present experiments. Thus, at 3.3 mM and 16.7 mM glucose, respectively, glucose utilization amounted to 1155 ± 100 (5) and 2700 ± 214 (5) pmol/2 hr/25 islets in the absence and to 1185 ± 82 (5) and 2731 ± 280 (5) pmol/2 hr/25 islets in the presence of RHC 80267 (35 μM). Likewise, at 3.3 mM and 16.7 mM glucose, re-

spectively, glucose oxidation amounted to 179 ± 31 (5) and 710 ± 28 (5) pmol/2 hr/25 islets in the absence and to 190 ± 25 (5) and 648 ± 65 (5) pmol/2 hr/25 islets in the presence of RHC 80267 (35 μM). The reason for this deviation from previous results is not known. Likewise, ACA (100 μM) and diazoxide (100 μM) did not inhibit glucose metabolism (results not shown).

Effects of AA on Insulin Secretion

Addition of AA at a total concentration of 200 μM in Krebs-Ringer medium containing 2 mg/mL albumin did not induce insulin secretion from islets at 3.3 mM glucose, but led to potentiation of insulin secretion at 16.7 mM glucose (Fig. 3). In these experiments, AA was added at a free concentration of approx. 10 μM [17, 18]. This is well below the concentration at which fatty acids may exert detergent effects on cell membranes [27, 28]. At this concentration, AA failed to counteract RHC 80267 (35 μM)-mediated inhibition of glucose (16.7 mM)-induced insulin secretion (Fig. 3). Similar results were obtained for ACA (100 μM) and diazoxide (100 μM) (results not shown). AA did, however, prevent inhibition of glucose (16.7 mM)-induced insulin secretion by the ATP-sensitive, Ca^{2+}-insensitive phospholipase A_2 inhibitor HELSS. Thus, glucose (16.7 mM)-induced insulin secretion, which in the absence and presence of HELSS (100 μM) amounted to 3.97 ± 0.58 (5) and 1.68 ± 0.30 (5) ng/hr/5 islets ($P < 0.025$), was stimulated by 10 μM of free AA to 5.04 ± 0.90 (5) and 6.43 ± 1.79 (5) ng/hr/5 islets, respectively.

Inhibition of diacylglycerol lipase by RHC 80267 may lead to a conditional increase in phosphatidic acid synthesis from diacylglycerol. However, inclusion of the diacylglycerol kinase inhibitor R 59022 did not prevent RHC 80267-mediated inhibition of glucose-induced insulin secretion in the absence or presence of AA (200 μM), suggesting that phosphatidic acid may not be the cause of inhibition of glucose-induced insulin secretion by RHC 80267 (Fig. 3).

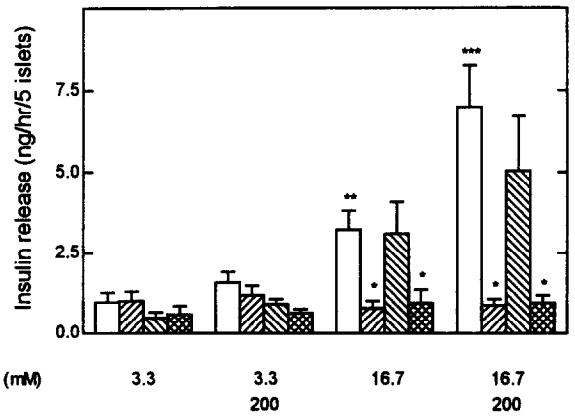

FIG. 3. Effects of AA on insulin secretion. Insulin release was determined with additions as indicated and in the presence of RHC 80267 (35 μM) (▨); R 59022 (50 μM) (▧); or RHC 80267 (35 μM) + R 59022 (50 μM) (■). Results are means ± SEM (n = 5–12). *$P <$ 0.025 vs control without inhibitors; **$P < 0.025$ vs 3.3 mM glucose; ***$P < 0.025$ vs 16.7 mM glucose.

Effects of RHC 80267, ACA, and Diazoxide on $^{86}Rb^+$ Efflux

At 3.3 mM glucose, RHC 80267 (35 μM) and to a smaller degree ACA (100 μM) stimulated $^{86}Rb^+$ efflux from islets (Figs. 4a and 5a). TPA (0.16 μM), which *per se* caused a slight transient stimulation of $^{86}Rb^+$ efflux, prevented stimulation of $^{86}Rb^+$ efflux by these agents (Figs. 4b and 5b). RHC 80267 and ACA thus mimicked the effect of diazoxide (100 μM) on $^{86}Rb^+$ efflux, which also appeared to be prevented by TPA (0.16 μM) (Fig. 5c and d).

RHC 80267-mediated stimulation of $^{86}Rb^+$ efflux did not appear to involve a consequential increase in phosphatidic acid synthesis in response to inhibition of diacylglycerol lipase, because R 59022 (50 μM), an inhibitor of diacylglycerol kinase, failed to prevent stimulation of $^{86}Rb^+$ efflux by RHC 80267 (35 μM) (Fig. 4c).

Stimulation of $^{86}Rb^+$ efflux by RHC 80267 at 3.3 mM glucose persisted in Ca^{2+}-free medium, suggesting that the stimulation may represent an opening of ATP-sensitive K^+ channels and not activation of Ca^{2+}-sensitive K^+ channels in islets (Fig. 4d).

An increase in glucose concentration from 3.3 to 16.7 mM led to a reduction in $^{86}Rb^+$ efflux (Fig. 6a). This reduction was abolished by RHC 80267 (35 μM), which led to a stimulation of $^{86}Rb^+$ efflux (Fig. 6b). Thus, RHC 80267 appeared to counteract glucose-mediated inhibition of ATP-sensitive K^+ channels.

Effects of AA on $^{86}Rb^+$ Efflux

Addition of AA (200 μM), corresponding to a free concentration of 10 μM, led to stimulation of $^{86}Rb^+$ efflux (Fig. 7a). This stimulation by AA was not dependent on AA metabolism by the cyclooxygenase or lipoxygenase pathway. Thus, AA stimulation of $^{86}Rb^+$ efflux persisted in the presence of the cyclooxygenase inhibitor indomethacin (50 μM) (Fig. 7b), and in the presence of the lipoxygenase inhibitor NDGA (50 μM) (Fig. 7c).

At variance from RHC 80267 and ACA, AA (200 μM) stimulation of $^{86}Rb^+$ efflux was attenuated in Ca^{2+}-free medium (Fig. 7d), likely implicating Ca^{2+}-sensitive K^+ channels in AA stimulation of $^{86}Rb^+$ efflux.

DISCUSSION

According to the present experiments, the diacylglycerol lipase inhibitor RHC 80267 and the phospholipase A_2 inhibitor ACA may inhibit glucose-induced insulin secretion by opening of ATP-sensitive K^+ channels. Thus, both RHC 80267 and ACA caused a specific inhibition of nutrient-induced insulin secretion, which is critically dependent on closure of ATP-sensitive K^+ channels [29], but failed to

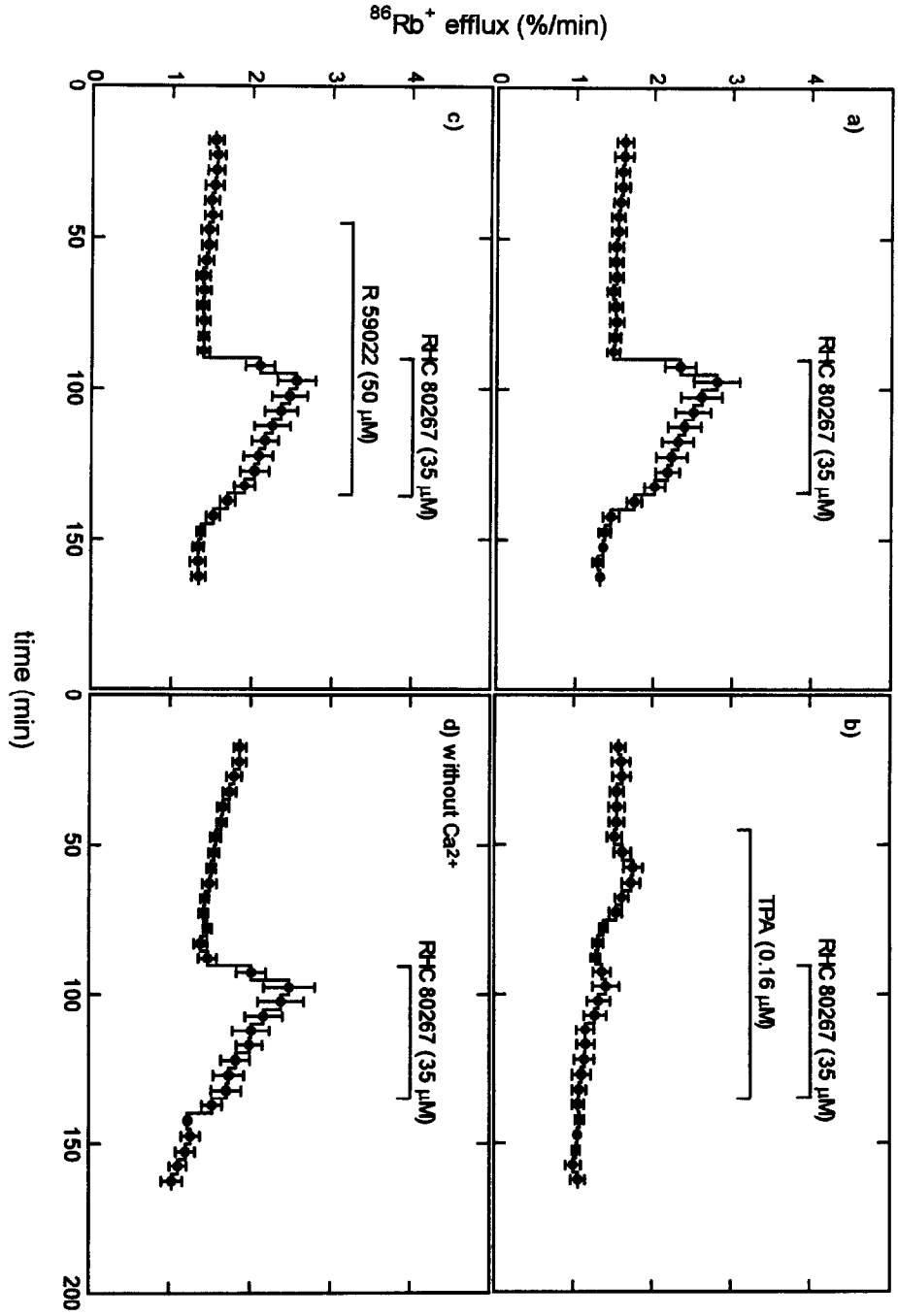

FIG. 4. Effects of RHC 80267 on $^{86}Rb^+$ efflux from preloaded, perifused islets. (a) RHC 80267 (35 µM) alone; (b) in the presence of TPA (0.16 µM); (c) in the presence of R 59022 (50 µM); or (d) in the absence of extracellular Ca^{2+} as indicated. All experiments were performed at 3.3 mM glucose. Results are means ± SEM (n = 3–4).

inhibit insulin secretion induced by K^+ or TPA, which may stimulate insulin secretion independently of a closure of these channels [21, 30]. In addition, both RHC 80267 and ACA mimicked the inhibitory effects of diazoxide, a well-established activator of ATP-sensitive K^+ channels [23], on insulin secretion and the stimulatory effects of diazoxide on $^{86}Rb^+$ efflux from islets.

A specific activation by RHC 80267 and ACA of ATP-sensitive K^+ channels was further emphasized by the ability of K^+ to counteract inhibition of glucose-induced insulin secretion by these agents. Thus, K^+, which causes membrane depolarization and Ca^{2+} influx independently of a closure of ATP-sensitive K^+ channels, obliterated the inhibitory effects of RHC 80267, ACA, and diazoxide on glucose-induced insulin secretion. A similar abolition of RHC 80267-mediated, ACA-mediated, or diazoxide-mediated inhibition of glucose-induced insulin secretion was observed upon addition of TPA, but not of forskolin, probably reflecting the ability of TPA to prevent opening of ATP-sensitive K^+ channels by these agents.

The ability of TPA to fully counteract inhibition of glucose-induced insulin secretion by RHC 80267, ACA, or diazoxide was unexpected. The synergistic effect of TPA and glucose on insulin secretion is normally ascribed to the combined actions of protein kinase C activation by TPA and stimulation of Ca^{2+} influx by glucose [31]. TPA may,

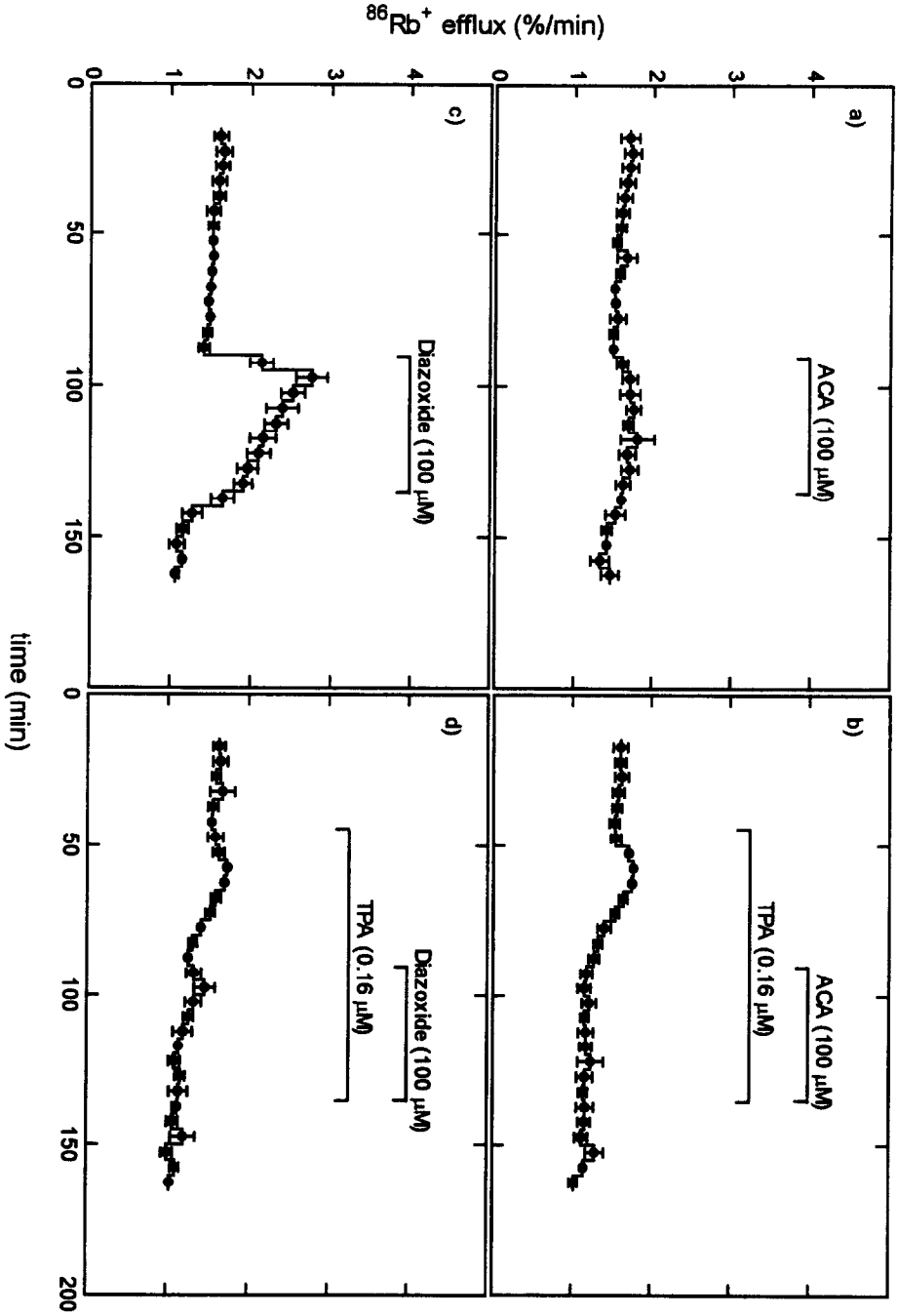

FIG. 5. Effects of ACA and diazoxide on $^{86}Rb^+$ efflux. (a) ACA (100 µM) alone; or (b) in the presence of TPA (0.16 µM); and (c) diazoxide (100 µM) alone; or (d) in the presence of TPA (0.16 µM) as indicated. In all experiments, the perifusion medium contained 3.3 mM glucose. Results are means ± SEM (n = 3).

therefore, counteract activation of ATP-sensitive K^+ channels by RHC 80267, ACA, or diazoxide and alleviate inhibition of glucose-stimulated Ca^{2+} influx by these agents. Indeed, the data do show that TPA prevents stimulation of $^{86}Rb^+$ efflux by RHC 80267, ACA, or diazoxide. It cannot be excluded, however, that the ability of TPA to prevent stimulation of $^{86}Rb^+$ efflux by these agents may be indirect, and probably not related to a direct effect on ATP-sensitive K^+ channels. Previous data have not been able to clarify the exact role of protein kinase C in regulation of ATP-sensitive K^+ channels. Thus, TPA has been shown to either open or close ATP-sensitive K^+ channels in insulinoma cells [32–34], but not to affect membrane potential in normal islet cells at nonstimulatory or stimulatory glucose concentrations [30]. In addition, TPA may have other yet-unresolved effects on K^+ permeability, leading to a paradoxical increase in $^{86}Rb^+$ efflux in Ca^{2+}-free medium containing a stimulatory concentration of glucose [30].

Alternatively, glucose might be suggested to potentiate TPA-induced insulin secretion irrespective of an opening of ATP-sensitive K^+ channels by RHC 80267, ACA, or diazoxide, and independently of concomitant Ca^{2+} influx. Thus, TPA induces insulin secretion despite a lowering of cytoplasmic Ca^{2+} [29], and lowers the requirement for Ca^{2+} influx for glucose stimulation of insulin secretion [24]. Furthermore, glucose may potentiate insulin secretion inde-

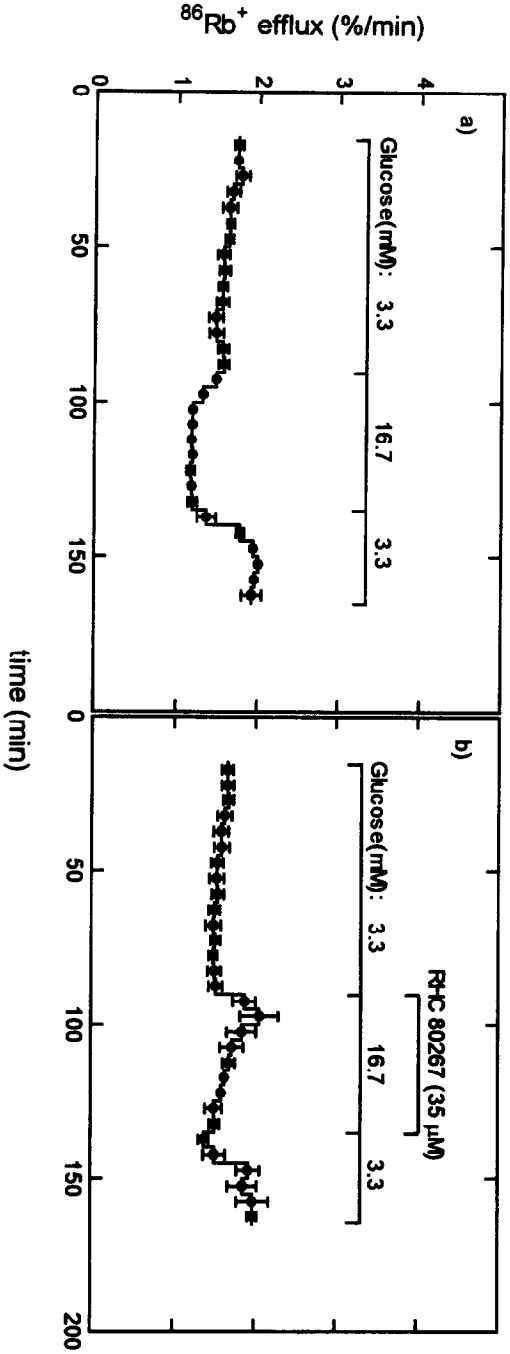

FIG. 6. Effects of glucose on $^{86}Rb^+$ efflux. (a) glucose (16.7 mM) alone; or (b), in the presence of RHC 80267 (35 µM) as indicated. Results are means ± SEM (n = 3).

pendently of its action on ATP-sensitive K^+ channels and voltage-dependent Ca^{2+} channels [21]. According to the present experiments, however, glucose stimulation of TPA-induced insulin secretion was dependent on Ca^{2+} influx through verapamil-sensitive Ca^{2+} channels. Thus, TPA appears to compensate for the opening of ATP-sensitive K^+ channels by RHC 80267, ACA, or diazoxide, but not for the direct inhibition of Ca^{2+} influx by verapamil. The degree to which TPA actually prevents an opening of ATP-sensitive K^+ channels by RHC 80267, ACA, or diazoxide must, therefore, be examined in future studies and address the exact role of protein kinase C in regulation of K^+ permeability.

An explanation of the mechanism involved in the stimulation of $^{86}Rb^+$ efflux by RHC 80267 and ACA is not readily at hand. Both RHC 80267 and ACA block glucose-induced AA accumulation in islets. However, AA did not prevent RHC 80267 or ACA from inhibiting glucose-induced insulin secretion; indeed, AA *per se* led to stimulation and not inhibition of $^{86}Rb^+$ efflux. RHC 80267, which inhibits diacylglycerol lipase, may cause an increase in diacylglycerol and consequently an increase in phosphatidic acid. However, RHC 80267-mediated inhibition of insulin secretion and stimulation of $^{86}Rb^+$ efflux was not prevented by the diacylglycerol kinase inhibitor R 59022. Furthermore, a stimulatory effect of diacylglycerol *per se* on $^{86}Rb^+$ seems unlikely because in a previous study, diacylglycerol led to inhibition of $^{86}Rb^+$ efflux [35], and because the diacylglycerol analog TPA in the present study did not mimic the effects of RHC 80267. It is possible that other yet-undefined fatty acids may have specific roles in regulation of ATP-sensitive K^+ channels or that RHC 80267 and ACA, in addition to diazoxide, may cause a direct activation of ATP-sensitive K^+ channels.

In a recent study, AA was suggested to close ATP-sensitive K^+ channels in HIT cells [6]. This study contradicted a previous study, where AA was demonstrated to open ATP-sensitive K^+ channels in HIT cells, an effect attributed to protein kinase C activation [36]. According to the present study, AA activates $^{86}Rb^+$ efflux from normal islets. This stimulation does not reflect an opening of ATP-sensitive K^+ channels in response to protein kinase C activation, because AA does not activate protein kinase C in islets [37], and because AA did not mimic the effect of TPA in preventing RHC 80267, ACA, or diazoxide inhibition of insulin secretion. AA-mediated opening of ATP-sensitive K^+ channels has been suggested to be mediated by prostaglandins, because it was prevented by indomethacin, an inhibitor of cyclooxygenase [6]. According to the present results, AA stimulation of $^{86}Rb^+$ efflux may be caused by AA *per se*, because neither indomethacin nor NDGA, an inhibitor of lipoxygenase, prevented AA stimulation of $^{86}Rb^+$ efflux. Previous studies with islets have demonstrated that AA may stimulate Ca^{2+} mobilization from intracellular stores [3, 5] and increase cytosolic Ca^{2+} [38]. It is, therefore, tempting to speculate that AA may activate Ca^{2+}-dependent K^+ channels in islets. In accordance, AA stimulation of $^{86}Rb^+$ efflux was attenuated in Ca^{2+}-free medium. The degree to which AA may fulfil a role in regulation of ATP-sensitive K^+ channels is, therefore, uncertain, but most likely stimulation of $^{86}Rb^+$ efflux by RHC 80267 and ACA is not mediated by inhibition of AA accumulation in islets.

According to the present results, the inhibitory actions of RHC 80267 and ACA coincide, because both agents may inhibit insulin secretion by opening of ATP-sensitive K^+

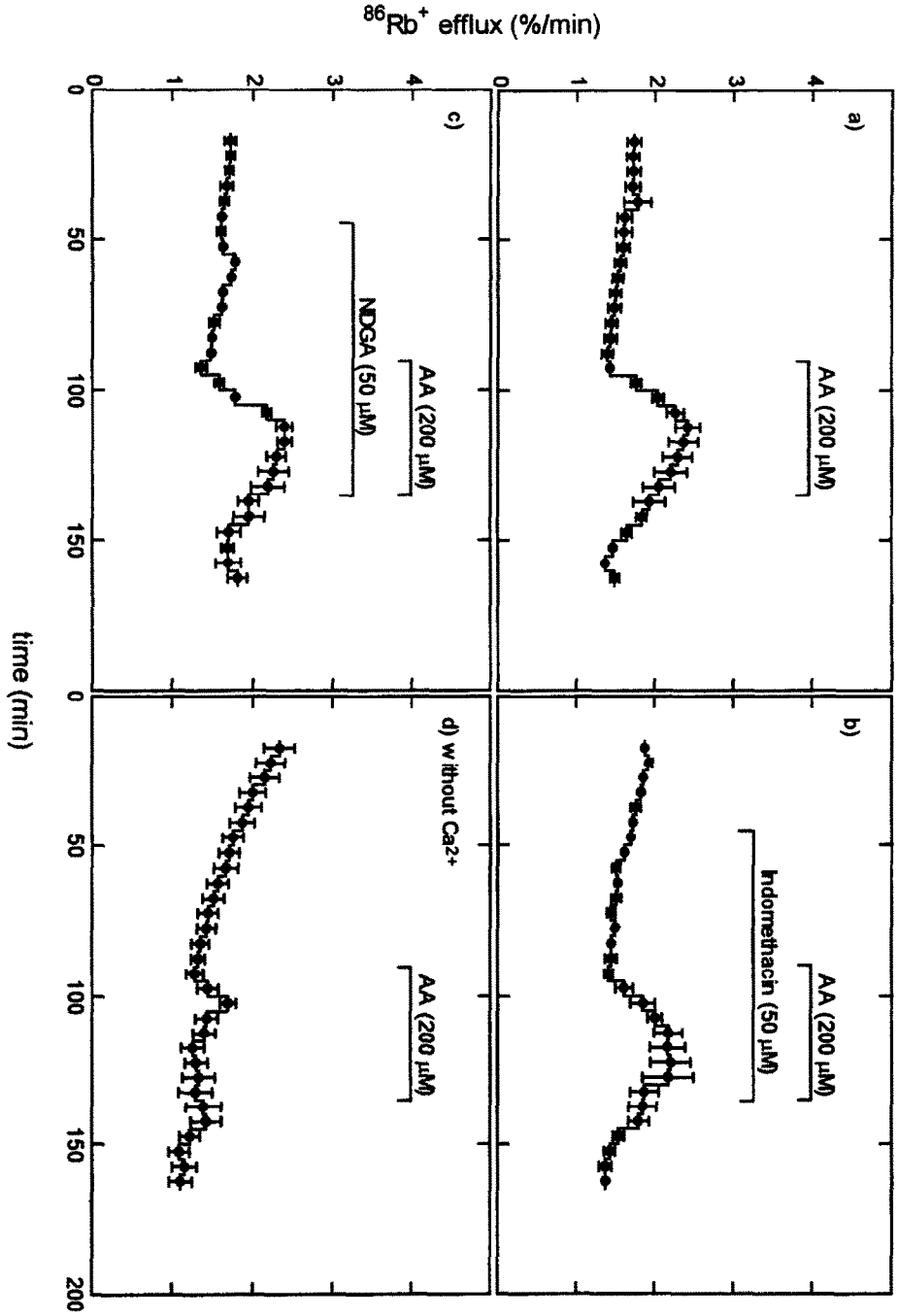

FIG. 7. Effects of AA on $^{86}Rb^+$ efflux. (a), AA (200 µM) alone; (b) in the presence of indomethacin (50 µM); (c) in the presence of NDGA (50 µM); or (d) in the absence of extracellular Ca^{2+}, as indicated. The glucose concentration was 3.3 mM. Results are means ± SEM (n = 3–4).

channels. The inability of added AA to counteract the effects of RHC 80267 and ACA, however, questions the role of a lowering of AA in these responses to RHC 80267 and ACA. Although AA may be of significance for regulation of cytosolic Ca^{2+} in islets [8, 38], RHC 80267 and ACA may, therefore, not be suitable choices in future studies examining the specific roles of AA in insulin secretion.

In conclusion, the present data have clearly demonstrated that the diacylglycerol lipase inhibitor RHC 80267 and the phospholipase A_2 inhibitor ACA inhibit glucose-induced insulin secretion by stimulation of K^+ efflux, a phenomenon most likely reflecting opening of ATP-sensitive K^+ channels.

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